THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOBEHAVIORAL HEALTH

THE EFFECT OF CAFFEINE ON SERUM DHEA-S LEVELS AFTER AN ACUTE STRESSOR IN INDIVIDUALS WITH A FAMILY HISTORY OF HYPERTENSION

SHARMILA SANDIRASEGARANE
SPRING 2015

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree in Biobehavioral Health
with honors in Biobehavioral Health

Reviewed and approved* by the following:

Laura Cousino Klein
Professor of Biobehavioral Health
Thesis Supervisor

David J. Vandenbergh
Associate Professor of Biobehavioral Health
Honors Adviser

Sheila G. West
Professor of Biobehavioral Health
Faculty Reader

* Signatures are on file in the Schreyer Honors College.
ABSTRACT

The levels of the hormone dehydroepiandrosterone-sulfate (DHEA-S) after an acute stressor were evaluated to assess the interaction with caffeine. DHEA-S has been hypothesized to be involved in protective actions that oppose the physiological tolls typically associated with the stress hormone, cortisol. Although caffeine use is ubiquitous in the United States, the health effects of caffeine intake are not well understood. Serum DHEA-S levels in response to stressor and caffeine exposure has not been studied. We examined the influence of caffeine and stress on serum DHEA-S levels in 26 men and 26 women (aged 18-29 years) with a confirmed family history of hypertension, which placed them at particular risk of the health consequences of caffeine exposure under stress. After half of the participants were administered 3 mg/kg caffeine or placebo, all participants underwent a lab-based stressor. Serum DHEA-S levels were measured before and 2 times after the stressor. Stress and caffeine interacted with sex such that caffeine exposure reduced serum DHEA-S levels in women but not men. These findings support a growing body of research that suggests that women with a family history of hypertension may be particularly vulnerable to the health consequences of caffeine exposure in the presence of stress.
TABLE OF CONTENTS

LIST OF FIGURES .......................................................................................................................... v
LIST OF TABLES ............................................................................................................................ vi
ACKNOWLEDGEMENTS .................................................................................................................... vii

Chapter 1 - Introduction .................................................................................................................. 1

Synthesis of DHEA-S .................................................................................................................. 2
Physiological Effects of DHEA/DHEA-S .................................................................................. 3
DHEA Supplementation ............................................................................................................. 7
Differences between DHEA and DHEA-S ................................................................................ 7
Interactions with Cortisol and Stress ....................................................................................... 8
Caffeine Interactions with DHEA/DHEA-S and Cortisol .......................................................... 10

Chapter 2 - Methods ...................................................................................................................... 13

Participants .................................................................................................................................. 13
Laboratory Session .................................................................................................................... 13
Caffeine Administration and Baseline ..................................................................................... 14
Stressor Administration ............................................................................................................. 14
Serum DHEA-S and Cortisol Assay ........................................................................................ 15
Data Analyses ............................................................................................................................ 15

Chapter 3 - Results ....................................................................................................................... 17

DHEA-S ........................................................................................................................................ 17
Cortisol ........................................................................................................................................ 17

Chapter 4 - Discussion .................................................................................................................. 18

Effect of Caffeine on DHEA-S Levels in Women ................................................................. 19
Effects of Caffeine and Stress .................................................................................................. 20
Health Effects of Lower DHEA-S Levels .............................................................................. 21
Cortisol/DHEA Ratio .................................................................................................................. 22
Limitations ................................................................................................................................... 25
Future Directions ....................................................................................................................... 26
Conclusion .................................................................................................................................... 27

REFERENCES ................................................................................................................................. 37

Appendix A: Institutional Review Board Approval and Consent Forms ......................... 52
Appendix B: Serum DHEA-S ELISA Protocol ........................................................................... 56
LIST OF FIGURES

Figure 1. Diagram of DHEA-S synthesis and metabolism, redrawn from (Sawalha & Kovats, 2008; Traish et al., 2011)............................................................32

Figure 2. Hypothesized mechanism of DHEA/DHEA-S rise after caffeine administration

Figure 3. Experimental Timeline, used with permission from (Bayly, 2014)..............34

Figure 4. DHEA-S (µg/mL) levels for sex and caffeine groups over time. Means ± SEM.

Figure 5. Cortisol levels (µg/dL) for sex and caffeine groups over time. Means ± SEM. 36
LIST OF TABLES

Table 1. Mean (±SEM) raw values for serum hormone levels across laboratory session by sex and caffeine treatment groups .............................................................30
ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance of Dr. Laura Cousino Klein. I met her a few weeks after I started at Penn State, nervous but excited about starting research. She introduced me to research protocol in several fields related to stress, including mindfulness, work-life balance, and diabetes. She also introduced me to Dr. Joseph Cannon at the Georgia Health Sciences University, with whom I was able to conduct summer research regarding the inflammatory effect of irisin, a hormone released during aerobic activity, in blood monocytes. Dr. Klein has provided me with guidance well beyond the sphere of research throughout undergrad, about which I am extremely grateful.

Dr. Joseph Gyekis played an integral role in my thesis process. Through honors options in BBH 310 and 411W, he patiently gave me time, recommendations, and criticisms. His commitment to helping me learn about the research writing process was much valued. He has honed my skeptical eye in research, and I am infinitely thankful for this perspective.

Within the Biobehavioral Health Studies Laboratory, I have had several key mentors. I had a wonderful time running assays with Mrs. Mary Curran, who was quick to share both her skills and her humor. Thank you to Dr. Kim Walter and Dr. Alicia Revitsky, who let me assist with their research projects while sharing advice from their professional experiences. I was also able to find an incredible friend and role model in Jennifer Bayly, an honors student one year senior to me. A special thank you goes to Dr. Jeanette Bennett for letting me work with her data set and samples from her original study.

There are several faculty members who have helped me as an honors student in Biobehavioral Health. I have been lucky enough to have had two talented honors advisers. Thank
you to Dr. Lori Francis and Dr. David Vandenbergh for keeping me on top of my deadlines and providing networking opportunities with other honors students in the major. I appreciate Dr. Sheila West for serving as the second reader for my thesis. Dr. Michele Stine has also been an excellent mentor and academic adviser to me. As the Global Health Minor Coordinator, Dr. Melina Czymoniewicz-Klippel has helped me develop writing and critical thinking skills, which have transferred into my thesis. There are innumerable faculty, staff, students, and friends who have contributed to academic experience at Penn State. I would also like to thank my family for their interest in my thesis work, while providing me continual encouragement and motivation. I am inexplicably grateful for the all of the support that I have received.
Chapter 1 - Introduction

During times of stress, drinking coffee or tea is a common response. Frequently cited stressors among individuals living in the United States include issues of money, work, and relationships (American Psychological Association, 2011). There is also ample exposure to caffeine in the U.S.; in a study of over thirty-seven thousand participants aged 2 years old and above, 85% consumed at least one caffeinated beverage per day (Mitchell et al., 2014). Prior studies suggest that the biological (e.g. cardiovascular, salivary α-amylase) effects of caffeine only are revealed under conditions of stress (Klein et al., 2011; Klein et al., 2014). The health effects of caffeine and stress have never been studied with dehydroepiandrosterone (DHEA). DHEA has been observed to have anti-glucocorticoid effects, which may work to counteract some of the major effects of stress. Considering that DHEA is the most abundant steroid in mammals (Shealy, 1995) and caffeine is the most commonly consumed psychoactive substance, the effects of caffeine on DHEA levels under acute stress were examined in this thesis. Further, in light of inconsistent findings regarding the role that caffeine intake plays in cardiovascular disease (Chou, 1992), I focused my research on participants with a family history of hypertension. This medical history may make them particularly susceptible to the potentially negative health consequences of caffeine (e.g. Lovallo et al., 1989).

DHEA, an anabolic steroid, has been shown to have physiological effects on the central nervous system (CNS), endothelial function, and immune function (Traish, Kang, Saad, & Guay, 2011). DHEA serves as precursor to biologically active androgens and estrogens in tissues including liver and adipose tissue (Witchel, 2007). While high fetal DHEA production serves as
a precursor to placental estrogen biosynthesis, childhood concentrations of DHEA and its sulfated derivative, DHEA-S, are low (Witchel, 2007). DHEA and DHEA-S levels increase from adrenarche to ages 20-30 years, with a subsequent decline across the lifespan (Witchel, 2007). In both men and women, DHEA/DHEA-S plays a role in puberty, followed by a significant decline in serum and saliva levels from early adulthood (Nawata et al., 2002; Heaney et al., 2012). DHEA/DHEA-S is higher among men than women (Traish et al., 2011, & Whetzel & Klein, 2010). Across the day, DHEA levels are characterized by a flat pattern of secretion with a progressive decline three hours after awakening (Heaney et al., 2012, Hammer et al., 2005).

Although prior research suggest a lack of a DHEA-S circadian rhythm (Zhai et al., 2011), serum levels have an observed peak in the afternoon (16:00) for healthy men aged 30-60 years (Zhao et al., 2003) and in young individuals aged 19-43 years (Ferrari et al., 2001). In another study of men and women aged 18-30 years, salivary DHEA-S levels were higher in the morning (08:00-09:00 hrs) than in the evening (16:00-17:00 hrs) (Whetzel & Klein, 2010).

**Synthesis of DHEA-S**

Both DHEA and DHEA-S are regulated by adrenocorticotropic hormone (ACTH) (Kalimi et al., 1993). Once ACTH reaches its receptor on the adrenal cortex, the secondary messenger cAMP is produced, which activates the protein kinase A pathway, which leads to intracellular cholesterol uptake and mobilization (Vanparys et al., 2012). Cholesterol is the precursor for the biosynthesis of steroids including DHEA (Vanparys et al., 2012). DHEA/DHEA-S levels are affected by hypothalamic-pituitary-adrenal (HPA) axis activity, as evidenced by decreased concentrations of DHEA-S among individuals with impaired HPA axis pathways from liver disease (Witchel, 2007).
DHEA is released from the adrenal inner zona reticularis, with regulation from type II 3β-hydroxysteroid dehydrogenase (HSD3B2), cytochrome b5 (CYB5), and steroid sulfotransferase (SULT2A1) (Endoh et al., 1996; Witchel, 2007; Rainey, & Nakamura, 2008; Figure 1). DHEA is sulfonated in the adrenal cortex, liver, brain, and steroidogenic organs, where SULT2A1 is expressed (Ou et al., 2014; O’Shaughnessy et al., 2013; Kojima & Degawa, 2014; Knapstein et al., 1968). DHEA sulfonation to DHEA-S reduces the bioavailability of DHEA, which is the direct precursor to androgens and estrogens (Witchel, 2007; Haring et al., 2012; Figure 1). While DHEA is primarily secreted in the adrenal glands, production also occurs in the gonads, gastrointestinal tract, and the brain (Sawalha & Kovats, 2009).

