Sugar Consumption Alters Perception of and Response to Stress in Undergraduate Students: Understanding the "Freshman Fifteen"

By

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Abstract

Psychological stress is a common part in everyday life that directly affects the body through the nervous system and neuroendocrine hormones. A perceived stressor leads to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, and the synthesis and release of the glucocorticoid hormone cortisol from the adrenal cortex. Studies have linked the release of cortisol during high-stress periods to an increased intake of sugary and fatty foods, consistent with a suspected glucocorticoid-metabolic-brain-negative pathway, with high sugar consumption leading to lower stress and subsequently cortisol levels. In this study, undergraduate students' diets were supplemented with either a high sugar drink or water for one week and then subjected to the Trier Social Stress Test (TSST). Subjects' perceived stress was measured with the Revised Undergraduate Student Hassle Scale (RUSHs) in terms of academic, social, and personal categories, and saliva samples were collected pre and post TSST to analyze salivary cortisol levels using an enzyme-linked immunosorbent assay (ELISA). A consistent correlation was observed between sugar and water group participants' perceived stress and baseline cortisol levels, along with statistically significant differences in perceived stress and cortisol response to the TSST. The results of this study indicate the need for specific stress-reduction interventions for college students to reduce unhealthy stress-induced changes in dietary habits.

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Introduction

The "freshman fifteen" refers to the prevailing myth that exists among undergraduates that when a student goes through their first year of college, they will gain an average of 15 pounds from their original pre-college weight. Vadeboncoeur, Townsend, & Foster (2015) conduced a meta-analysis of studies done on students transitioning from high school to college, and found that 60.9% of the students included in the study gained an average of 7.5 pounds during their freshman year of college. They also discovered that one in ten students did gain an average of 15 pounds, which served to provide somewhat of a base for the myth of the "Freshman 15." Potential risk factors that may be contributing to first year weight gain include unhealthy dieting choices, interrupted sleeping patterns, increase in alcohol consumption, decline in physical activity, and newly introduced psychological factors in academic, personal, and social parts of the student's life (Vadeboncoeur, Townsend, & Foster, 2015). The academic portion refers to psychological stress created through high academic pressure coursework in undergraduate classes, such as upcoming exams and projects, deciding a major, grades, etc. Personal psychological stress may include adjusting to living with a roommate or in a dorm, being away from home, discrimination from peers, and adjusting lifestyle to college. Making friends, pressure to go out, balancing friends from hometown and college are a few that could be considered in the psychological social stress for a college student. Individuals with a higher BMI entering college were more likely to gain weight when under high stress (Boyce and Kuijer, 2015).

Stress is defined as any uncomfortable "emotional experience accompanied by predictable biochemical, physiological, and behavioral changes." (Baum, 1990). It can be

divided into three separate categories: environmental, psychological, and biological stress. The psychological category specifically places an emphasis on the individual and their own ability to cope, and is enormously important in terms of mental health as it has been strongly linked to mental illnesses like depression, schizophrenia, and anxiety disorders (Herbert 1997 & Cohen, Kessler, and Gordon, 1995). In addition to mental health, stress has the ability to affect one's physical health. Segerstrom and Miller (2004) compiled a comprehensive report that examined different types of stress and their effects on the immune system. They found that chronic stress, such as unemployment or being a caregiver, was associated with the suppression of both cellular and humoral processes of the immune system. The physical toil from stress in common day life has become widespread in recent times, with the American Psychological Association reporting three out of four Americans have visited the the doctor for stress related ailments (The American Institute of Health).

Stress is considered to be a multifactorial element and can be presented in many different forms. A chronic stressor (as mentioned above) would involve matters present for a long time, such as circumstances in one's home life. An example of an acute stressor would be something immediate such as giving a speech or receiving an injury. There are also varying degrees of how severe the stressor is, the frequency at which the exposure takes place, as well as taking into account a person's own perception of the stress and how it differs between individuals (Lucassen, Pruessner, & Sousa, 2013). Studies have highlighted the direct effect of stress on the body, weight loss and gain being one. Harris et al. (1998) demonstrated that when using a moderate psychological and physical stressor of restraint on rats, the repeated restraints induced an initial period of weight loss and

reduced food intake, followed by higher fat accumulation than non-restrained rats. The study indicated that the induced metabolic state favored energy storage as fat rather than protein, with similar effects being observed in humans due to sharing a common pathway activated by stress.

An individual's perception of an internal or external factor as unpleasant triggers a series of psychological and biological processes that is meant to evaluate and then respond to the stressor, in order to restore body homeostasis (Lucassen et al, 2013). For balance to be restored, the stress response incorporates the nervous system and the endocrine system, each playing a crucial role in triggering and maintaining the body's response to a perceived stress (Widmaier, Raff, & Strang, 2013). The nervous system is split into two separate parts: the central nervous system and the peripheral nervous system. The central nervous system consists of the brain and spinal cord, while the peripheral nervous system contains the somatic nervous system and the autonomic nervous system (ANS) (Kandel, Schwartz, & Jessel, 2000). The ANS is responsible for control of the involuntary bodily functions, such as breathing, heart rate, and digestion, and helps to relay signals from internal organs to parts of the brain, specifically the medulla, pons, and the hypothalamus (Streeton, National Dysautonomic Research Foundation). The ANS is split into two divisions: the sympathetic and the parasympathetic. These pathways allow for a direct route for autonomic nerves to convey unconscious, necessary changes as a reaction to stimuli, such as stress (Streeton). When a stressor is perceived by an individual, the ANS pathway, through the sympathetic division, is the first to be activated in the immediate "flight or flight" response. The adrenal medulla releases the hormones epinephrine and norepinephrine, which work to ready the body in a physical response by raising heart,

respiratory and metabolic rate, as well as directing blood flow towards crucial parts of the body that would be necessary in a physical response (Lucassen et al, 2013). This release of hormones in response to stress brings into play the relationship between the nervous and endocrine system, as the second portion of the stress response involves the hypothalamic-pituitary-adrenal (HPA) axis.

The HPA axis is extermely important to the stress response to both chronic and acute stress. Once an individual registers a stressor, the HPA axis is activated by the hypothlamus releasing corticotrophin-releasing hormone (CRH). The release of CRH then increases the production of adrenocorticotropic-hormone (ACTH) in the anterior pituitary, which subsequently stimulates the adrenal cortex to produce the glucocorticoid hormone cortisol (Yau & Potenza, 2013). The presence of the steroid hormone cortisol in the body then creates a negative feedback loop that leads to the hypothalamus and anterior pituitary to cease production of CRH and ACTH, respectively, and allows for the body to regain homeostasis after the perceived stressor. This exchange of information that occurs between the hypothalamus and the pituitary, and the resulting effects, is known as the neuroendocrine system; meaning it involves the stimulation of the nervous system and the secretion of hormones from the endocrine system (Medical Dictionary). Hormones play a

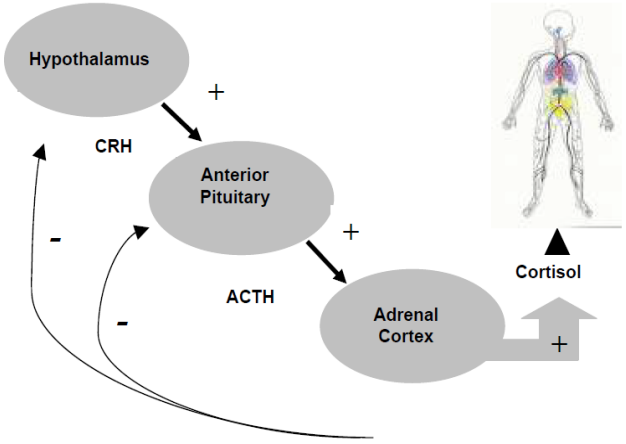

Figure 1. The HPA axis and its regulation (Source: Adam Bender 2014 Student Research Grant, Union College. 2014)

critical role in maintaining the stress response, and are crucial in initiating the necessary physiological changes, and come in a variety of forms with different functions.

