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# The Effect of Circadian Rhythm on Cortisol and Perceived Stress Correlations

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The Effect of Circadian Rhythm on Cortisol and Perceived Stress Correlations

By

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Submitted in partial fulfillment

of the requirements for

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UNION COLLEGE

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ABSTRACT

BENDER, ADAM          The Effect of Circadian Rhythm on Cortisol and Perceived Stress Correlations. Department of Biological Sciences, June 2015.

ADVISOR: Brian D. Cohen

        Stress is a known trigger of the Hypothalamus-Pituitary-Adrenal (HPA) Axis which leads to the production and secretion of the catabolic steroid cortisol by the adrenal gland. Since cortisol production is affected by stress, it follows that a high self-perception of stress would be correlated with high blood and saliva cortisol levels. Literature generally shows a gap in identifying this correlation, perhaps because of the interconnected nature of endocrine pathways. New experimental methods that control for the effects of circadian rhythm have shown limited success in demonstrating this correlation. Our purpose is to continue exploring the relationship between cortisol, perceived stress, and circadian rhythm. This will be done by comparing the results of the Trier Inventory for Chronic Stress (TICS) survey, to the cortisol concentration in saliva samples taken during the cortisol awakening response (CAR). When these results are analyzed, based on individual chronotype (whether someone is a morning or evening person based on the Lark or Owl Questionnaire), we hypothesize that a stronger, positive correlation between perceived stress and cortisol concentration change will be exhibited. When the salivary cortisol concentration changes, during the CAR, were compared to participant perceived stress and chronotype, no statistical significance was determined. Despite conflicting correlative trends, based on comparing results from different time points during the CAR, this research has shown evidence towards a negative correlation between perceived stress and CAR reactivity, and parallel correlative trends between perceived stress and chronotype.

**Introduction**

There has been a vast amount of research focusing on the human endocrine system, which controls the flow of information between different tissues and cells throughout the body. This is accomplished through the internal secretion of chemical messengers, hormones, which circulate through the bloodstream, or which act locally by binding to specific hormone receptors. Binding subsequently causes receptor conformation to alter, which, depending on the hormone, leads to activation or inhibition of post receptor events that influence cellular functioning (Webb et al. 2007).

Cortisol is one aspect of the endocrine system that has been extensively examined, and, while there is still much to learn, the basic regulation and functions of this hormone are well understood. Cortisol is a naturally produced catabolic steroid, which was classified as a glucocorticoid due to the initial discovery of its role in glucose regulation. Freely circulating cortisol is biologically active, and able to pass through cellular membranes in order to bind to the cytosolic glucocorticoid receptor proteins found in almost all bodily tissues (Aron et al. 2007). Cortisol is one of the byproducts of the hypothalamic-pituitary adrenal (HPA) axis, a major endocrine information pathway involving the hypothalamus, the anterior pituitary gland and the adrenal cortex, represented in Figure 1. Central nervous system input causes the hypothalamus to release corticotropin releasing hormone (CRH), which binds to receptors in the anterior pituitary gland. When this occurs, the anterior pituitary gland stimulates the release of adrenocorticotropic hormone (ACTH), a tropic hormone that travels through the bloodstream to this system's periphery organ, the adrenal cortex. ACTH binds to plasma membrane receptors found in two areas of the adrenal cortex, the zona fasiculata and the zona reticularis, and causes the conversion of cholesterol to cortisol through processing by multiple enzymes (Dockray 2007).
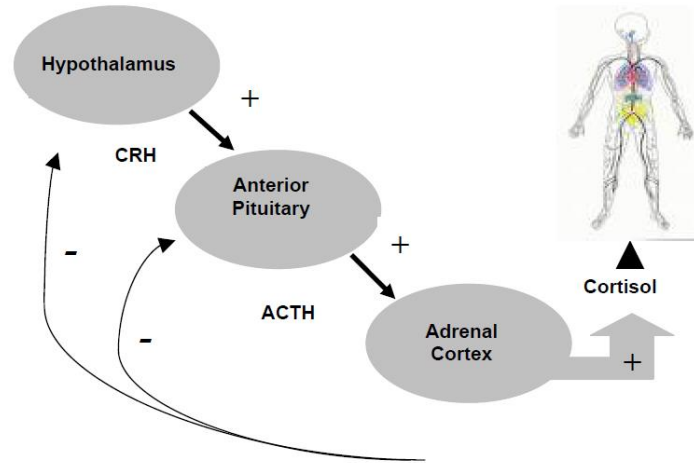
Figure 1. The Hypothalamus Pituitary Adrenal (HPA) axis (Dockray 2007).