SULT2A1 genetic variants do not appear to affect DHEA and DHEA-S levels or their respective ratio, which illustrates a consistent sulfonation capacity between the variants (Haring et al., 2012). However, hepatic SULT2A1 mRNA levels appear to be moderated by androgen concentration, as male pigs with higher androgen levels had lower mRNA levels than female pigs (Kojima & Degawa, 2014). Landrace pigs, with low levels of serum androgens, had no such sex differences (Kojima & Degawa, 2014). While DHEA sulfonation is considered reversible (Sawalha & Kovats, 2009), in both in vivo and in vitro studies, there is no evidence that SULT2A1 converts DHEA-S to DHEA (Hammer et al., 2005). After the administration of oral DHEA, both DHEA and DHEA-S increased from baseline, while intravenous DHEA-S administration only increased DHEA-S, not DHEA (Hammer et al., 2005).

**Physiological Effects of DHEA/DHEA-S**

Numerous studies have suggested anti-glucocorticoid effects of DHEA. Adipocyte proliferation is related to intracellular activation of cortisol with the catalysis of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (McNelis et al., 2013). DHEA, but not
DHEA-S, significantly inhibited preadipocyte proliferation and 11β-HSD1 oxoreductase activity in a human subcutaneous cell line (McNelis et al., 2013). DHEA coincubation with cortisol had a diminished inhibitory effect on preadipocyte proliferation, while DHEA significantly enhanced insulin-dependent glucose uptake (McNelis et al., 2013). This selective inhibition of 11β-HSD1 may aid treatment of obesity and type 2 diabetes (McNelis et al., 2013). Hepatic tyrosine aminotransferase activity was inhibited after DHEA administration in male Swiss-Webster mice (Browne et al., 1992). A similar response occurred after DHEA-S, but to a lesser extent. Other glucocorticoid-induced enzymes were also inhibited by DHEA in a dose- and time-dependent manner, including liver and kidney ornithine (Browne et al., 1992). Five days of DHEA administration reduced glucocorticoid receptors in the rat liver by 50% (Kalimi et al., 1990; Crudo, 1989). Taken together, DHEA serves to inhibit glucocorticoid related actions, while downregulating receptors.

Possible antihypertrophic effects may also occur from DHEA. In bovine aortic endothelial cells, physiological concentrations of DHEA dilated vascular tissue through a G-protein coupled receptor, which activates endothelial nitric oxide synthase (eNOS), more so than the vasodilator bradykinin increased eNOS levels (Liu & Dillon, 2004). The activation of nitric oxide synthesis in human endothelial cells occurs as quickly as 10 min after DHEA treatment, while nitric oxide increase is dose-dependent on the concentration of DHEA administered (Simoncini et al., 2003). In a meta-analysis, elevated levels of plasma DHEA/DHEA-S have been associated with reduced obesity and abdominal fat, but the results are inconsistent (Traish et al., 2011). DHEA has been found to stimulate resting metabolic rate and lipid oxidation, while increasing glucose disposal in fat cells through increasing the expression of GLUT-1 and GLUT-4 on plasma membranes (De Pergola, 2000, Nakashima et al., 1995). DHEA stimulates the
expression and activity of adipose triglyceride lipase and hormone-sensitive lipase, with promotes lipid absorption in adipose tissue (Karbowska & Kochan, 2012). With vascular dilation, metabolic stimulating, and lipid oxidation properties, normal DHEA levels may contribute to lower risk of CVD.

DHEA/DHEA-S may also have immunoenhancing properties. In immune-mediated inflammatory diseases, including systemic lupus erythematosus and Crohn’s diseases, lower circulating levels of DHEA-S have been observed (Kasperska-Zajac et al., 2009). DHEA/DHEA-S may regulate T helper cells by promoting a Th1 response after a suppressed Th2 response (Reed, 1995). By playing a role in the regulation of the Th1/Th2 balance, DHEA/DHEA-S could impact the cytokine pathways of these immune cells (Reed, 1995). In NZB/W mice that spontaneously develop autoimmune disease, treating two-month old female mice with DHEA-S delayed the onset of the disease and prolonged survival, but the treatment did not affect symptoms (Norton et al., 1997). There was no change in mortality rates in the female mice that were treated at six months of age (Norton et al., 1997).

Considering that there are higher concentrations of DHEA/DHEA-S in the brain than in plasma and the suggested antidepressant effects of the steroid (e.g. Morsink et al., 2007), its neurobiological effects are of interest (Maninger et al., 2008). DHEA/DHEA-S levels are increased during the sexual maturation stage of adrenarche, in which DHEA and testosterone relate to cortical maturation (Nguyen et al., 2013). In a four-year longitudinal study, DHEA levels were associated with increased cortical thickness in regions including the left dorsolateral prefrontal cortex in girls and boys aged 4-13 (Nguyen et al., 2013). DHEA acts in the CNS through GABAA, N-methyl-D-aspartate (NMDA), androgen, and σ1 receptors (Mo et al., 2004; Maninger et al., 2008). DHEA tends to act as a noncompetitive antagonist for the GABAA
receptor, which has an inhibitory neurotransmitter as its ligand, with DHEA-S having even more potent effects as an antagonist (Baulieu, 1998; Imanura & Prasad, 1998; Maninger et al., 2008). DHEA/DHEA-S furthermore prompts excitatory responses, by acting as an σ1 receptor agonist and potentiating NMDA receptor function, in a dose-dependent manner (Bergeron et al., 1996; Maninger et al., 2008). Neurosteroids like DHEA/DHEA-S do not have steroid hormone nuclear receptors; instead, they have ion-gated neurotransmitter receptors (Compagnone & Mellon, 2000).

Reviewing preclinical and clinical data from various studies, the neurobiological action of DHEA/DHEA-S may be mediated through signaling pathways involving specific membrane receptors of DHEA derivatives (Traish et al., 2011). There has not been a specific nuclear receptor protein found that is specific to DHEA, but a membrane-bound receptor protein has been identified as having a high affinity with DHEA in the endothelium, heart, liver and kidney, prompting downstream transcriptional regulation (Traish et al., 2011; Liu & Dillion, 2004; Simonici et al., 2003). DHEA-S bound to a plasma membrane preparation of human uterine cervical fibroblasts has been detected, potentially having a modulating effect on enzymes and second messenger pathways (Imai, Ohno, & Tamaya, 1992). In cultured cervical fibroblasts, DHEA-S increased levels of interleukin-8 and the expression of its receptor in a time- and dose-dependent manner, while DHEA a slight effect (Kanayama et al., 1998). DHEA/DHEA-S appears to directly or indirectly affect various physiological pathways, but there is still much research to be done to further investigate the mechanisms through which the hormone functions.

Despite the evidence of potential psychophysiological benefits from high DHEA levels, there is possibly an increased risk of breast cancer in post-menopausal women with elevated levels of DHEA/DHEA-S (Fang et al., 2014). However, decreased levels of DHEA/DHEA-S are
associated with Th2-type cytokines, some of which are shown to stimulate the activity of enzymes that are involved in estrogen synthesis in breast cancer cells (Reed, 1995). At this point, the influence of DHEA on the progression of breast cancer is unclear and needs further investigation.

**DHEA Supplementation**

Over-the-counter use of DHEA has been popular in recent years, but there is little evidence suggesting its efficacy in improving lipid-lipoprotein profile or body fat distribution (Tchernof & Labrie, 2004). Serum DHEA and DHEA-S levels increase with increasing doses and plateau with supplementation at 300 mg/day (Tummala & Svec, 1999). In men and women aged 40-70 years, the administration of 50 mg oral DHEA over six months doubled serum levels of androgens in women and resulted in a small rise in androstenedione in men (Morales et al., 1994). There was an increase in both physical and psychological wellbeing reported in the majority of male and female participants (67% and 82%, respectively), with less than 10% reporting change in the placebo group (Morales et al., 1994). In another study with men and women, minimal benefit or adverse effects from supplements have been found (Nair et al., 2006). However, in patients with mild to moderate lupus, an auto-immune inflammatory disease, DHEA supplementation may increase quality of life measurements and glucocorticoid requirements. Studies involving DHEA supplement usage and endogenous DHEA levels suffer from scientific weaknesses including low statistical power, confounding factors, and differences in the populations studied (Tchernof & Labrie, 2004).

**Differences between DHEA and DHEA-S**

Although related, endogenous DHEA and DHEA-S have distinctive biological properties. DHEA-S has an abundant plasma concentration of 5 µM to 7 µM, compared to DHEA
concentration of 0.01 µM to 0.02 µM (Kalimi et al., 1993). DHEA-S, but not DHEA, has increased superoxide generation in primed human neutrophils by activating recombinant protein kinase C-β (Radford et al., 2010). DHEA has been shown to stimulate adrenal cells to release nitric oxide into circulation, which decreases the responsiveness to platelet cells to physiological agonists including thrombin and collagen (Bertoni et al., 2011). DHEA-S, but not DHEA, inhibited platelet activation, in a dose-dependent manner, after a mild stimulus (Bertoni et al., 2011). DHEA-S is more abundant in circulation than DHEA is; it is the androgen that is secreted in the greatest quantity from the adrenal glands (Kannisto et al., 2001).

**Interactions with Cortisol and Stress**

Both cortisol and DHEA are secreted from adrenal glands after ACTH promotes synthesis from their shared progrenolone precursor (Kalimi et al., 1993, Katsumata, 2012). DHEA/DHEA-S is commonly considered to have anti-glucocorticoid effects. While cortisol plays a role in neuronal death and survival, serotonin biosynthesis, and norepinephrine uptake, DHEA-S enhances neuronal and glial survival, with evidence of improving memory and learning performance (Ferrari & Magri, 2007; Baulieu, 1998). Male rats with subcutaneous pellets of DHEA displayed increased cell formation in the dentate gyrus of the hippocampus and antagonized suppressive effects of corticosterone, while DHEA precursors pregnenolone or androstenediol did not have the same cell formation effect (Karishma & Herbert, 2002). DHEA pellets did not have as strong of an effect on neurogenesis in older rats (12-14 months old) in comparison to younger rats (3 months) (Karishma & Herbert, 2002). In rats put under two hours of immobilization stress every day for 60 days, the daily administration of 5 mg DHEA was associated with a significant inhibition of body weight gain, an increase in glucocorticoid
receptor in the liver, thymus and spleen, an increase in adrenal weight, and decreased plasma triglyceride levels, compared to the stressed group without DHEA (Hu et al., 2000).