Steroid hormones, and their role between the brain and endocrine system, were discovered by Geoffrey Harris when pioneering research on communication between the hypothalamus and the pituitary through steroid hormones (Harris, 1970). Synthesized from cholesterol, the steroid hormones are produced by endocrine glands to develop and drive physiological responses to stimuli. The secretion of adrenal steroid hormones, one of which being cortisol, are highly involved in maintaining the homeostasis of the body through glucose metabolism and the stress response with the HPA axis (Evans, 1988). Guillemin and Rosenberg (1955) lent support to the existence of the HPA axis using the pituitary of a rat, and demonstrating that CRH from the hypothalamus led to the release of ACTH from the pituitary. The HPA axis is mediated by a multitude of factors that include neurotransmitters, neuropeptides, and steroid hormones, and work to activate and control different aspects of the pathway. Neuropeptides are chemical signals in the brain, and defined as small proteinaceous substances produced and released by neurons through the regulated secretory route and acting on neural substrates (Burbach, 2011). Stress activates different neuropeptides such as CRH, vasopressin, neuropeptide Y, and ghrelin, each of which exerts different functions on the brain to influence a certain physiological response to the stressor (Joels & Baram, 2009). The activation of the HPA axis and involved neurotransmitters is accompanied by the release of cortisol, arguably the most important hormone involved in the stress response.

Edward C. Kendall and Philip S. Hench first discovered cortisol, also known as hydrocortisone, in the 1930's through their methods of extracting hormones from bovine

adrenal glands (Saenger, 2010). Being one of the six hormones extracted, it is defined as being a steroid hormone by its four ring linked structure, three rings with six carbons and one with five (Figure 2). Cortisol is produced by stimulation of the zona fasciculata of the adrenal cortex by ACTH. It belongs to the glucocorticoid family, whose receptors are known to be involved in regulating metabolism of almost every vertebrate (Adam & Epel, 2007; Pelt, 2011). Glucocorticoids are particularly known for their anti-inflammatory actions by inhibiting the expression of inflammatory genes, and increasing transcription of anti-inflammatory genes (Barnes, 1998). In addition to its effects on the immune system, cortisol alters the body's metabolism by elevating blood glucose levels through glycogenolysis in the liver and breaking down proteins and fat, along with decreasing the size of blood vessels to raise blood pressure and therefore heart rate (Dickerson & Kemeny, 2004). The raised glucose levels result in an increase of energy that can be used to respond to the perceived stressor. Although cortisol is always present in the stress response, a prolonged presence of the hormone can be detrimental. Due to the fact that cortisol is associated with the suppression of the immune system, its presence during chronic stress weakens the body's defense against disease and infection. Cortisol also directly affects fat storage, as stress-induced cortisol secretion has been implicated in visceral fat storage, with studies finding that a higher BMI is associated with social stressors over a period of time (Epel, McEwen, & Seeman, 2000). Furthermore, continuous cortisol levels during periods of chronic stress has been connected to more serious health issues. Bergmann, Gyntelberg, & Faber (2014)

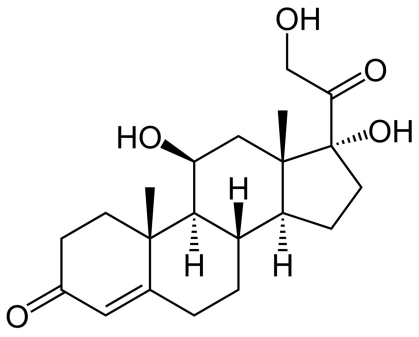

Figure 2. Structure of cortisol

found evidence that chronic psychological stress is a risk factor for developing metabolic syndrome, a health concern that is characterized by a large waistline, high triglyceride level, lower HDL cholesterol, high blood pressure, and high fasting blood sugar. Along with being linked to higher BMI and obesity, cortisol has been implicated in causing changes in eating patterns once the stress response pathway has been initiated. The immediate "fight or flight" response with noepinephrine and epinephrine is correlated with lowering appetite, while the activation of the HPA axis ending in cortisol is accompanied with increasing appetite and higher calorie intake, especially on the day a stressor takes place (Torres & Nowson, 2007; Epel et al., 2000). Cortisol being released leads to a cascade involving other neuropeptides and processes in the body, which work to inhibit and activate hormones influencing hunger. Increased cortisol secretion leads to higher levels of neuropeptide Y, and the down-regulation of leptin, a hormone involved in reducing food intake (Torres & Nowson, 2007; Girard, 1997). Neuropeptide Y (NPY) receptors have been found in areas of the brain such as the hypothalamus, the limbic system, the pons, and the brain stem, all of which are involved in emotional, autonomic, and endocrine processes in the body. Plasma NPY is positively correlated with the release of cortisol and negatively correlated with psychological distress (Morgan et al., 2002). High levels of NPY stimulate hunger

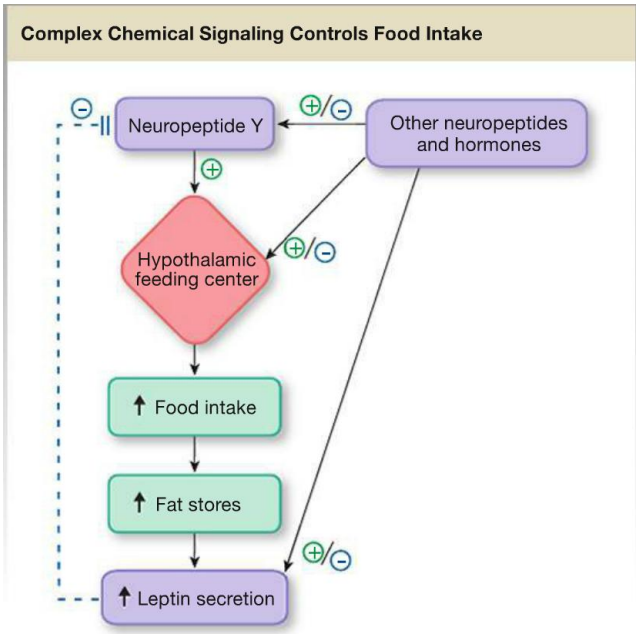

Figure 3. NPY and leptin regulation from cortisol release

and food-seeking behavior, and provide a connection between the gut and brain with how stress influences hunger and the resulting consequences (Hirsch & Zukowska, 2012). In addition to food intake and food seeking behaviors being manipulated by NPY and cortisol, the type of food eaten during a period of stress has been determined to be altered from that of a relatively stress free time. McCann, Warnick & Knopp (1990) found that when comparing groups of people with differing levels of workload, those that believed they had a larger workload and more stress consumed greater amounts of fatty foods, further emphasizing the fact that cortisol secretion influences eating habits. In studies of patients with Binge Eating Disorders (BED), participants were more likely to crave sweet things and have the desire to binge after being exposed to the stressor than those without BED. Additionally, after exposure to a Trier Social Stress Test (TSST), a laboratory technique designed to simulate acute psychological stress, those individuals in the BED group had cortisol levels that were positively correlated with higher binge scores (Rosenberg, Block, & Avi, 2013). In summary, the secretion of cortisol leads to higher levels of NPY, which results in increased hunger and a greater desire for fatty and sugary foods. Diggins, Woods-Giscombe, and Waters (2015) analyzed the existence of emotional eating and stress in African American college women, with emotional eating being defined as the consumption of foods during times of increased stress, especially high sugar and high fatty foods. Their results indicated that an association existed between emotional eating and perceived stress, and that higher perceived stress was a positive indicator for emotional eating among the college women.