This HPA axis is highly regulated through three major neuroendocrine controls. The first control is that CRH secretion, so ACTH and cortisol secretion, is based on an individual's circadian rhythm. This circadian rhythm is controlled by the suprachiasmatic nucleus in the hypothalamus. While each person's rhythm may be affected by sleep patterns, light exposure and stressors, the general population follows a very reliable, diurnal pattern of cortisol secretion as seen in Figure 2. Beginning at midnight, the plasma cortisol levels are extremely low, and in some cases undetectable. The levels increase steadily throughout the night, and experience a sharp increase upon waking. This waking spike has a greater response when waking occurs around 8 in the morning. After this sharp increase, the plasma cortisol levels slowly decrease throughout the day (Chan & Debono 2010). The second major neuroendocrine control centers around the physiological and psychological stress, such as pain or hypoglycemia, responses originating in the central nervous system. CRH is released in response to serotonergic and cholinergic systems in the central nervous system that are brought on by the physical arousal of stress, leading to an increase in ACTH and cortisol secretion (Dockray 2007). The final neuroendocrine control of the HPA axis is through negative feedback at both the hypothalamus and pituitary levels. When ACTH is secreted, it binds to receptors in the hypothalamus, a short loop mechanism, which inhibits secretion of CRH. Similarly, when cortisol is secreted in high enough concentrations, it binds to receptors in the

hypothalamus that inhibits CRH secretion, and it also binds to receptors in the anterior pituitary gland that will inhibit ACTH secretion. Since suppression of both CRH and ACTH will, in turn, inhibit the release of cortisol, these multiple forms of negative feedback allows cortisol concentrations to return to basal levels once the active mechanism eliciting the concentration increase ends (Aron et al. 2007).
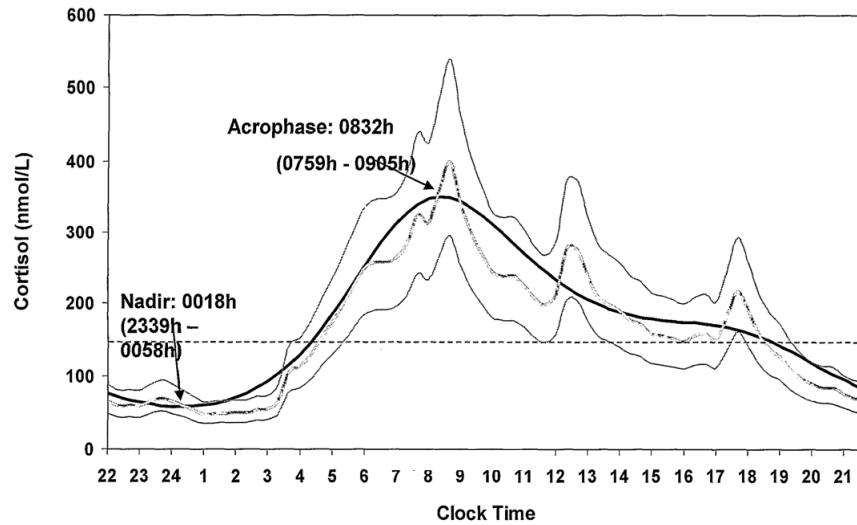


Figure 2. The average cortisol circadian rhythm of 33 healthy individuals, represented by the black line. Cortisol levels peak around 8:30 am and are at their lowest point around midnight (Chan & Debono 2010).

Due to the fact that glucocorticoid receptors are located in almost every tissue throughout the body, increased cortisol concentrations lead to many different physiological effects. Cortisol accelerates catabolism in carbohydrates and proteins in peripheral tissues, and accelerates the breakdown of glycogen. All of which increases the amount of substrate available for increased gluconeogenesis, which allows for an increase in blood glucose levels. Cortisol also increases the strength and frequency of heart contractions, causes blood vessel constriction, causes increases in blood pressure and causes inhibition of the immune inflammation response. The increase in blood sugar levels combined with the

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interactions with the circulatory system, metabolism, the immune system, and the central nervous system allows the body to be prepared to respond efficiently to whatever stressor is being experienced.