Cortisol has been shown to directly stimulate the secretion of DHEA in human adrenocortical cells (Topor et al., 2010). In an in vitro adrenal cell line, cortisol competitively inhibited 3-β hydroxysteroid dehydrogenase Type II (3β-HSD2), which led to the stimulation of DHEA secretion, in a dose-dependent manner (Topor et al., 2010). The hydroxysteroid 3β-HSD2 metabolizes DHEA after cortisol binds and alters its secondary structure, which leads to the binding of 3β-HSD2 in the intermitochondrial space as a membrane-associated protein (Thomas et al., 2014).

A relationship appears to exist between cortisol and DHEA levels have been observed in various lab-based studies. Immediately after the Trier Social Stress Test (TSST), salivary DHEA levels increased by an average of 60% in a group of healthy men (n=33), with a moderate correlation to cortisol (n=33) (Izawa et al., 2008). Postmenopausal women (n=40) who had greater increases in negative affect and anxiety after TSST exposure also displayed higher levels of plasma ACTH, cortisol, and serum DHEA (Fang et al., 2014). In healthy men (n=20) and women (n=19), DHEA and DHEA-S levels were significantly elevated in response to the TSST (Lennartsson, Kushnir, Bergquist, Jonsdottir, 2012). The magnitude of change for serum DHEA was positively correlated with magnitude of changes in plasma ACTH, serum cortisol, and heart rate (Lennartsson, Kushnir, Bergquist, Jonsdottir, 2012). Despite these consistent increases in DHEA/DHEA-S after acute stressors, there is a large inter-individual difference in the extent of hormone response, among both men and women (Lennartsson, Kushnir, Bergquist, Jonsdottir, 2012). For chronic stressors, mean DHEA-S levels were 23% lower in men and women who reported stress at work, compared to the non-stressed group (Lennartsson et al., 2013).
Caffeine Interactions with DHEA/DHEA-S and Cortisol

Caffeine is related to sympathetic nervous system activity, like cortisol. Caffeine and its metabolites similarly lead to vasoconstriction by activating the renin angiotensin system (Bennett, Rodrigues, & Klein, 2013). The drug non-selectively inhibits adenosine receptors, which exist in cells throughout the body. The A1 and A2A receptors modulate serotonin release. The inhibition of these receptors results in the psychoactive and stimulating effects of caffeine. A2A receptors are localized in the hypothalamus, the source of corticotropin-releasing hormone (CRH) for the initial activation of the pituitary gland in the HPA axis response (Ferré, 2007). Therefore, when caffeine stimulates the hypothalamus, HPA axis activation could also result. Administration of caffeine before the TSST has been shown to increase systolic blood pressure and cortisol, in men and women, compared to controls (Bennett, Rodrigues, & Klein, 2013). Following high dosages of caffeine (30 mg/kg), plasma levels of adrenocorticotrophic hormone (ACTH) and cortisol increased in rats, suggesting caffeine’s role in activating the HPA axis (Patz, Day, Burow, & Campeau, 2006). In healthy men, the administration of 5 mg/kg of caffeine elevated ACTH and cortisol during non-rapid eye movement sleep (Lin et al, 1998).

In fetal rats, the administration of caffeine to mothers inhibited the development of the HPA axis, as indicated from decreased CRH and cortisol levels. (Xu et al., 2012). This fetal HPA axis may downregulation may have resulted from elevated maternal glucocorticoid levels from caffeine intake (Xu et al., 2012). In rats undergoing maternal separation stress, A2A receptors increased (Batalha et al., 2013). Blocking the A2A receptors brought hippocampal glucocorticoid receptors and plasma cortisol down to normal levels. Although these findings suggest that caffeine, an A2A receptor antagonist, decreases cortisol levels, the rats appear to have been undergoing HPA axis downregulation from chronic stress (Batalha et al., 2013). Caffeine also
affects the immune response in a dose-dependent manner, as indicated by decreased antibody production in cultures of mouse spleen cells after 4 days of antigenic stimulation (Laux & Klesius, 1973). The mechanism for this action appears to be from caffeine depressing protein synthesis, by potentially inhibiting enzymes involved in the process (Laux & Klesius, 1973).

There is little evidence that caffeine has any direct effect on DHEA/DHEA-S levels, but the studies conducted were not executed under stressful conditions. Considering that caffeine significantly stimulates cortisol levels under conditions of stress (particularly in vulnerable populations, e.g. family history of hypertension, Lovallo et al., 1989), these factors may be necessary to observe a relationship between caffeine and DHEA-S. In one study, after an intraperitoneal injection of caffeine in rats, increases of pregnenolone, and progesterone in the cerebral cortex and plasma were dose- and time-dependent (Concas et al., 2000). While caffeine increased the plasma concentration of corticosterone, there was a slight, but insignificant increase in brain or plasma concentrations of DHEA (Concas et al., 2000). In a sample of 16 male and female healthy adults with a history of caffeine consumption, 200 mg of caffeine was orally administered twice a day, for 7 days (MacKenzie et al., 2007). There was no difference in serum DHEA levels throughout the study (MacKenzie et al., 2007). In another study overweight, coffee consumers were randomized to consume five 6-ounce cups of cafffeinated coffee, decaffeinated coffee, or water for eight weeks (Wedick et al., 2012). In the cafffeinated group, there was an increase of total testosterone in men, and a decrease of total testosterone in women, but there were no significant changes in DHEA-S levels in either sex (Wedick et al., 2012). The results for all of these studies could have been significant under stressful conditions.

The purpose of the present thesis was to examine the effects of caffeine and stress on serum DHEA-S, in healthy, young individuals under an acute stressor, with a family history of
hypertension. I hypothesized that caffeine activates the HPA axis, which leads to an increase in serum DHEA-S and cortisol release from the adrenal gland (Figure 2). In healthy male and female participants with a family history of hypertension, I hypothesized that there should be a heightened rise in DHEA-S after TSST for the group that received caffeine, compared to the caffeine free group. I also predicted that the male participants would have higher levels of DHEA-S than the female participants. DHEA/DHEA-S may have cardiovascular protective effects, while caffeine has been shown to increase glucocorticoid levels as a sympathetic stimulant. Therefore, DHEA/DHEA-S levels may be protective toward the potentially damaging cardiovascular implications for high caffeine consumers or those with a family history of hypertension. Considering that caffeine is the most widely consumed psychoactive drug worldwide and DHEA/DHEA-S is the most abundant steroid, the relationships between caffeine and DHEA-S under an acute stressor was examined in men and women with a family history of hypertension.
Participants

The effect of caffeine on DHEA-S after an acute stressor was investigated in a population of 52 healthy adults (N=26 women, 26 men) with a confirmed family history of hypertension as part of a larger study reported elsewhere (Bennett et al., 2013). The participants were 18-29 years old (21.4 ± 0.3 years). Flyers around the Penn State campus and local community were used to recruit participants. Potential participants were screened for health history by a trained researcher. Individuals were excluded if they could not provide evidence of at least one parent having hypertension and receiving medication in the past year. Exclusion criteria also included significant health problems, the use of medications that would interfere with BP and blood marker data, body mass index (BMI) over 30, and daily caffeine consumption outside of the range of 100-500 mg. Women were in the late luteal phase of their menstrual cycle, as assessed from their self-reported last period and cycle length. Female participants were excluded if they took hormonal medication within the past year. These criteria were evaluated during the screening laboratory visit at the Penn State General Clinical Research Center (GCRC). The women participated in the study during the late luteal phase of their menstrual cycles, as confirmed by estradiol and progesterone assay. The Pennsylvania State University Institutional Review Board reviewed and approved the study (see Appendix A for letter of approval).

Laboratory Session

The study used a 2 × 2 mixed model experimental design, with sex (M, F) and drug (caffeine, placebo) as the independent variables and stress as the within-subjects variable. All of
the participants followed the same laboratory protocol. Participants ate a low-fat lunch and abstained from caffeine for 4 h before the laboratory session. All sessions were conducted in the GCRC at 1300 h to control for diurnal rhythms of hormones. Participants provided informed consent at the GCRC upon their arrival (Figure 3). Height, weight, and health status assessments were conducted by a nurse practitioner to verify participant eligibility. Participants then completed questionnaires about caffeine intake and mood. The nurse then inserted an indwelling catheter into the participant’s non-dominant arm and placed a standard blood pressure (BP) cuff on the participant’s dominant arm (Dinamap Compact Blood Pressure Monitor, Critikon, Tampa, FL). Blood pressure and heart rate (HR) were collected every 2 min during the study.

**Caffeine Administration and Baseline**

Next, participants were instructed to sit quietly for 30 min. Participants were then given white grapefruit juice (Unsweetened White Grapefruit Juice, Giant® brand, Landover, MD), with anhydrous caffeine (Spectrum Chemical Corporation, Gardena, CA) mixed in the drink for half of the participants (caffeine group). The other half of the sample was not administered any caffeine (place group). The caffeine was prepared and administered in a double-blind manner by a nurse such that neither the nurse nor the experimenter was aware of the drug condition. The amount of caffeine administered for the treatment group was based on body weight (3.3 mg caffeine/kg) (e.g., Hartley et al., 2000; Lovallo et al., 1989, 1991). In order to allow for caffeine absorption, participants rested for 20 min before the baseline blood draw.

**Stressor Administration**

A modified Trier Social Stress Test (TSST) was conducted on all participants. In front of an experimenter who was believed to be a psychologist, participants completed a public speaking task and a mental arithmetic challenge (Kirshbaum, 1993). Participants prepared for a speech
about a personal failure for 10 min and delivered the videotaped speech for 3.5 min. They were
told that the speech would be evaluated by psychologists. Participants then completed a 12 min
serial subtraction task with negative feedback and time pressure from the experimenter.
Participants were told that the subtraction task experimenter was a psychologist who was
assessing performance.