Higher desire for sugary foods in the presence of stress presents the risk for developing a dependence on sugar during stressful times. Sugar intake activates dopamine

pathways of the brain, a neurotransmitter of the brain involved in experiencing pleasure (Wideman, Nadzam, & Murphy, 2005). The activation of dopamine receptors in the nucleus accumbes of the brain is characteristic to drugs frequently involved in addiction, and this repeated activation of receptors was discovered in rats that consumed sugar in a binge-like manner. The mRNA levels of the D2 dopamine receptor were decreased, along with the expression of genes preproenkephalin and preprotachykinin, while the D3 dopamine receptor mRNA was increased (Colantuoni, Schwenker & McCarthy, 2001). Dopamine's role in the brain is involved in reward seeking behavior and reinforcement, and addictive drugs such as cocaine, amphetamines, opiates, alcohol, and nicotine all directly or indirectly stimulate its release into the brain (Hadad & Knackstedt, 2014). Its involvement with sugar consumption highlights dopamine's role in shaping eating patterns when the stress response pathway is activated. A recent study indicated that with sugar consumption came lower cortisol levels, as a glucocorticoid-metabolic-brain-negative pathway could be activated by sugar intake, thus leading to lower basal cortisol levels and sugar becoming an addicting and crucial part in relieving stress (Tryon et al., 2015).

Based on these previous studies, we developed the hypothesis that sugar consumption would result in lower basal cortisol levels. This was tested using experimental and control groups where the experimental group consumed excess sugar in the form of sugary drinks, with the control group being asked to refrain from high sugar drinks. An acute stressor, the Trier Social Stress Test (TSST) was applied after seven days of either the sugar or non-sugar diet regime to induce a cortisol spike in individuals (Rosenberg et al., 2013). Participants provided saliva samples throughout each portion of the TSST and salivary cortisol levels were analyzed by enzyme-linked immunosorbent assay (ELISA).

The goal of the study was to test whether sugar consumption affected cortisol levels, as well as examining the effect of sugar consumption on perceived stress, in hopes of providing more clarification to existing literature in the relationship between sugar and stress.

Materials and Methods

Participants

23 participants, 10 males and 13 females, were recruited by researchers on a volunteer basis with compensation of nine dollars. All were Union College undergraduates at an average age of 21, and were asked their preference on being assigned to the sugar or non-sugar (control) consumption portion of the study. Any participant with a family history of diabetes, sugar intolerance, high blood pressure (140/90mm Hg), kidney or liver disease, elevated triglycerides, or on any medications related to controlling blood sugar were automatically placed into the group abstaining from sugary drinks. A total of nine people were used in the sugar consumption leg of the study, with 14 in the control (water) group. The participants' anonymity was ensured by randomly assigning them a number, which would begin with "55" and end with either "1" or "2" to identify if placed in the water or sugar group, respectively.

The Trier Social Stress Test

The Trier Social Stress Test was used to induce stress in the participants, subsequently causing a change in their cortisol levels. After completing all assigned surveys, participants were told that they would have five minutes to prep a five-minute speech to say in front of a panel of three judges, on explaining to an employer why a liberal arts education at Union College would be help them earn a job. They were not allowed to write down their speech, and had to stay in front of the judges for the entire five minutes. In order to give them incentive to make an effort with their speech, they were told that a prize would be given out at the end of the study to the person with the best speech. The panel consisted of members from Professor Cohen's research lab, who were instructed to

act as though they were truly evaluating the participants' performance. After the five-minute speech, a member of the panel would instruct the participant that they would be undergoing an arithmetic problem for five minutes, and count backwards from 1022 by intervals of 13. Whenever the participant made a mistake, a member of the panel would say "1022" and indicate that they start from the beginning at 1022. After the five minutes, participants were allowed to leave the room, and were immediately briefed by the researcher.

Materials

Participants were given a total of four surveys to complete before and after the TSST. Before the TSST, they were asked to rate themselves on a scale of one to ten on how stressed they felt, if they had assignments or sporting events occurring that same week, and their food preferences. They also completed the Undergraduate Student Hassle Scale (RUSHS), a survey of 57 questions in order to gauge the stress present in academic, social, and personal parts of their lives. Post TSST, they were once again asked to rate themselves on a scale of one to ten, their stress during any portions of the test, and their food preferences. Participants then filled out a demographic survey to collect information on their gender, major, body data, eating, sleeping and exercise habits, and any medication they were currently taking. Each participant was also asked to keep a food log for the seven days prior to the TSST, and write down their meals and beverages consumed to properly assess the sugar content in their diet.

Procedure

Seven days prior to coming into the lab, participants were asked to begin their sugar or non-sugar diet through the consumption of sugary or non-sugary beverages, and record

their diet. Participants were asked to come into the laboratory between the hours of 6pm-7:30pm for the total of an hour, in order to control for changes in cortisol levels that occur periodically throughout the day. After arriving in the lab located on the second floor in Wold, participants were asked to give a saliva sample and fill out two surveys. Once all required tasks were completed, they were led into the adjoining lab and received instructions to begin the TSST. Immediately after finishing the math portion of the TSST, participants were brought back into the original lab and asked to give a second saliva sample. They were debriefed and informed the purpose of the TSST and the study, and asked to give a third saliva sample after a 30-minute "cool down" period.

Enzyme-Linked Immunosorbent Assay (ELISA)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard #1 400ng/mL			Standard #9 2.5ng/mL			Sample #5			Sample #13		
B	Standard #2 320ng/mL			Standard #10 1.25ng/mL			Sample #6			Sample #14		
C	Standard #3 160ng/mL			Standard #11 0ng/mL			Sample #7			Sample #15		
D	Standard #4 80ng/mL			BLANK			Sample #8			Sample #16		
E	Standard #5 40ng/mL			Sample #1			Sample #9			Sample #17		
F	Standard #6 20ng/mL			Sample #2			Sample #10			Sample #18		
G	Standard #7 10ng/mL			Sample #3			Sample #11			Sample #19		
H	Standard #8 5ng/mL			Sample #4			Sample #12			Sample #20		

Figure 4. Typical layout for 96-well microtiter plate used for ELISAs

The saliva samples were analyzed for their cortisol level content using the competitive binding technique with cortisol HRP-conjugate in an enzyme-linked immunosorbent assay (ELSA). All deviations from the ELISA protocol (Appendix A) are listed. Corning Easy Wash microtiter plates were coated with rabbit anti-cortisol polyclonal antibody in coating buffer at 1:15,000 dilution instead of 1:30,000, as a much more concentrated antibody dilution yielded more accurate results on cortisol levels in the saliva. The dilution of cortisol HRP in EIA buffer was increased from 1:6,000 to 1:10,000, with

the ratio between antibody and cortisol-HRP significantly improving the standard curve. The amount of standards and samples added was increased from 2.5ul/well to 25 ul/well, along with a pooled IEV cortisol sample in the very last three rows of the plate for a positive cortisol control. The standards, ranging from 400ng/ml – 1.25ng/ml, were adjusted slightly in concentration in order to develop a more precise standard curve. Saliva samples from participants were centrifuged at 12,000rpm for five minutes, keeping the supernatant and discarding the pellet. Each pre-TSST, post-TSST, and 30-minute saliva sample were placed in separate sections on the plate with three wells each, with a total of nine wells corresponding to one participant. 7.5milliliters of Solution A and Solution B were used for the TMB Peroxidase per plate, and one kinetic run and one endpoint run were run for each plate, as one endpoint was deemed sufficient enough to collect just as exact results as two.