The strong biological ties between the physiological and psychological stress response with the regulation of the HPA axis, which increases plasma cortisol concentrations and allows for efficient management of stressors, suggests that people undergoing stressful activities will have higher free cortisol concentrations. This correlation is supported by various evaluations of stress responses elicited by daily hassles (Jacobs et al. 2007). In a study conducted by Jacobs, women were required to complete self-assessment Likert forms related to current thoughts, context, context appraisal and mood during unpredictable times within each of the  10 ninety minute intervals between 07:30 and 22:30, over five consecutive days. When these responses were compared to the cortisol concentrations of corresponding saliva samples, it was determined that there was a positive correlation between minor stressors, increased negative affect and cortisol concentrations (Jacobs et al. 2007). In an additional study, men and women were asked to submit a saliva sample, at 11:00, 15:00 and 18:00, and answer questions regarding daily task performance pressure, performance failure and questions about negative affect levels dealing with being worn out, tense, unhappy and angry, during each time. This process was repeated on a second day, 3 months apart. It was found that momentary performance pressure was positively correlated to momentary cortisol levels, and that this relationship was accentuated with trait anxiety. However, a negative correlation was seen between task failure and daily cortisol concentrations, and state negative affect was not found to have a mediating role between anxiety and cortisol (Schlotz et al. 2006).

While a great deal of research supports the fact that cortisol levels increase when participants are subjected to stressors, there is conflicting research that points to an absence of a correlation between perceived stress and cortisol concentrations. One study was conducted by Marleen van Eck, in

which white collar working males were asked to collect saliva samples and respond to questionnaires covering major life events, long term difficulties, psychosomatic symptoms, depression and trait anger. This data collection occurred at random times during 10 ninety minute intervals throughout the day, over a course of 5 days. When the results were compared between high stress workers and low stress workers, as determined by the perceived stress scale, there was no correlation between perceived stress and cortisol concentrations. Despite this, there was a correlation between the occurrences of stressful events and salivary cortisol concentrations (Van Eck et al. 1996). A similar phenomenon was seen when the salivary cortisol concentrations, global perceived stress, acute perceived stress and stressful events of undergraduate students were compared between exam and non-exam week experiences. Although both the salivary cortisol concentrations, the number of stressful events and acute perceived stress did increase in the exam period, compared to the non-exam period, the global perceived stress, measured through the perceived stress scale, did not (Murphy et al. 2010). Both of these studies suggest that there may be a greater correlation between acute or momentary perceived stress and cortisol concentrations than the correlations related to a global scale, yet this can't be easily concluded when the cortisol response to stress itself is not always consistent between groups experiencing different levels of trait anxiety (Schlotz et al. 2006) or negative affect (Jacobs et al. 2007).

The vast range of research results dealing with cortisol measurement related to the stress response suggests that there are many different mechanics that affect individual cortisol concentration responses. This is further illustrated in a study that compared the acute cortisol stress responses between men and women who were in different phases of their menstrual cycles or who were using hormone birth control methods (Kirschbaum et al. 1999). One aspect of the Kirschbaum study involved measuring the HPA axis response following the Trier Social Stress Test (TSST), a public 15 minute arithmetic oral exam. Men, women on birth control, women in the follicular phase or women in the luteal phase of their menstrual cycle experienced the TSST and saliva samples were obtained at 1, 10,

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20, 30, 45, and 60 minutes after the test. The samples collected showed that men had a greater salivary ACTH concentration then women did. Results also showed that salivary cortisol concentrations differed; women in the luteal phase and men had the highest concentration, while the women in the follicular phase and in the oral contraceptive groups had lower concentrations.