Blood was collected 15 and 45 min after the stressor. Blood was drawn into
anticoagulant-free (serum) tubes. Tubes sat at room temperature for 15 min, and then were
centrifuged at 4 °C at 1500 × g for 15 min and frozen at -80 °C for cortisol and DHEA-S
assessment. Participants were paid and debriefed about the caffeine administration and video-
taping procedure after the final blood draw.

**Serum DHEA-S and Cortisol Assay**

Serum DHEA-S and cortisol levels were measured in the Biomarker Core Lab at the
Pennsylvania State University. Using commercially-available enzyme linked immunosorbent
assay (ELISA) kits, DHEA-S and cortisol were measured (ALPCO, Salem, New Hampshire;
Diagnostic Systems Laboratories, Inc., Webster Texas), respectively. The DHEA-S assay had a
minimum sensitivity of 0.005 and an upper range of 10 µg/mL. The cortisol ELISA kit had a
minimum sensitivity of 0.1 mg/dL and upper range of 60 mg/dL. Average of the duplicate values
were used in analyses.

**Data Analyses**

DHEA-S and cortisol levels were not normally distributed; therefore, values were log
transformed (e.g., Bennett et al., 2013). Separate repeated-measures analyses of variance (RM-
ANOVA) were used to test the effects of sex and caffeine on cortisol and DHEA-S. Separate
RM-ANOVAs were conducted to test any significant group × time interactions. All tests were
two-tailed, with statistical significance considered to be $\alpha = 0.05$. The table presents means (±SEM) of untransformed (raw) data values (Table 1). Caffeine and sex were independent variables, with time as a within subject variable. The dependent variables were DHEA-S and cortisol levels.
Chapter 3 - Results

DHEA-S

Overall, DHEA-S levels were elevated in men in comparison to women (F_{1,47} = 12.51, p < 0.05; Figure 4). Serum DHEA-S showed the expected peak following the TSST in all groups as shown by a significant quadratic effect (F_{1,47} = 5.74, p < 0.05). There was no main effect of caffeine on DHEA-S levels. However, caffeine did interact with sex and time (F_{2,47} = 3.00, p = 0.05). Specifically, women exposed to caffeine displayed lower DHEA-S levels at the baseline time point, which was 20 min after drug administration (Table 1). DHEA-S levels then rose to reach control levels by the recovery period among women exposed to caffeine. Among women in the placebo group, there was a negative linear pattern from baseline to stressor (F_{1,23} = 6.09, p < 0.05). In men, there was no time or time × caffeine effect.

Cortisol

Cortisol levels significantly increased over the session (F_{2,94} = 19.62, p < 0.05; Figure 5). There was also a significant quadratic pattern time effect for cortisol (F_{1,47} = 34.16, p < 0.05). The cortisol rise after the stressor was significantly more pronounced in men (F_{2,94} = 4.20, p < 0.05). There was also a significant linear time × caffeine effect (F_{2,94} = 4.02, p < 0.05). Participants in the caffeine group displayed elevated cortisol levels during the stressor, and higher levels remained in the recovery period. There were no between-subject sex differences in cortisol.
Chapter 4 - Discussion

This study examined the how caffeine affected DHEA-S levels during and after the TSST in healthy, young individuals with a family history of hypertension. The most promising finding was that women who consumed caffeine displayed lower levels of DHEA-S at baseline than did the women who had not consumed caffeine (Figure 4). Considering that the women all had a family history of hypertension, decreased levels of DHEA-S could further aggravate CVD risk. The cortisol response validated the stressful condition of the study (Figure 5). While there were 13 women in the sample that had been administered caffeine, all of the participants were screened for specific criteria, including age between 18-29, minimal caffeine consumption, and BMI below 30. There is a greater likelihood that the randomly assigned groups were normalized, with the high specificity of the group criteria.

Serum DHEA-S levels were elevated in men relative to women, as consistent with the literature (Traish et al., 2011). There was no difference in average serum cortisol levels between men and women, also consistent with the literature (Kudielka & Kirshbaum, 2004). Men had a higher cortisol peak after the TSST, compared to women. The study sessions started at 1300, while DHEA-S levels peak in the afternoon (Zhao et al., 2003; Ferrari et al., 2001). While the DHEA-S levels in the participants may have been in the process of reaching a peak due to the diurnal rhythm, there was a significant quadratic effect from the stressor. Therefore, the TSST still resulted in an increase in average serum DHEA-S levels, while the levels then decreased, after recovery (Table 1). The DHEA-S levels at this final blood draw were back at baseline, indicating that the recovery was complete.
The proposed mechanism for the rise in DHEA-S after the TSST is the increase of ACTH release from the anterior pituitary gland. ACTH stimulates cholesterol uptake in the adrenal gland, which leads to the production of both glucocorticoids and DHEA-S (Vanparys et al., 2012). In this experiment, cortisol and DHEA-S similarly changed over the course of the lab session, but F-statistic indicates a subtler rise and fall for DHEA-S ($F = 5.74$) than for cortisol ($F = 34.16$). There are many potential factors that affected this result. During times of stress, the adrenal gland shifts toward glucocorticoid production, instead of DHEA/DHEA-S (Kalimi et al., 1993). DHEA is also sulfonated in regions other than the adrenal glands; there is expression of the sulfotransferase gene, SULT2A1, in the liver, brain, and steroidogenic organs (Ou et al., 2014; O’Shaughnessy et al., 2013; Kojima & Degawa, 2014; Knapstein et al., 1968). While ACTH stimulates DHEA/DHEA-S production, DHEA needs to be synthesized before the sulfotransferase acts to sulfonate DHEA. The 15 minutes between the stressor and the blood draw associated with the TSST may not have been sufficient to indicate the extent to which DHEA-S production was stimulated. Considering the various locations that DHEA can be sulfonated, perhaps more time was required to complete the sulfonation of the newly produced DHEA molecules.

**Effect of Caffeine on DHEA-S Levels in Women**

While there was no main effect of caffeine on DHEA-S levels, caffeine interacted with sex and time. It has been reported that there is no sex differences in the pattern of DHEA or DHEA-S levels after acute psychosocial stress (Lennartsson et al., 2013), but caffeine has never been added to such a study. There were suggestively lower DHEA-S levels in women who had been administered caffeine at baseline (Figure 4). Although caffeine was hypothesized to activate the production of ACTH and the subsequent production of DHEA-S, caffeine may have
a dampening effect on DHEA-S levels, but only in women. However, the mechanism behind this action is unclear. The adenosine receptors that caffeine antagonizes are primarily located in the brain, with no evidence of receptors on the zona reticularis of the adrenal gland. There is SULT2A1 expression in the brain, which allows for the potential of caffeine to have an inhibitory effect on sulfotransferase activity. This inhibition could lead to the decrease of DHEA-S levels after caffeine consumption. Women already have lower levels of DHEA-S levels than do men; therefore, the potential dampening effect of caffeine on DHEA-S may only be observed in women due to potentially different mediators by which DHEA-S is processed.

**Effects of Caffeine and Stress**

There were contradictory results relating to DHEA-S levels during the stressor between the caffeine and placebo groups. In the caffeine group and male placebo group, DHEA-S levels increased after the stressor, while there was a decline from baseline after the stressor for the female placebo group. Despite the dampening effect that caffeine initially appeared to have, there was still a peak in DHEA-S levels after the TSST. As originally hypothesized, caffeine could have helped stimulate ACTH production, and thus resulted in the subsequent increase in DHEA-S.

Among women in the placebo group, we examined the time course of the DHEA-S response to stress in the absence of caffeine. The adrenal gland may have shifted from DHEA-S production to glucocorticoid production in response to the stressor (Kalimi et al., 1993), which resulted in decreased DHEA-S levels. There is evidence that increased cortisol levels suppress DHEA-S. In post-menopausal women, a glucocorticoid treatment for asthma, beclomethasone dipropionate, suppressed DHEA-S (Smith et al., 1994). Furthermore, school-aged children
administered either glucocorticoid, budesonide or fluticasone, had inhibited DHEA-S production, in a dose-dependent manner (Kannisto et al., 2013).

**Health Effects of Lower DHEA-S Levels**

With both caffeine and stress, there are risk factors for health associated with lower DHEA-S levels. Specifically for cardiovascular health, DHEA/DHEA-S has antiatherogenic effects. DHEA stimulates endothelial cells to release nitric oxide (Bertoni et al., 2013). In blood from healthy donors DHEA-S inhibits thrombin-induced platelet aggregation (Bertoni et al., 2012). In cultured mouse macrophage cells, DHEA reduced the accumulation of cholesteryl ester by 30% compared to controls (Taniguchi et al., 1996). The effect of DHEA was both dose- and time-dependent, with effects observable from 6 h to 48 hr (Taniguchi et al., 1996).

Lower adrenal androgen levels can contribute to long-term bone loss (Smith et al., 1994). In women aged 45-69 years, serum DHEA-S levels were significantly lower in the group with low bone density (Szathmári et al., 1994). Low DHEA-S levels can also adversely impact physical and mental capabilities. In a cohort of nearly one thousand individuals over 65 years, DHEA-S decline was measured over a 9 year period (Sanders et al., 2010). In women, greater DHEA-S decline was associated with a greater decrease in gait speed and cognitive function tests (Sanders et al., 2010). The relationship between cognitive function and DHEA-S was further investigated in a study including 295 women aged 21-77 years (Davis et al., 2008). DHEA-S was independently associated with higher scores on tests that measured executive function, simple concentration, and working memory (Davis et al., 2008).

DHEA/DHEA-S levels also have been associated with depression, but the results are contradictory. In 699 postmenopausal women in a community setting, DHEA-S levels were significantly and negatively correlated with depressed mood, while DHEA levels were not
Depressed women had significantly lower plasma DHEA-S levels than age-matched women who were not depressed (Barrett-Connor et al., 1999). Similarly, in men and women with major depressive disorder who were not on antidepressants, DHEA-S levels, but not DHEA levels, were lower than in healthy participants (Scott et al., 1999). In a study including 2855 men and women aged 70-79 years, DHEA-S levels were also associated with depressive symptoms, after adjustment for covariates (Morsink et al., 2007).