Results

RUSHS scores and Perceived Stress in Sugar vs. Water Group

Comparing the scores from the Revised Student Undergraduate Scale (RUSHS), survey between sugar consumption and non-sugar consumption (water) group, revealed higher RUSHS scores for the sugar group than the water group in terms of academic, social, personal, and overall perceived stress participants (Figure 6). The difference was significant for the academic ($p= 0.027$) and overall ($p= 0.037$).

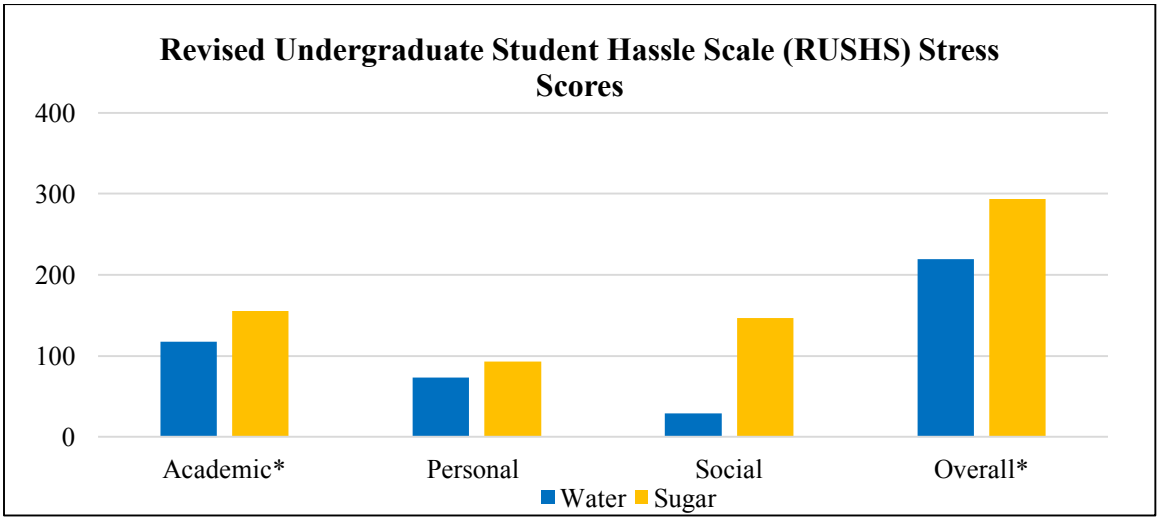

Figure 5. The Revised Undergraduate Student Hassle Scale (RUSHS) scores for academic, personal, social, and overall stress for participants in the sugar consumption vs. non-sugar consumption (water) group. Academic and overall stress were significant with $p< 0.05$.

Change in Cortisol Levels: Pre-TSST to Post-TSST

Analysis of the change in cortisol levels from pre-TSST (cortisol 1) to post-TSST (cortisol 2) indicated a difference from cortisol baseline levels to the post-stressor period. The sugar consumption group displayed a significantly higher percent change in cortisol levels than the water group ($p< 0.05$). The higher percent change demonstrates that the

participants consuming excess sugar collectively had a larger increase in cortisol levels after the TSST than the water group (Figure 6).

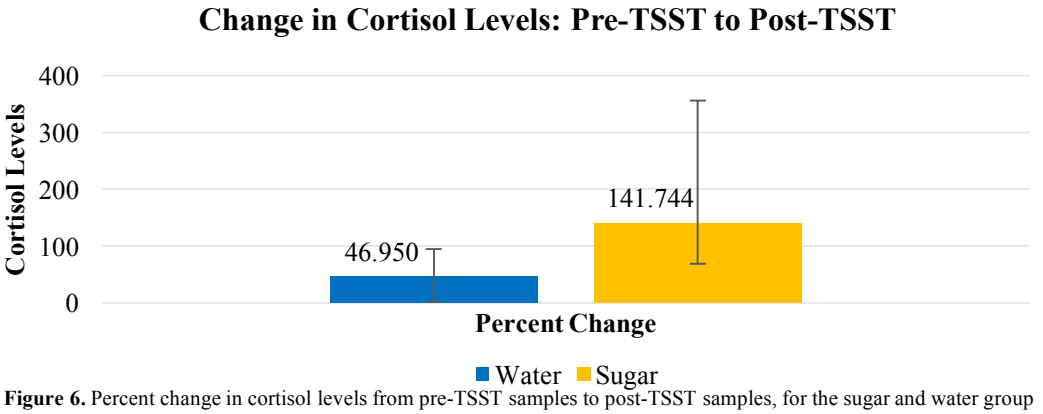

Figure 6. Percent change in cortisol levels from pre-TSST samples to post-TSST samples, for the sugar and water group participants ($p<0.05$).

RUSHS Scores in Perceived Stress vs. Baseline Cortisol Levels

Baseline cortisol levels for Cortisol 1 samples were compared with the perceived stress of participants in both groups, as indicated by their RUSHS scores for academic, personal, social, and overall stress. A consistent trend was present throughout each part of RUSHS analyzed against Cortisol 1 samples. Sugar group participants displayed higher stress and RUSHS scores and lower baseline cortisol levels, while water group participants displayed higher stress and higher baseline cortisol levels (Figures 7-10). The correlations did not reveal significant values, with R-squared values being less than .5 and p-values > 0.05.