Conversely, in a group of 13 medicated, depressed patients, morning and evening levels of DHEA-S were significantly elevated compared to matched controls (Assies et al., 2004). In 26 un-medicated, severely depressed patients, plasma samples were collected every 30 min for 24 hours (Heuser et al., 1998). There was a parallel increase in mean DHEA, cortisol, and ACTH levels for the depressed patients, compared to controls (Heuser et al., 1998). There was an increased diurnal minimum of DHEA plasma concentrations, but no difference in maximum plasma concentrations or diurnal amplitude (Heuser et al., 1998). While relationship between DHEA and depression appears to be mediated by factors including age, medication, and sex, supplementation in depressed individuals has been shown to be significantly effective (Wolkowitz et al., 1997). In six middle-aged or elderly patients, DHEA administration for 4 weeks increased plasma levels, while depression ratings and aspects of memory performance improved (Wolkowitz et al., 1997).

**Cortisol/DHEA Ratio**

A cortisol/DHEA ratio approach has been reported in other studies to examine the potential health protective effects that DHEA may have to counteract glucocorticoid action from stress (Hecher, Grossman, & Chatterton, 1997). The measure aims to calculate a value for steroid levels in an individual in an effort to demonstrate the body’s ability to mount a physiological
stress response (Whetzel, 2008, Maninger et al., 2009). It is hypothesized that low levels of DHEA-S compared to cortisol would indicate hormone imbalance in the catabolic direction, which would make individuals more susceptible to stress-related disease (Whetzel, 2008). For example, a high cortisol/DHEA-S ratio has been associated with chronic stress, depression, and cognitive disorders, as predicted (Lennartsson, 2013, 2013; Jeckel et al., 2010; Young et al., 2002; Ferrari et al., 2001). An elevated cortisol/DHEA ratio during TSST was significantly and moderately correlated to negative mood during and after the stressor in a sample of healthy men (N=33) (Izawa et al., 2008). In management employees (N=103) of a German airplane manufacturing plant, increasing levels of job demands were associated with decreasing cortisol/DHEA-S ratios, with an interaction with job control (Gadinger et al., 2011). Very high and very low levels of job control were associated with the highest cortisol/DHEA-S ratios, while lower ratios were found among those who reported moderately low or high levels of job control (Gadinger et al., 2011). Among the employees without management responsibilities (N=493), having high job demands and low control did not predict the cortisol/DHEA-S ratio (Gadinger et al., 2011).

Although it is initially appealing to use a cortisol/DHEA ratio in an effort to examine how these biomarkers might work together to create a protective or disadvantageous response to stress, the usefulness of the ratio is also questionable for several factors. These determinants include time of day, inconsistency between DHEA and DHEA-S, interconnectedness of the two biomarkers, and sex differences. The timing of cortisol and DHEA collection may affect significance of ratio (Goodyer, Herbert, & Altham, 1998). For 8-16 year olds (n=68), higher cortisol/DHEA ratios (above sixtieth percentile) at 20:00 and 24:00 predicted major depression and disappointing life events. A high ratio at 8:00, 12:00, and 16:00 only predicted disappointing
life events (Goodyer, Herbert, & Altham, 1998). Either DHEA or DHEA-S is not consistently used as measure in the literature when constructing the ratio. The ratio is valued for consideration of lowered DHEA compared to cortisol, but higher levels of cortisol with normal levels of DHEA can also increase the ratio. DHEA-S has a large vascular pool, with a half-life of 10-20 hours, while cortisol has a half-life of 2 hours, which makes cortisol prone to more fluctuation compared to DHEA-S (Kannisto et al., 2013).

Furthermore, DHEA/DHEA-S decreases after puberty, while there is no observed change in concentration for ACTH and cortisol during aging (Nawata et al., 2002). DHEA and cortisol are synthesized by separate enzyme activities of cytochrome P450c17, 17,20-lyase and 17-α-hydroxylase, respectively (Nawata et al., 2002). In bovine adrenal glands, the 17,20-lyase activity was significantly lower in the aged sample (10-12 years old, n=4) compared to the younger sample (1 year old, n=6), meaning that the cortisol production remains consistent over time, while there was a decreased DHEA synthesis potential (Nawata et al., 2002). There are many details regarding the biomechanics of the enzymes involved in cortisol and DHEA production that need to be considered before determining that the cortisol/DHEA ratio correctly represents the catabolic to anabolic hormonal balance. Lastly, on average, men have higher levels of DHEA/DHEA-S than do women, which further skews the validity of the ratio, between the sexes (Traish et al., 2011, & Whetzel & Klein, 2010).

With the present study, a more sophisticated statistical model is necessary to consider the significance of the cortisol/DHEA-S ratio. With the different circadian rhythms of the two biomarkers, the value of the ratio is uncertain with the given timing of the blood draws. Also, as the most highly circulating hormone in the body, DHEA-S has very high baseline concentrations in comparison to cortisol. The number of DHEA-S molecules to indicate a significant increase in
DHEA-S levels from the baseline to stressor time points would need to be greater than the number of molecules that would be required for cortisol to show a significant increase.

**Limitations**

While the conclusions about caffeine and stress inhibiting DHEA-S levels may be subject to further investigation, there are several limitations to the study. All of the participants consumed no more than 100-500 mg of caffeine a day. With low caffeine consumption, the participants could have been more susceptible to the effects of the drug as opposed to high caffeine consumers. There is also potential for type I error with the results. The null hypothesis could have been rejected incorrectly, especially considering that one of the major findings had borderline significance, with a p-value of 0.05. The results may not be clinically significant during the circadian incline of DHEA-S, as DHEA-S levels tended to return to near baseline during recovery. Also, due to the high background levels of DHEA-S, a larger sample size compared to cortisol was necessary to address the signal to noise ratio. With a possible type II error, there could have been significant effects of caffeine on DHEA-S that were not detected. There may simply have not been enough time for the sulfonation process from DHEA to DHEA-S to occur. After ACTH stimulation of adrenal glands in 14 young men, plasma DHEA levels rose acutely one hour after ACTH treatment, but DHEA-S levels only rose twelve hours after ACTH administration (Griffing et al., 1985). The sulfonation process appears to be less rapid than previously anticipated, which may have contributed to the low F-statistics of the DHEA-S patterns over time.

The DHEA-S metabolism could have been affected by the usage of grapefruit juice as a medium to administer dissolved caffeine. When grapefruit is ingested, serum levels of estrogen have been observed to rise in women (Monroe, Stanczyk, Besinque, & Pike, 2013). Whole
grapefruit juice significantly increased endogenous estrone-3-sulfate and DHEA-S, while fresh and bottle juiced significantly lowered estradiol (Monroe et al., 2013). All participants were given grapefruit juice, so results are controlled for possible grapefruit juice effect. However, the manner in which grapefruit juice affected individual women could have altered the results of the study.

**Future Directions**

In a follow-up study, there are several conditions that could be altered to improve the validity of the results. Regarding grapefruit juice, the conditions could be repeated by using another form of caffeine ingestion, including another beverage or intravenous administration. The grapefruit effect may in fact be another point of investigation; there could be a matched subjects design in which one group is administered caffeine through grapefruit juice, and the other group receives another method of caffeine administration, both with appropriate placebos. The effect of grapefruit juice on DHEA-S under stress has not been studied, nor has the respective interaction with caffeine.

In terms of the general design of the study, there would need to be a pre-caffeine baseline collected in order to verify the dampening effect of caffeine on DHEA-S levels in women. While the women were randomly selected for placement in the caffeine or non-caffeine groups, there could have been between-group differences that accounted for the lower DHEA-S levels in the caffeine group. Having a larger sample size could also address this methodological issue. The current sample size should be recognized as the minimum to recognize factors that interact with caffeine, sex and DHEA-S.

Investigating a dose-dependent response of caffeine on DHEA-S levels is another point of potential analysis. Requiring even more groups, there could be at least two increasing caffeine
conditions, along with the placebo, to study whether there is a greater dampening or even an excitatory effect of caffeine on DHEA-S between groups. An excitatory effect should be studied considering evidence that DHEA-S may act as an σ1 receptor agonist, thereby potentiating NMDA receptor function, as previously discussed (Bergeron et al., 1996; Maninger et al., 2008). Adrenal gland production of DHEA is required before conversion to DHEA-S occurs, therefore DHEA levels at baseline, caffeine baseline, stressor, and recovery should be measured. Regular caffeine consumers could also be included to see if caffeine effects on the HPA axis vary, compared to low caffeine consumes. The conversion from DHEA to DHEA-S may also be unidirectional (Hammer et al., 2005), which could be studied further based on assaying for both DHEA and DHEA-S.

In a different study design, the circadian rhythms of DHEA-S in chronically stressed individuals in daily life should be investigated in order to understand the long-term, clinically significant effects of stress. Studying both high and low caffeine consumers could indicate the longitudinal effects of caffeine on DHEA/DHEA-S levels, under stress. The relationship between caffeine and DHEA/DHEA-S should also be studied in specific clinical populations, including individuals who have diagnosed hypertension. The effects of acute stressors and caffeine on DHEA/DHEA-S levels may not be clinically significant due to the fast return to baseline; therefore, a longitudinal study could better explain the potential for low-DHEA/DHEA-S levels, sustained over repeated exposure, to have negative health effects especially in women.

Conclusion

Regardless of the study limitations, this is the first study to examine the influence of caffeine administration on DHEA-S levels in men and women with a family history of hypertension, under stress. There are preliminary findings that caffeine suppresses DHEA-S
levels in women with this medical history, at least 20 min after drug administration. Considering caffeine’s sex-based interaction with DHEA-S and the health effects of both molecules, this area of research deserves more attention. There is ample opportunity for further research regarding DHEA/DHEA-S, caffeine, and stress.
Table 1. Mean (±SEM) raw values for serum hormone levels across laboratory session by sex and caffeine treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/kg (N=13)</td>
<td>3.3 mg/kg (N=13)</td>
<td>0 mg/kg (N=13)</td>
</tr>
<tr>
<td><strong>DHEA-S (µg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.25±0.25</td>
<td>2.80±0.35</td>
<td>2.49±0.72</td>
</tr>
<tr>
<td>Stress</td>
<td>3.40±0.30</td>
<td>2.91±0.37</td>
<td>2.29±0.59</td>
</tr>
<tr>
<td>Recovery</td>
<td>3.18±0.24</td>
<td>2.67±0.33</td>
<td>1.98±0.50</td>
</tr>
<tr>
<td><strong>Cortisol (µg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>10.5±1.2</td>
<td>8.27±0.66</td>
<td>11.0±1.4</td>
</tr>
<tr>
<td>Stress</td>
<td>15.8±2.6</td>
<td>17.0±1.8</td>
<td>11.5±1.0</td>
</tr>
<tr>
<td>Recovery</td>
<td>10.6±1.0</td>
<td>12.4±0.92</td>
<td>8.79±0.79</td>
</tr>
</tbody>
</table>
Figures
Figure 1. Diagram of DHEA-S synthesis and metabolism, redrawn from (Sawalha & Kovats, 2008; Traish et al., 2011)
Figure 2. Hypothesized mechanism of DHEA/DHEA-S rise after caffeine administration
Figure 3. Experimental Timeline, used with permission from (Bayly, 2014)
Figure 4. DHEA-S (µg/mL) levels for sex and caffeine groups over time. Means ± SEM.
Figure 5. Cortisol levels (µg/dL) for sex and caffeine groups over time. Means ± SEM.
REFERENCES


psychosocial stress and its correlations with biological and psychological changes.


Appendix A: Institutional Review Board Approval and Consent Forms
Date: July 13, 2004

From: Mary B. Becker, IRB Administrator

To: Isabella M. Rodrigues

Subject: Results of Review of Proposal - Full Review (IRB #190000)
Approval Expiration Date: June 16, 2005
"Effects of Caffeine on Cardiovascular Risk Markers"

The Biomedical Institutional Review Board has reviewed and approved your proposal for use of human participants in your research. By accepting this decision, you agree to obtain prior approval from the IRB for any changes to your study. Unanticipated participant events that are encountered during the conduct of this research must be reported in a timely fashion.

Enclosed is/are the dated, IRB-approved informed consent(s) to be used when recruiting participants for this research. Participants must receive a copy of the approved informed consent form to keep for their records.

If signed consent is obtained, the principal investigator is expected to maintain the original signed consent forms along with the IRB research records for this research at least three (3) years after termination of IRB approval. For projects that involve protected health information (PHI) and are regulated by HIPAA, records are to be maintained for six (6) years. The principal investigator must determine and adhere to additional requirements established by the FDA and any outside sponsors.

If your study will extend beyond the above noted approval expiration date, the principal investigator must submit a completed Continuing Progress Report to the Office for Research Protections (ORP) to request renewed approval for this research.

On behalf of the committee and the University, thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of human participants.

MBB/slk
Enclosure
cc: Laura C. Klein

Please Note: The ORP encourages you to subscribe to the ORP listserv for protocol and research-related information. Send a blank email to: l-ORP-Research-1-subscribe-request@lists.psu.edu.
INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY

Title of Project: The Effects of Caffeine on Cardiovascular Risk Markers

Principal Investigator: Isabella M. Rodrigues
315 East Health and Human Development
Penn State University
University Park, PA 16802
Telephone: (814) 863-5845
Email: imr104@psu.edu

Other Investigator: Laura Cousino Klein, Ph.D.
315 East Health and Human Development
Penn State University
University Park, PA 16802
Telephone: (814) 865-8813
Email: lklein@psu.edu

This is to certify that you, ____________________________ , have been given the following information regarding your participation as a volunteer in a program of investigation under the supervision of Isabella M. Rodrigues and Laura Cousino Klein in the Department of Biobehavioral Health.

Purpose of the study:
The purpose of this study is to see how stress and caffeine affect blood markers of heart disease. Changes in hormones and blood markers of heart disease will be measured in blood samples collected during the study. We also are examining these changes in men and women to see how they compare in their responses to challenge and caffeine. These changes will provide important information about men and women’s vulnerability to stress and caffeine.

You understand that you are being asked to participate in this study because you are a healthy adult (at least 18 years old) who does not have any of the following health conditions or problems:

1. high blood pressure;
2. a history of angina (chest pain due to heart problems) or arrhythmia (fast, slow, or irregular heart beats that require medication);
3. diabetes (excessive amounts of sugar in your blood);
4. any neurological disorder (e.g., stroke);

6-28-04
Page 1 of 6

Initials __________
Date

Dear NAME

Your son/daughter, ________________, is participating in a research project in the department of Biobehavioral Health at Penn State University, under the supervision of Dr. Laura Klein and approved by the Penn State University Office of Research Protections. This project is examining the relationship among caffeine, stress, blood pressure, and risk for development of heart disease. Your son/daughter has sent you this letter because one important aspect of our research is to verify the cardiovascular health of our research volunteer’s parents. Specifically, we are interested in knowing about whether or not you have ever been diagnosed or treated for hypertension or high blood pressure. If this is okay with you, then please take a couple of minutes to complete the enclosed form and return it in the postage-paid envelope.

Your completion of this form is voluntary and you may withdraw at any time or refuse to answer any questions; there are no risks involved by participating in this survey. Your assistance with this information will be useful in helping us understand factors related to hypertension and cardiovascular disease. Completion of this form implies your consent to answer these questions, and to keep a copy of this letter for your records.

Please know that the information you provide is completely confidential. This information is identified by a code number and your responses to these questions will remain anonymous. Thank you for helping us with our research.

If you have questions about your rights as a participant, please contact the Office for Research Protections in reference to IRB #19000 at (814) 865-1775.

Sincerely,

Laura C. Klein, Ph. D.
Assistant Professor and Investigator
The Biobehavioral Health Studies Laboratory
Phone: (814) 865-8813
Email: lcklein@psu.edu

Isabella Rodrigues, B.A., B.S.
Graduate Researcher and Investigator
The Biobehavioral Health Studies Laboratory
Phone: (814) 863-5845
Email: irm104@psu.edu
Appendix B: Serum DHEA-S ELISA Protocol
Dehydroepiandrosterone Sulfate ELISA
(DHEA-S)
For the quantitative determination of Dehydroepiandrosterone sulfate (DHEAS) by enzyme immunoassay in human serum

For “In Vitro Diagnostic” use within the United States of America.
This product is for “Research Use Only” outside of the United States of America.

Catalog Number: 11-DHEHU-E01
Size: 96 Wells
INTENDED USE
For the direct quantitative determination of DHEAS by enzyme immunoassay in human serum.
For in vitro diagnostic use only.

PRINCIPLE OF THE TEST
The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, controls and samples) and an enzyme-labeled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stop solution. The absorbance is measured on a microtitre plate reader. The intensity of the color formed is inversely proportional to the concentration of DHEAS in the sample. A set of standards is used to plot a standard curve from which the amount of DHEAS in samples and controls can be directly read.

APPLICATIONS
Dehydroepiandrosterone sulfate (DHEAS) is produced by the adrenals and gonads. As a result, the determination of the level of DHEAS in serum is important in the evaluation of the functional state of these glands. DHEAS is a precursor of testosterone and estrone. Besides the adrenals in females, the ovaries have been shown to be an important source of DHEAS. It has been reported that there is a fluctuation day by day of DHEAS in women during the ovulatory cycle.
The principle production of testosterone in females is from conversion of other related androgens, especially DHEAS. An abnormal testosterone level in women should be accompanied by the estimation of serum DHEAS. The use of serum testosterone determination in conjunction with the ELISA of DHEAS can be used to determine if the source of excess androgen production is ovarian or adrenal.

PROCEDURAL CAUTIONS AND WARNINGS
1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials or serum pools should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
10. The substrate solution (TMB) is sensitive to light and should remain colorless if properly stored.
11. When dispensing the substrate and stop solution, do not use pipettes in which these liquids will come into contact with any metal parts.
12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS
1. All the reagents within the kit are calibrated for the determination of DHEAS in human serum. The kit is not calibrated for the determination of DHEAS in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Only calibrator A may be used to dilute any high serum samples. The use of any other reagent may lead to false results.
5. The results obtained with this kit should never be used as the sole basis for a clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should include all aspects of a patient’s background including the frequency of exposure to animals/products if false results are suspected.

**SAFETY CAUTIONS AND WARNINGS**

**POTENTIAL BIOHAZARDOUS MATERIAL**
Human serum that may be used in the preparation of the standards and controls has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. However, if the test kit is used, complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

**CHEMICAL HAZARDS**
Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

**SPECIMEN COLLECTION AND STORAGE**
Approximately 0.1 ml of serum is required per duplicate determination. Collect 4-5 ml of blood into an appropriately labeled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

**SPECIMEN PRETREATMENT**
This assay is a direct system; no specimen pretreatment is necessary.

**REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED**
1. Precision pipettes to dispense 25, 50, 200, 150 and 300 µl
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microwell plate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater* (see assay procedure step 10).

**REAGENTS PROVIDED**
1. Rabbit Anti-DHEAS Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.
   Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant.
   Storage: Refrigerate at 2-8°C
   Stability: 12 months or as indicated on label.

2. DHEAS-Horseradish Peroxidase (HRP) Conjugate Concentrate - Requires Preparation.
   Contents: DHEAS-HRP conjugate in a protein-based buffer with a non-mercury preservative.
   Volume: 0.8 ml/ml
   Storage: Refrigerate at 2-8°C
   Stability: 12 months or as indicated on label.
   Preparation: Dilute 1:50 in assay buffer before use (e.g., 40 µl of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 0.5 ml of HRP in 25 ml of assay buffer. Discard any that is left over.
3. **DHEAS Calibrators - Ready To Use.**

Contents: Seven vials containing DHEAS in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of DHEAS. 

*Listed below are approximate concentrations, please refer to vial labels for exact concentrations.*

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Concentration</th>
<th>Volume/Vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0 µg/ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>B</td>
<td>0.005 µg/ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>C</td>
<td>0.02 µg/ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>D</td>
<td>0.1 µg/ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>E</td>
<td>0.5 µg/ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>F</td>
<td>2.5 µg/ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>G</td>
<td>10 µg/ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Storage: Refrigerate at 2-8°C

Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. **Controls - Ready To Use.**

Contents: Two vials containing DHEAS in a serum-based buffer with a non-mercury preservative. Prepared by spiking serum with defined quantities of DHEAS. Refer to vial labels for the acceptable range.

Volume: 0.5 ml/vial

Storage: Refrigerate at 2-8 °C

Stability: 12 months in unopened vial or as indicated on label. Once opened, the control serum should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. **Wash Buffer Concentrate - Requires Preparation.**

Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.

Volume: 50 ml/bottle

Storage: Refrigerate at 2-8 °C

Stability: 12 months or as indicated on label.

Preparation: Dilute 1:10 in distilled water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of distilled water.