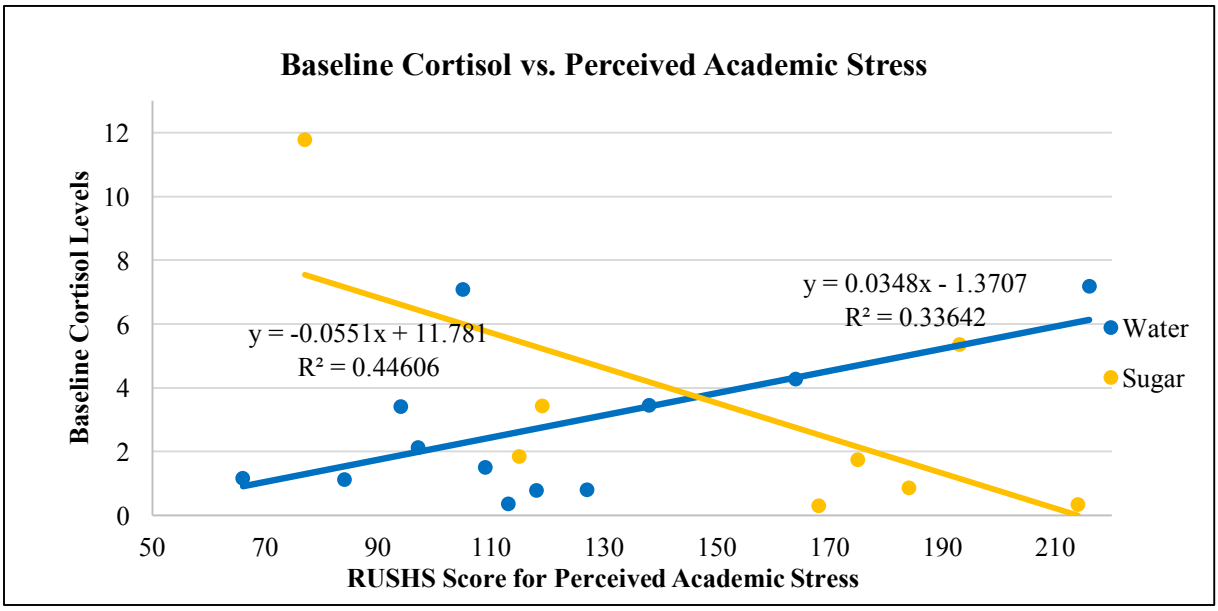

Figure 7. RUSHS scores for perceived academic stress vs. baseline cortisol levels for sugar and non-sugar (water) consumption participants

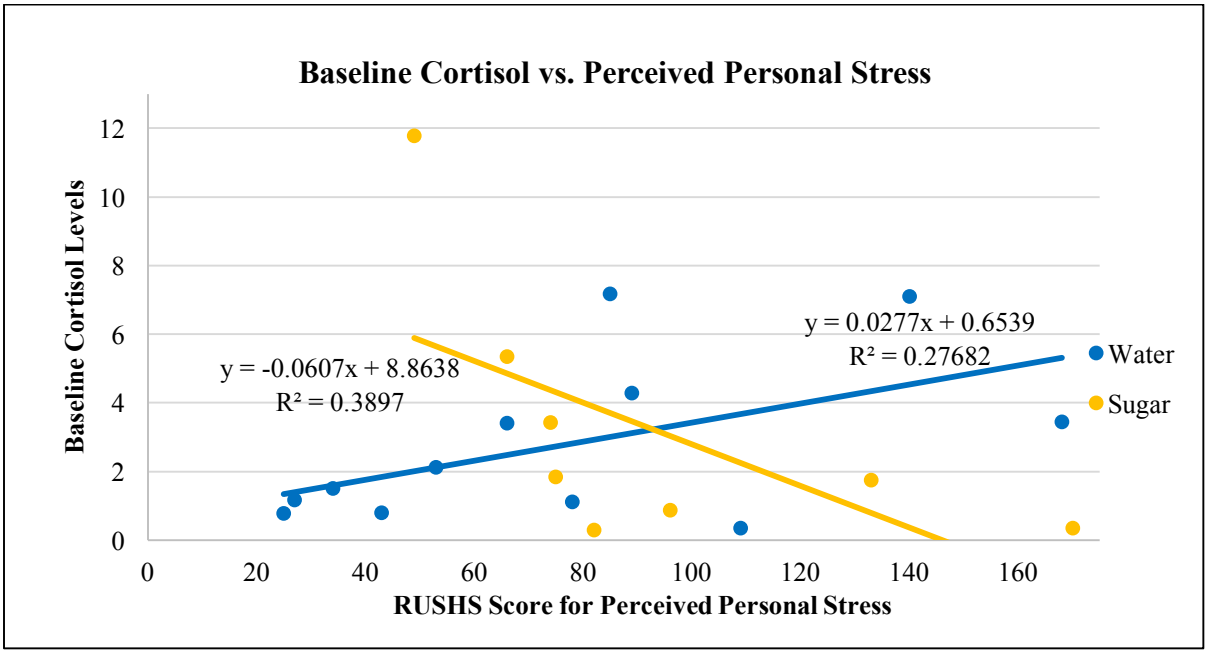

Figure 8. RUSHS scores for perceived personal stress vs. baseline cortisol levels for sugar and non-sugar (water) consumption participants

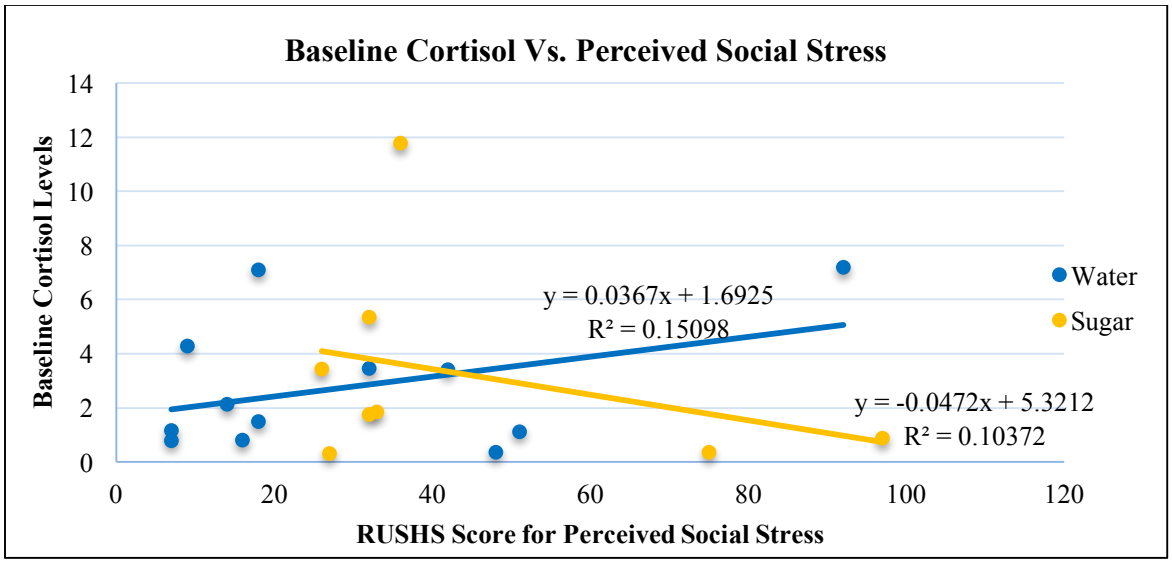

Figure 9. RUSHS scores for perceived social stress vs. baseline cortisol levels for sugar and non-sugar (water) consumption participants