6. **Assay Buffer - Ready To Use.**

Contents: One vial containing a protein-based buffer with a non-mercury preservative.

Volume: 30 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

7. **TMB Substrate - Ready To Use.**

Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.

Volume: 16 ml/bottle

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.
8. Stop Solution - Ready To Use.
Contents: One vial containing 1M sulfuric acid.
Volume: 6 ml/vial
Storage: Refrigerate at 2-8 °C
Stability: 12 months or as indicated on label.

ASSAY PROCEDURE
Specimen Pretreatment:
None.

All reagents must reach room temperature before use. Calibrators, controls and samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the DHEAS-HRP conjugate and wash buffer.
2. Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 25 μl of each calibrator, control and specimen sample into correspondingly labeled wells in duplicate.
4. Pipette 200 μl of the conjugate working solution into each well (The use of a multichannel pipette is recommended).
5. Incubate on a plate shaker (approximately 200 rpm) for 45 minutes at room temperature.
6. Wash the wells 3 times with 300 μl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).
7. Pipette 150 μl of TMB substrate into each well at timed intervals.
8. Incubate on a plate shaker for 15-20 minutes at room temperature (or until calibrator A attains dark blue color for desired OD).
9. Pipette 50 μl of stop solution into each well at the same timed intervals as in step 7.
10. Read the plate on a microwell plate reader at 450nm within 20 minutes after addition of the stop solution.
   *If the OD exceeds the upper limit of detection or if a 450nm filter is unavailable, a 405 or 415nm filter may be substituted. The optical densities will be lower; however, this will not affect the results of samples/controls.

CALCULATIONS
1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoeassay software is being used, a 4-parameter or 5-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 10 μg/ml then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.
TYPICAL TABULATED DATA

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>OD 1</th>
<th>OD 2</th>
<th>Mean OD</th>
<th>Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.106</td>
<td>2.000</td>
<td>2.083</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>1.910</td>
<td>1.863</td>
<td>1.887</td>
<td>0.005</td>
</tr>
<tr>
<td>C</td>
<td>1.638</td>
<td>1.769</td>
<td>1.703</td>
<td>0.02</td>
</tr>
<tr>
<td>D</td>
<td>1.398</td>
<td>1.382</td>
<td>1.390</td>
<td>0.1</td>
</tr>
<tr>
<td>E</td>
<td>0.966</td>
<td>0.838</td>
<td>0.952</td>
<td>0.5</td>
</tr>
<tr>
<td>F</td>
<td>0.496</td>
<td>0.479</td>
<td>0.488</td>
<td>2.5</td>
</tr>
<tr>
<td>G</td>
<td>0.250</td>
<td>0.252</td>
<td>0.251</td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.690</td>
<td>0.688</td>
<td>0.689</td>
<td>1.20</td>
</tr>
</tbody>
</table>

TYPICAL CALIBRATOR CURVE
Sample curve only. Do not use to calculate results.

PERFORMANCE CHARACTERISTICS

SENSITIVITY
The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the DHEAS ELISA kit is 0.005 µg/ml.

SPECIFICITY (CROSS-REACTIVITY)
The following compounds were tested for cross-reactivity with the DHEAS ELISA kit with DHEAS cross-reacting at 100%.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEAS</td>
<td>100</td>
</tr>
<tr>
<td>Andosterone</td>
<td>16.0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>1.7</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.9</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.6</td>
</tr>
<tr>
<td>DHT</td>
<td>0.5</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The following steroids were tested but cross-reacted at less than 0.001%: 17β-Estradiol, Estrone, Estrone-Sulfate and Pregnanolone.
**INTRA-ASSAY PRECISION**
Three samples were assayed ten times each on the same calibrator curve. The results (in µg/ml) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.24</td>
<td>0.02</td>
<td>7.5</td>
</tr>
<tr>
<td>2</td>
<td>2.02</td>
<td>0.18</td>
<td>9.1</td>
</tr>
<tr>
<td>3</td>
<td>9.54</td>
<td>0.11</td>
<td>11.5</td>
</tr>
</tbody>
</table>

**INTER-ASSAY PRECISION**
Three samples were assayed ten times over a period of four weeks. The results (in µg/ml) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.13</td>
<td>0.02</td>
<td>15.3</td>
</tr>
<tr>
<td>2</td>
<td>1.11</td>
<td>0.09</td>
<td>8.1</td>
</tr>
<tr>
<td>3</td>
<td>6.33</td>
<td>0.27</td>
<td>4.2</td>
</tr>
</tbody>
</table>

**RECOVERY**
Spiked samples were prepared by adding defined amounts of DHEAS to three serum samples. The results (in µg/ml) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs.Result</th>
<th>Exp.Result</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unsiked</td>
<td>0.67</td>
<td>0.77</td>
<td>109.1</td>
</tr>
<tr>
<td>+0.1</td>
<td>0.84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+1.0</td>
<td>1.97</td>
<td>1.67</td>
<td>117.9</td>
</tr>
<tr>
<td>+5.0</td>
<td>5.80</td>
<td>5.67</td>
<td>102.3</td>
</tr>
<tr>
<td>2 Unsiked</td>
<td>1.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+0.1</td>
<td>1.40</td>
<td>1.16</td>
<td>120.7</td>
</tr>
<tr>
<td>+1.0</td>
<td>1.82</td>
<td>2.06</td>
<td>86.3</td>
</tr>
<tr>
<td>+5.0</td>
<td>5.25</td>
<td>6.06</td>
<td>86.8</td>
</tr>
<tr>
<td>3 Unsiked</td>
<td>1.73</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+0.1</td>
<td>1.90</td>
<td>1.83</td>
<td>103.8</td>
</tr>
<tr>
<td>+1.0</td>
<td>2.21</td>
<td>2.73</td>
<td>86.0</td>
</tr>
<tr>
<td>+5.0</td>
<td>5.79</td>
<td>6.73</td>
<td>86.0</td>
</tr>
</tbody>
</table>
LINEARITY
Three serum samples were diluted with calibrator A. The results (in μg/ml) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs. Result</th>
<th>Exp. Result</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>1.48</td>
<td>1.42</td>
<td>104.2</td>
</tr>
<tr>
<td>1.4</td>
<td>0.61</td>
<td>0.71</td>
<td>114.3</td>
</tr>
<tr>
<td>1.8</td>
<td>0.46</td>
<td>0.36</td>
<td>127.8</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6.32</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td>3.16</td>
<td>100.3</td>
</tr>
<tr>
<td>1.4</td>
<td>1.63</td>
<td>1.56</td>
<td>103.2</td>
</tr>
<tr>
<td>1.8</td>
<td>0.76</td>
<td>0.79</td>
<td>98.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>7.12</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td>3.66</td>
<td>86.6</td>
</tr>
<tr>
<td>1.4</td>
<td>1.78</td>
<td>1.78</td>
<td>100</td>
</tr>
<tr>
<td>1.8</td>
<td>0.80</td>
<td>0.59</td>
<td>89.9</td>
</tr>
</tbody>
</table>

EXPECTED NORMAL VALUES
As for all assays each laboratory should collect data and establish their own range of expected normal values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Range (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>0.39-4.63</td>
</tr>
<tr>
<td>Females</td>
<td>0.46-2.75</td>
</tr>
<tr>
<td>Postmenopausal Females</td>
<td>0.48-2.08</td>
</tr>
</tbody>
</table>

REFERENCES
Appendix C: Serum Cortisol ELISA Protocol
Cortisol ELISA
For the quantitative determination of cortisol in human serum.

For Research Use Only. Not for Use in Diagnostic Procedures.

Catalog Number: 11-CORHU-E01
Size: 96 wells
INTENDED USE
For the direct quantitative determination of Cortisol by enzyme immunoassay in human serum. For Research Use Only. Not For Use in Diagnostic Procedures.

PRINCIPLE OF THE TEST
The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, controls and samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microtiter plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stop solution. The absorbance is measured on a microtiter plate reader. The intensity of the color formed is inversely proportional to the concentration of cortisol in the sample. A set of standards is used to plot a standard curve from which the amount of cortisol in samples and controls can be directly read.

INTRODUCTION
Cortisol is the most abundant circulating steroid and the major glucocorticoid secreted by the adrenal cortex. Cortisol is physiologically effective in blood pressure maintenance and anti-inflammatory activity. It is also involved in calcium absorption, gluconeogenesis as well as the secretion of gastric acid and pepin.
Measurement of blood cortisol levels can be used as an indicator of adrenal function and the differential diagnosis of Addison’s and Cushing’s diseases as well as adrenal hyperplasia and carcinoma.
Most circulating cortisol is bound to cortisol binding globulin or transcortin. Therefore, the free cortisol concentration excreted in the urine is very small, and the 24-hour collection of urine is a must in order to obtain an accurate measurement of urinary cortisol. Cortisol in blood shows a diurnal rhythm with the highest levels in the morning and the lowest levels at night.

PROCEDURAL CAUTIONS AND WARNINGS
1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials or serum pools should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
10. The substrate solution (TMB) is sensitive to light and should remain colorless if properly stored. Instability or contamination may be indicated by the development of a blue color; in which case it should not be used.
11. When dispensing the substrate and stop solution, do not use pipettes in which these liquids will come into contact with any metal parts.
12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS
1. All the reagents within the kit are calibrated for the direct determination of cortisol in human serum. The kit is not calibrated for the determination of cortisol in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Only calibrator A may be used to dilute any high serum samples. The use of any other reagent may lead to false results.

SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL
Human serum that may be used in the preparation of the standards and controls has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. However no test method can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

CHEMICAL HAZARDS
Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE
Approximately 0.1 ml of serum is required per duplicate determination. Collect 4-5 ml of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT
The assay is a direct system; no specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED
1. Precision pipettes to dispense 20, 50, 100, 150 and 300 μl
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater* (see assay procedure step 10).
REAGENTS PROVIDED

1. Rabbit Anti-Cortisol Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.
   Contents: One 96 well (12x8) polyclonal antibody coated microwell plate in a resealable pouch with desiccant.
   Storage: Refrigerate at 2-6°C
   Stability: 12 months or as indicated on label.

   Contents: Cortisol-HRP conjugate in a protein-based buffer with a non-mercury preservative.
   Volume: 300 µl/vial
   Storage: Refrigerate at 2-6°C
   Stability: 12 months or as indicated on label.
   Preparation: Dilute 1:100 in assay buffer before use (eg. 20 µl of HRP in 2 ml of assay buffer).
   If the whole plate is to be used, dilute 120 µl of HRP in 12 ml of assay buffer. Discard any that is left over.