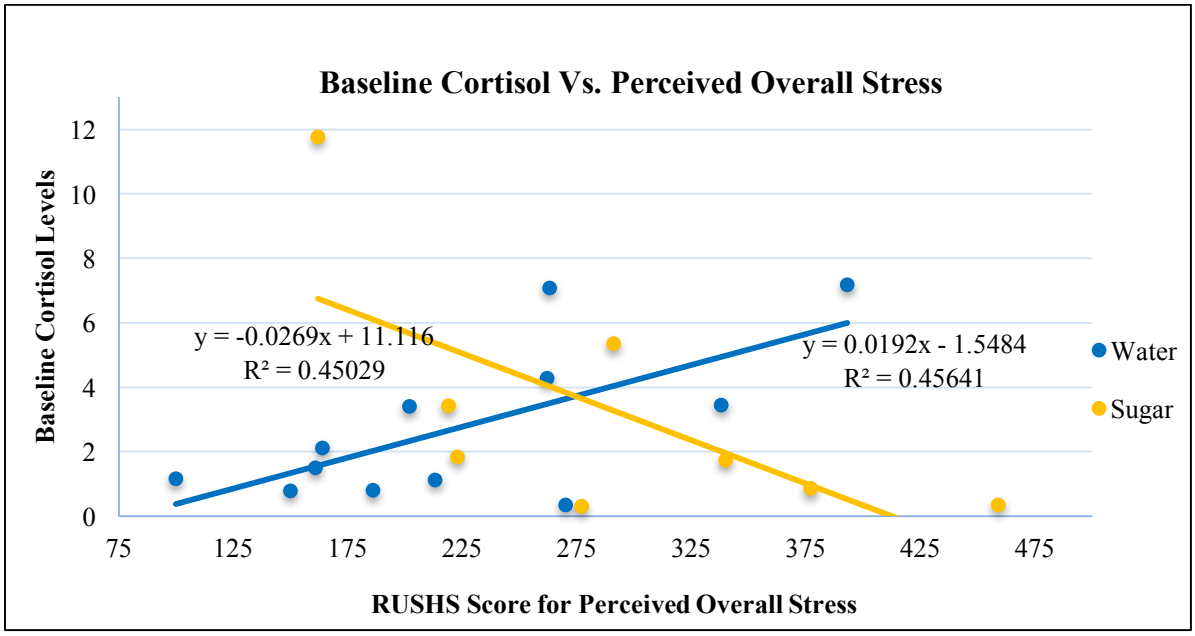

Figure 10. RUSHS scores for perceived overall stress vs. baseline cortisol levels for sugar and non-sugar (water) consumption participants

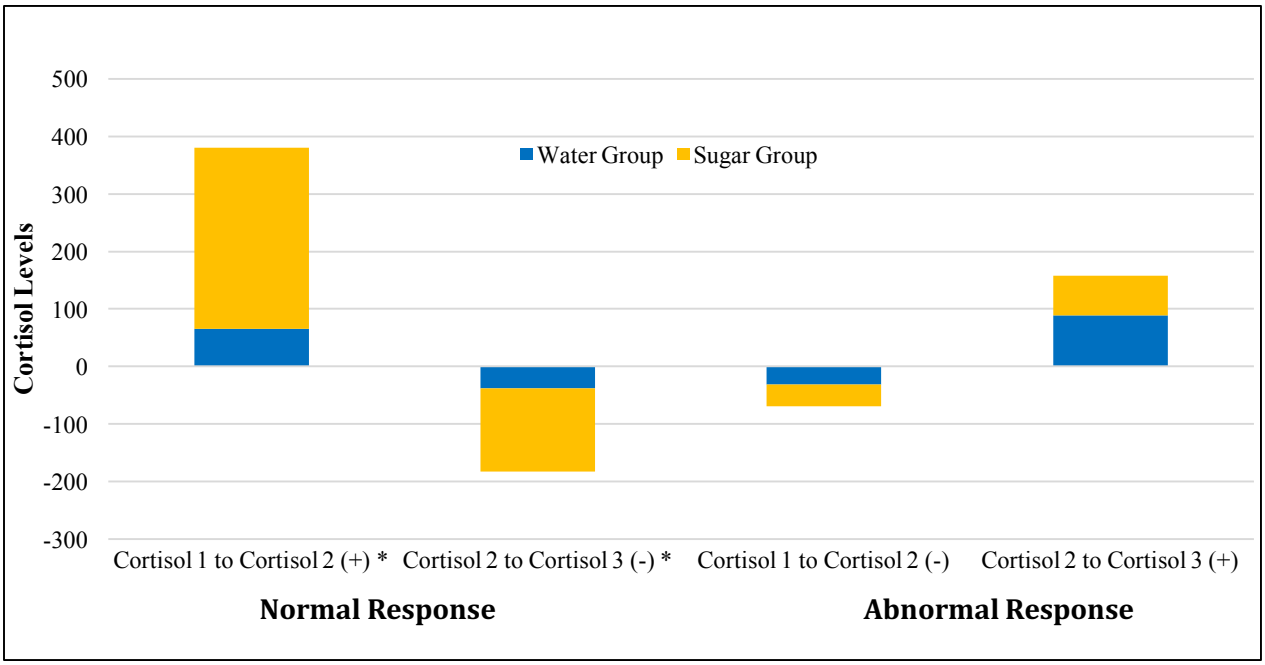

Figure 11. Abnormal and normal responses of cortisol levels in participants. Cortisol 1 represents pre-TSST, Cortisol 2 represents directly after TSST, and Cortisol 3 is 30 minutes post-TSST.

Normal vs. Abnormal Response to Stress

A normal response to stress was determined to be a rise in cortisol levels from Cortisol 1 (pre-TSST) to Cortisol 2 (post-TSST), and a decrease in cortisol levels from Cortisol 2 to Cortisol 3 (30-minute cool down period). An abnormal response to stress was defined as anything varying from that, including cortisol levels decreasing from Cortisol 1 to Cortisol 2 or rising from Cortisol 2 to Cortisol 3 (Figure 11). Participants were placed into a normal or abnormal response to stress based on their cortisol levels (Figure 11). The results for a normal response to stress revealed that the sugar consumption group showed a significantly larger increase in cortisol levels from Cortisol 1 to Cortisol 2 than the water group ($p < 0.024$). Sugar group participants also showed a significantly greater decrease in their cortisol levels when compared to the water group ($p<0.048$).

Discussion

Based on previous literature concerning sugar consumption and cortisol levels, we developed the hypothesis that sugar consumption would result in lower basal cortisol levels. A correlation was observed in the data between the perceived stress and cortisol levels of the subjects for both the experimental and control group. Sugar participants reported higher perceived stress in terms of RUSHS, with ELISA assays revealing lower baseline cortisol levels. On the other hand, water participants presented with higher RUSHS scores and perceived stress, along with higher baseline cortisol levels when compared to the sugar consumption group. Although the association was not statistically significant, the trend was consistent through reported academic, personal, social, and overall stress when comparing baseline cortisol and RUSHS scores.

In order to examine the data more in depth and differentiate between individuals that had or had not experienced the normal stress response, with increased salivary cortisol levels after the TSST and decreasing cortisol levels 30 minutes later, the sugar and water groups were split into normal and abnormal stress response. The separation eliminated any individuals who had not been stressed by the TSST in terms of their cortisol levels, and aided in sorting out those who may have self-reported not feeling distressed but had increased cortisol levels that revealed otherwise. The sugar group had statistically significant differences from the water group both directly after the TSST and 30 minutes later, with sugar consumption participants showing a much larger increase in cortisol levels directly after undergoing the stressor, and displaying lower cortisol levels and a faster “cool down” than water participants in the 30 minute period. Their levels differed from the water group as they had lower baseline cortisol levels, a greater

increase in cortisol levels pre-TSST, and then a more rapid return to lower cortisol levels post-TSST in the "cool down" portion of the procedure. The variations between the sugar and non-sugar consumption group suggest that ingesting sugar as a part of their regular diet aided participants in their physiological response to the psychological stress induced by the Trier Social Stress Test, and the sugar could have played a significant role in blunting their stress response and lowering cortisol levels at a much swifter rate post-stressor. By restricting the water participants from consuming sugar to a minimal amount or completely absent, their response to stress was missing the regulation that could have been gained from sugar.