3. Cortisol Calibrators - Ready To Use.
   Contents: Seven vials containing cortisol in a human serum-based buffer with a non-mercury preservative. Prepared by spiking serum with a defined quantity of cortisol.
   *Listed are approximate concentrations, please refer to vial labels for exact concentrations.
   Storage: Refrigerate at 2-8°C
   Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Concentration</th>
<th>Volume/Vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator A</td>
<td>0 µg/dl</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Calibrator B</td>
<td>0.5 µg/dl</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Calibrator C</td>
<td>2 µg/dl</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Calibrator D</td>
<td>5 µg/dl</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Calibrator E</td>
<td>10 µg/dl</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Calibrator F</td>
<td>30 µg/dl</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Calibrator G</td>
<td>60 µg/dl</td>
<td>0.3 ml</td>
</tr>
</tbody>
</table>

4. Controls - Ready To Use.
   Contents: Two vials containing cortisol in a human serum-based buffer with a non-mercury preservative. Preparred by spiking serum with defined quantities of cortisol. Refer to vial labels for the acceptable range.
   Volume: 0.3 ml/vial
   Storage: Refrigerate at 2-6 °C
   Stability: 12 months in unopened vial or as indicated on label. Once opened, the controls serum should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Wash Buffer Concentrate - Requires Preparation.
   Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 ml/bottle
Storage: Refrigerate at 2-8 °C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:10 in distilled water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of distilled water.

6. **Assay Buffer - Ready To Use.**
Contents: One vial containing a protein-based buffer with a non-mercury preservative.
Volume: 15 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

7. **TMB Substrate - Ready To Use.**
Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume: 16 ml/bottle
Storage: Refrigerate at 2-8 °C
Stability: 12 months or as indicated on label.

8. **Stop Solution - Ready To Use.**
Contents: One vial containing 1M sulfuric acid.
Volume: 6 ml/vial
Storage: Refrigerate at 2-8 °C
Stability: 12 months or as indicated on label.

**ASSAY PROCEDURE**

**Specimen Pretreatment:** None.

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the cortisol-HRP conjugate and wash buffer.

2. Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.

3. Pipette 20 µl of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.

4. Pipette 100 µl of the conjugate working solution into each well (The use of a multichannel pipette is recommended).

5. Incubate on a plate shaker (approximately 200 rpm) for 45 minutes at room temperature.

6. Wash the wells 3 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).

7. Pipette 150 µl of TMB substrate into each well at timed intervals.
8. Incubate on a plate shaker for 15-20 minutes at room temperature (or until calibrator A attains dark blue color for desired OD).

9. Pipette 50 µl of stop solution into each well at the same timed intervals as in step 7.

10. Read the plate on a microplate reader at 450nm within 20 minutes after addition of the stop solution.

*If the OD exceeds the upper limit of detection or if a 450nm filter is unavailable, a 405 or 415nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of the samples or control.

**CALCULATIONS**

1. Calculate the mean optical density of each calibrator duplicate.

2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.

3. Calculate the mean optical density of each unknown duplicate.

4. Read the values of the unknowns directly off the calibrator curve.

5. If a sample reads more than 80 µg/dl then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

**TYPICAL TABULATED DATA**

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>OD 1</th>
<th>OD 2</th>
<th>Mean OD</th>
<th>Value (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.263</td>
<td>2.138</td>
<td>2.223</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>2.071</td>
<td>2.001</td>
<td>2.036</td>
<td>0.5</td>
</tr>
<tr>
<td>C</td>
<td>1.717</td>
<td>1.719</td>
<td>1.718</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>1.382</td>
<td>1.402</td>
<td>1.395</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>0.985</td>
<td>0.933</td>
<td>0.959</td>
<td>10</td>
</tr>
<tr>
<td>F</td>
<td>0.385</td>
<td>0.373</td>
<td>0.379</td>
<td>30</td>
</tr>
<tr>
<td>G</td>
<td>0.174</td>
<td>0.175</td>
<td>0.175</td>
<td>60</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.697</td>
<td>0.722</td>
<td>0.710</td>
<td>16.3</td>
</tr>
</tbody>
</table>

**TYPICAL CALIBRATOR CURVE**

Sample curve only. Do not use to calculate results.
PERFORMANCE CHARACTERISTICS

SENSITIVITY
The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the Direct Cortisol ELISA kit is 0.4 µg/dl.

SPECIFICITY (CROSS-REACTIVITY)
The following compounds were tested for cross-reactivity with the Cortisol ELISA kit with cortisol cross-reacting at 100%.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>%Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>100</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>13.6</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>7.6</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>7.2</td>
</tr>
<tr>
<td>Progesterone</td>
<td>7.2</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.2</td>
</tr>
<tr>
<td>Deoxycortisol</td>
<td>5.6</td>
</tr>
<tr>
<td>Prednisone</td>
<td>5.6</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.6</td>
</tr>
</tbody>
</table>

No cross reaction was detected with DHEAS and Tetrahydrocortisone.
Please note that there is an observed cross-reactivity of 13.6% with prednisolone. Since prednisone is converted to prednisolone in vivo, caution must be exercised when assaying the cortisol levels of individuals undergoing either therapy.

INTRA-ASSAY PRECISION
Three samples were assayed ten times each on the same calibrator curve. The results (in µg/dl) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.44</td>
<td>0.14</td>
<td>9.4</td>
</tr>
<tr>
<td>2</td>
<td>14.06</td>
<td>0.41</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>37.55</td>
<td>1.87</td>
<td>5.0</td>
</tr>
</tbody>
</table>

INTER-ASSAY PRECISION
Three samples were assayed ten times over a period of four weeks. The results (in µg/dl) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.60</td>
<td>0.13</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>15.01</td>
<td>0.74</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>38.18</td>
<td>1.43</td>
<td>3.8</td>
</tr>
</tbody>
</table>

RECOVERY
Spiked samples were prepared by adding defined amounts of cortisol to three serum samples (1:1). The results (in µg/dl) are tabulated below.
LINEARITY
Three serum samples were diluted with calibrator A. The results (in μg/dl) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs.Result</th>
<th>Exp.Result</th>
<th>Recovery%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.94</td>
<td>5.47</td>
<td>112.1</td>
</tr>
<tr>
<td>1:2</td>
<td>6.13</td>
<td>2.74</td>
<td>116.4</td>
</tr>
<tr>
<td>1:8</td>
<td>1.55</td>
<td>1.37</td>
<td>113.1</td>
</tr>
<tr>
<td>2</td>
<td>18.92</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>9.64</td>
<td>9.46</td>
<td>101.9</td>
</tr>
<tr>
<td>1:4</td>
<td>4.61</td>
<td>4.73</td>
<td>97.5</td>
</tr>
<tr>
<td>1:8</td>
<td>1.98</td>
<td>2.37</td>
<td>83.7</td>
</tr>
<tr>
<td>3</td>
<td>42.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>20.44</td>
<td>21.00</td>
<td>97.8</td>
</tr>
<tr>
<td>1:4</td>
<td>9.57</td>
<td>10.50</td>
<td>94.0</td>
</tr>
<tr>
<td>1:8</td>
<td>4.76</td>
<td>5.25</td>
<td>90.7</td>
</tr>
</tbody>
</table>

EXPECTED NORMAL VALUES

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (μg/dl)</th>
<th>Range (μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males and</td>
<td>15.59</td>
<td>3.95-27.23</td>
</tr>
<tr>
<td>Fathers – AM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males and</td>
<td>5.93</td>
<td>1.45-10.41</td>
</tr>
<tr>
<td>Mothers – PM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ACADEMIC VITA

Sharmila Sandirasegarane
226 Highland Ave. Apt. 405, State College, PA 16801 | sharmila383@gmail.com | 717-829-4644

Education
THE PENNSYLVANIA STATE UNIVERSITY UNIVERSITY PARK, PA
Schreyer Honors College, College of Health and Human Development
Bachelor of Science in Biobehavioral Health, May 2015
Minors in Global Health and Spanish

Research
RESEARCH ASSISTANT BIOBEHAVIORAL HEALTH BIOMARKER CORE LAB
January 2012 – Present, Pennsylvania State University
Acquired research experience and analyzing literature in process of writing honors thesis
Executed experimental protocol in clinical study regarding smoking, diabetes, and stress
RESEARCH ASSISTANT DR. JOSEPH CANNON’S PHYSIOLOGY LAB
May 2012 – July 2012, Georgia Health Sciences University
Analyzed inflammatory cytokines in Irisin Development Study

Leadership
MALINI FOUNDATION
Global Fellowships Program Director, September 2014 – Present
Organized student internships and summer programs with Sri Lankan nonprofit

BIOBEHAVIORAL HEALTH SOCIETY
President, April 2013 – April 2014
Oversaw professional development, fundraising, community service, and social events
Acted as student representative to Biobehavioral Health Alumni Affiliate Program Group
Social Chair, April 2012 – April 2013
Organized social events to unite society members of Biobehavioral Health major

SCHREYER HONORS COLLEGE STUDENT COUNCIL
Penn State Dance Marathon (THON) Chair, April 2013 – April 2014
Directed efforts of organization that contributed $19,094.09 to THON 2014
Supported pediatric cancer treatment and research through the Four Diamonds Fund
Public Relations Chair, April 2012 – April 2013
Promoted council’s events and meetings through social media and other advertisements

SCHREYER HONORS COLLEGE MENTORING
Career Development Program, September 2013 – May 2014
Served as professional development mentor of two first-year students
SHO TIME (Schreyer Honors Orientation) Mentor, August 2012
Led orientation activities for group of eight first-year students during three days

Work Experience
MID-STATE LITERACY COUNCIL STATE COLLEGE, PA
Tutor, September 2012 – Present
Developed curriculum and taught classes including “Global Conservations”

UNIVERSITY HOSPITAL AUGUSTA, GA
Volunteer, June 2013 – August 2014
Provided comfort care visits and wheelchair transportation for patients

Honors and Awards
Jean Phillips Shibley Memorial Health Education Scholarship, July 2014
Helen Skade Hintz Biobehavioral Health Scholarship, July 2014
College of Health and Human Development Academic Achievement Scholarship, July 2013