The results are consistent with previous research that has implicated sucrose consumption having an effect in decreasing cortisol levels before and after undergoing stress tests. Tryon et. al (2015) placed 19 female participants on a sucrose vs. aspartame 12-day diet, with the Montreal Imagine Stress Test (MIST) in order to measure both brain and cortisol level activity throughout the duration of the stressor. The researchers discovered that there was no difference between the two groups before the 12-day diet, but after the diet, the sucrose subjects displayed lower salivary cortisol levels before and after their stress intervention than the aspartame group. Furthermore, the aspartame group showed increased levels of cortisol response when compared to the tests before their 12-day diet. Another portion of their study involved monitoring brain activity of both groups via magnetic resonance imaging (MRI) pre- and post- stressor, and results showed the sucrose consumption group with statistically significantly higher activity in the hippocampal area. The hippocampus and its glucocorticoid receptor (GR), specifically in

the forebrain area, have been implicated in the feedback regulation required for the HPA axis response to stress (Furay, Bruestle, & Herman, 2008).

The TSST was designed to mimic an acute psychological stressor, which suggests that the hippocampus being involved is not entirely unexpected. The hippocampus can be split into two different portions: the dorsal and the ventral hippocampus. Forbes (2011) found that the dorsal part is typically implicated in learning and spatial memory, while the ventral part deals with emotion and motivation. Furthermore, the ventral hippocampus has been associated with controlling reward and emotional behavior through neural connections with areas such as the amygdala and prefrontal cortex, as well as the stress response with the regulation of the HPA axis (Forbes, 2011). In regards to our study, the first portion of the results indicated that there was a significant difference in perceived stress between the sugar and non-sugar consumption groups, with the sugar group at a higher perceived stress in terms of academic, personal, social, and overall parts of their lives. The relationship between previous studies with higher activation of the hippocampus, and its relation to emotion and controlling the stress response, could explain why the participants on the sugar consumption diet reported experiencing higher stress. The activation of the hippocampus could be manifesting itself in greater perceived stress in all aspects of their life for the sugar consumption individuals, while the water group was not affected, as they were not on a sugar consumption diet.

The cortisol level activity for the sugar consumption vs. the non-sugar participants could be explained by a proposed glucocorticoid-metabolic-feedback-pathway that is affected by sugar, or sucrose. Previous studies have suggested that normally in the body signals originating from ATP concentration typically inhibit glucose uptake into muscular

and adipose tissue, and streamline resources towards the blood-brain barrier (Peters, Schweiger, Pellerin, et. al, 2004). In the face of high stress, this pathway is reversed to favor catabolism and uptake of energy. Dallman et. al (2003) studied the eating habits and pronounced effects from adrenalectomized rats, including a sucrose vs. saline diet and how the HPA axis would respond. The results showed that the more sucrose ingested, the less corticotrophin releasing factor (CRF) mRNA was expressed. On the other hand, as the amount of mesenteric fat on the rats increased, the expression of CRF mRNA decreased as well, suggesting that both the amounts of sucrose and fat favor a decrease in activation of the HPA axis due to being interpreted as a large amount of energy storage. In regards to our study, the sugar could be interpreted as a source of high energy and helping to deactivate the HPA axis. This may explain the lower baseline levels and the exaggerated response to stress. The faster cortisol level cool down period from post-TSST to 30 minutes later for sugar consumption participants, rather than just water, could be explained by the high amount of sugar and energy still present, and the body using that energy to stop the stress response as evidenced by the low cortisol levels. This provides further evidence for a glucocorticoid metabolic feedback pathway as suggested in previous research, as the non-sugar consumption group did not have the same results with their cortisol level, and asked to obey a sugar-free diet.

Future directions for this study include increasing the sample size (n = 22), in order to control for potential outliers in the data. To control for confounding variables that could be present in each of the individuals being tested, the subjects would have to be restricted to simply one gender or females not taking birth control. After the methods had been implemented and the testing of subjects began, a study was published that stated

women taking oral contraceptives exhibited higher baseline cortisol levels pre-TSST and post-TSST (McQuaid, et al., 2016). Although participants were asked to list any sort of medication they were on, in addition to their gender while filling out demographic information, the effects that the birth control may have had on cortisol levels was not examined. The results of this study has implications on how to address undergraduate students during their years in college, in not only education for proper diet and stress management in the first year, but throughout the duration of their university or college career. With the growing amount of pressure being placed upon students in the coming age, and being held to a higher standard of academic success, it is essential that programs are implemented in order to ensure that programs highlighting healthy eating and stress reduction habits are put into place.

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Appendix A

ELISA Protocol

I. Buffer Preparation and reagent supplies

- Rabbit anti-cortisol, polyclonal antibody (cat.# 20-CR50, Fitzgerald Ind. Int'l, MA)
    - Stored at -20^{0}C (also, 1:100 dilutions stored at -20^{0}C)
    - * Currently used at ~1:30,000 dilution
    - ** Alternate Monoclonal AB: #E86220M, Meridian Life Sciences Inc., ME
- Cortisol-HRP conjugate; (cat. # 65-IC08, Fitzgerald Ind. Int'l, MA)
    - Liquid; Stored at 4^{0}C
    - * Currently used at ~1:6000 dilution in EIA Buffer
- TMB Microwell Peroxidase Substrate (cat #50-76-03; KPL/Kirkegaard & Perry)
- Bovine Serum Albumin (Sigma # A7030)
- Pressure Sensitive Film (Falcon #3073; from Sigma)
- Corning/Costar Easy Wash microtiter plates (#3369 Corning), Fisher # 07-200-642

* Exact dilution will vary with lot and should be determined before running assays.p

Coating Buffer (0.05M, pH 9.6):

15 mM Na_2CO_3	0.159 g	MW = 105.99 g/mol
35 mM $NaHCO_3$	0.294 g	MW = 84.01 g/mol
0.02% Sodium Azide	0.020 g	
dd H_2O	100 ml	

Add chemicals to 100 ml H_20; Store at 4^{o}C for no more than one week.

Phosphate Buffer Stocks (2X concentrated) for EIA Buffer and Wash Solution:

Solution A	0.2M NaH_2PO_4	12.0 g/500 ml	MW = 119.98 g/mol
Solution B	0.2M Na_2HPO_4	14.2 g/500 ml	MW = 141.96 g/mol

Wash Solution (10 X concentrated stock; store at 4^{o}C):

1.5M NaCl	87.66 g	MW = 58.44 g/mol
0.5% Tween 20 (liquid)	5.0 ml	
dd H_2O	1 L	

Alternate pre-made Wash Solution: 2mM imidazole, 0.02% Tween 20, 0.5 mM EDTA and 160 mM NaCl (20x concentrate; cat #50-63-00; KPL/Kirkegaard & Perry)

Wash Solution (1 X working solution): 0.1M PBS, 0.15M NaCl, 0.05% Tween 20

10X conc stock	100 ml
dd H_2O	400 ml
Solution A	195 ml
Solution B	305 ml

EIA Buffer (0.1M PBS)	for 100 ml:	for 200 ml:
Solution A	19.5 ml	39 ml
Solution B	30.5 ml	61 ml

0.15 M NaCl	0.877 g	1.754 g
0.1% BSA	0.1 g	0.2 g
ddH_2O	50 ml	100 ml

Adjust pH to 7.4; Store at 4°C.

HCl (0.5M) = 5.0 ml of 5 M HCl plus 50 ml dd H_2O

Ringers Solution (for preparation of standards)

140 mM NaCl	8.182 g/L	MW = 58.44 g/mol
10 mM $NaHCO_3$	0.84 g/L	MW = 84.01 g/mol
2mM NaH_2PO_4	0.24 g/L	MW = 119.98 g/mol
1mM $MgSO_4$	0.12 g/L	
*1mM $CaCl_2$	0.147 g/L	MW = 147.02 g/mol
4mM KCl	0.298 g/L	MW = 74.56 g/mol

Add to 1 L of dd H_2O; Adjust to pH 7.8

*Add after mixing other standard solutions and bringing up to at least half of the final volume in dd H_2O.

For Standards: Add 0.1% BSA at 1.0 g/L

II. Dilutions of Standards for Cortisol EIA

- Cortisol frozen stock solution 0.4 mg/ml in ethanol at -80°C.
- Use 0.1%BSA in Ringer's solution (see above)
- Aliquot standards to labeled tubes, store at -80 °C

Option #1: Dilute 0.4 mg/ml stock in EtOH to 0.1 mg/ml (250 µl stock + 750 µl EtOH), then follow dilutions below…

Concentration	µl of:	µl of Ringers
500 ng/ml	10 µl of 0.1 mg/ml	1,990 µl
400 ng/ml	1,600 µl of 500 ng/ml	400 µl
200 ng/ml	1,000 µl of 400 ng/ml	1,000 µl
100 ng/ml	1,000 µl of 200 ng/ml	1,000 µl
50 ng/ml	1,000 µl of 100 ng/ml	1,000 µl
25 ng/ml	1,000 µl of 50 ng/ml	1,000 µl
10 ng/ml	200 µl of 100 ng/ml	1,800 µl
5 ng/ml	1,000 µl of 10 ng/ml	1,000 µl
2.5 ng/ml	1,000 µl of 5 ng/ml	1,000 µl

Option #2: Alternate Standards (Lower range); no dilution of original stock…

Concentration	µl of:	µl of Ringers
400 ng/ml	5 µl of 0.4 mg/ml	5 ml
320 ng/ml	1,600 µl of 400 ng/ml	400 µl
160 ng/ml	1,000 µl of 320 ng/ml	1,000 µl
80 ng/ml	1,000 µl of 160 ng/ml	1,000 µl
40 ng/ml	1,000 µl of 80 ng/ml	1,000 µl
20 ng/ml	1,000 µl of 40 ng/ml	1,000 µl

10 ng/ml	1,000 µl of	20 ng/ml	1,000 µl
5 ng/ml	1,000 µl of	10 ng/ml	1,000 µl
2.5 ng/ml	1,000 µl of	5 ng/ml	1,000 µl
1.25 ng/ml	1,000 µl of	2.5 ng/ml	1,000 µl

III. Detailed protocol

DAY 1

1.) ANTI-BODY COATING:

a. Coat Corning Easy Wash microtiter plates with Rabbit anti-cortisol polyclonal antibody at 1:30,000 final dilution in Coating Buffer, 150 µl/well. (Use the Eppendorf repeater pipette set at 3, with a 2.5 ml tip – this will dispense 150 µl).
b. Tightly seal the plates with Pressure Sensitive Film. Incubate for 3 Hours at 37°C (NOTE: Plates can also be incubated overnight at 4°C)

2.) WASH THE PLATE 5X: Use 1X wash solution and Program 1 ("P1") on the plate washer. It is not necessary to empty the wells before placing on the washer, as its first step is to aspirate from the wells.

Plate Washer (Multi-Wash III, Tri Continent) instructions:

a. Turn the power switch on the back of the machine to on.
b. Prime by hitting the "Prime" button
c. After the line is primed, check that display reads "P1" (first program). Push "Select/Review" and then the up/+ buttons to select "P1" if it's not already set. Settings for this program are:

	P1	P3 (if needed)
• Dispense volume	300ul	300ul
• Dispense rate	300ul/sec	300ul/sec
• Soak time	0 sec	300 sec (5 min)
• Wash cycles	5	5
• Wash mode	"Strip Plate"	
• Plate type	"rnd" (round, not flat bottom)	

d. Make sure the number of rows is correct: Press the "Rows" button, and then the up/+ buttons to select the correct number (12 is max, counted left to right)
e. Uncover plate, place on washer, push start.
f. After wash is complete, snap plate briskly to dry (invert and pound on paper towels on counter).

3.) BLOCKING:

a. Plate 250 µl/well EIA Buffer (Use the multi-channel, repeater pipette) – this is the blocking solution. Let the plates block for 30 minutes at room temperature.
b. Aspirate the wells using the plate washer (Push the up/+ button to select "P2" – the second program, then push "Start")
c. If this is the last plate for the day, press "Rinse" on the washer, turn off power

4.) ADD BUFFER, SAMPLES AND STANDARDS:

a. Using the Repeater pipette, add 100 µl/well of cortisol-HRP conjugate in EIA Buffer at 1:6000 to each well except for the "Blanks"!
b. Add 100 µl EIA Buffer to each "Blank" well.
c. Add 2.5 µl/well of Standard or Sample.
d. Add 150µl/well EIA Buffer to the plate (Use the multi-channel, repeater pipette).

Follow your plate template to make sure you've done this correctly!

5.) OVERNIGHT INCUBATION – Seal the plate tightly with Pressure Sensitive Film and incubate overnight at 25°C.

DAY 2:

6.) Remove TMB Peroxidase substrate (KPL) from 4 °C 1.5 hours before use. Mix equal volumes of the two solutions – use plastic graduated cylinders and mix into a polypropylene beaker (DO NOT USE GLASS!)

7.) SET UP MICROPLATE READER: (VMax, Molecular Devices)
a. Open Windows; open Soft Max software (icon on desktop)
b. Open the Cortisol EIA template file – this will have 3 "plates" already set for you to use. Immediately SAVE to a new file for your particular experiment.
c. Choose the appropriate plate for the Kinetic, Endpoint #1 and Endpoint #2 runs, as listed below

8.) WASH THE PLATE 5X: Use 1X wash solution and Program 1 ("P1") on the plate washer. Pound dry as before.

9.) ADD TMB REAGENT: 150µl/well (Use the multi-channel, repeater pipette). NOTE: This step should be accomplished as quickly as possible (~ 1 min.) to minimize across the plate differences. (We place controls on each side of every plate in order to monitor this, such as the "0" standard and a pooled plasma sample.)

10.) KINETIC RUN: Place the plate in the reader, choose the Kinetic plate and click on "Read". Monitor the progression of the curves that appear on screen. The reaction time will vary with the freshness of the TMB used but should be ~10 minutes. The desired range is E_0=0.6-0.9. The plate settings for this run should be:

- Mode: Kinetic 1
- Wavelength 1: 650 nm (NOTE: this wavelength allows for monitoring the initial blue color development of the TMB)
- Runtime: 10 minutes
- Read interval: 10 seconds
- Automix: ON

11.) ENDPOINT RUN #1: At the end of the kinetic run, choose the plate for Endpoint Run #1 and click "Read". Reader settings:

- Mode: Endpoint 1
- Wavelength 1: 650 nm
- Automix: ON

12.) STOPPING THE REACTION: Remove the plate from the reader and, using the multichannel, repeater pipette, add 100 µl of 0.5 M HCl to each well to stop the color reaction. You will see a change from blue to a yellow color. Put plate back into the reader.

13.) ENDPOINT RUN #2: Choose the plate for Endpoint #2 and click on "Read". The HCl will increase the OD by 2-3 times. (E_0=1.8-2.0 is optimum). Reader settings:

- Mode: Endpoint 2
- Wavelength 1: 450 nm
- Automix: ON