In order to determine whether a reliable correlation between perceived stress and cortisol concentrations can be made, the influences of the many unknown, interacting factors related to cortisol must be controlled as effectively as possible. One possible way to do this would be to control for one of the neuroendocrine controls of cortisol secretion, specifically the effects of circadian rhythm. Recent studies have shown that measuring cortisol concentrations during the awakening spike response has a high intraindividual stability, when comparing multiple sample concentrations taken across trials (Pruessner et al. 2003). The importance of controlling for the circadian rhythmic patterns of the HPA axis is emphasized when slight differences in measurement times, even when stable characteristic of the rhythm, such as the awakening response, are present. In a study following male pilots, participants collected saliva samples at 0 minutes, 30 minutes, 2.5 hours, 8 hours, 12 hours after waking and then again at bedtime. This occurred during two days of morning shifts (04:26), late shifts (08:40) and rest days (07:52). When mood, sleep duration and stress were accounted for, the results showed that when pilots woke for early shifts, the cortisol concentrations were correlated with a greater awakening response and with higher daily levels than during late shift or rest days (Bostock et al. 2013).

A twin study conducted by Stefan Wust supports this measurement reliability. Wust had his participants collect saliva samples at 0, 30, 45 and 60 minutes after waking at 0800 hours, and had participants fill out questionnaires covering chronic stress, self-esteem and self-efficacy. Despite finding a significant impact on cortisol waking response due to genetic factors, multiple factors related to chronic stress, such as worry, social stress, and lack of recognition were associated with the waking

cortisol response (Wust et al. 2000). One aspect of the genetic factors that may have impacted the

waking response is an individual's chronotype. A chronotype is the classification of when a person is and

feels the most alert, awake and productive. A person can be classified as some level of a lark, an early

morning person, or an owl, an evening person (Horne et al. 1976). Horne developed the Owl-and-Lark-

questionnaire in order to determine this classification in individuals and found that an individual's

chronotype classification was correlated with their natural circadian rhythm, based on analyzing body

temperature measurements taken throughout a day. Research that examined cortisol circadian rhythm

in the light of participant chronotype has found that an individual's chronotype does affect the cortisol

awakening response. When participants measured their salivary cortisol concentrations at 0, 30, 45 and

60 minutes after waking, the cortisol awaking response was greater for those who were classified as the

lark chronotype. This held true when sleep duration and waking times were accounted for (Kudielka et

al. 2006).

The conflicting evidence for a correlation between perceived stress and cortisol concentrations,

suggested by past literary work, indicates that there are many interacting factors involved with cortisol

regulation. However, the biological understanding of the HPA axis and its three primary regulatory

forces suggest that if the interacting factors can be better controlled, then a correlation between

perceived stress and cortisol concentrations may exist. Specifically, if the effects of chronotype and

circadian rhythm on salivary cortisol concentrations can be controlled, then a positive correlation

between perceived stress and cortisol concentration will be observed.

**Methods**

*Participants*

The participants in this study are both male and female students from Union College, between the ages of 18 and 23, spanning from freshman to seniors. Participants completed an informed consent form approved by the Union College Human Subjects Institutional Review Board (see Appendix A), and reported no prior history of mental illnesses or prescribed medication that could affect circadian rhythm patterns or HPA activity. Of the 50 participants who began this research project, only 12 successfully completed the project, and each participant did so under complete anonymity.

*Sample Collections*

Each participant was asked to complete a series of salivary sample collections and to complete a packet of surveys that measured perceived stress and chronotype during one morning. In order to take into account the cortisol awakening response, each student was required to wake at 8 am, and to provide salivary samples upon waking, 30 minutes after waking, 45 minutes after waking and 60 minutes after waking. Each sample should contain approximately 1 mL of Saliva. Participants collected samples in previously labeled collection tubes, labeled with a unique research ID number matching their individual packet number, and were directed to freeze them immediately. Each participant refrained from eating, drinking and brushing their teeth before collecting samples in the privacy of their own dorm room. Participants followed specific directions intended to minimize confounding variables (see Appendix B), and were instructed to note any deviations. Frozen saliva samples, along with completed survey packets, were then delivered to the designated laboratory.

Each participant was given a pre-numbered survey packet, matching the ID numbers printed on the saliva collection tubes. The packet contained the Trier Inventory of Chronic Stress (TICS) survey (see

Appendix C), an acute stress scale, ranging from 1 – 10, attached to research instructions and a collection log (see Appendix B), the Owl-and-Lark-questionnaire (see Appendix D), and a general demographic questionnaire (see Appendix E). The TICS survey was designed to measure perceived chronic stress and was chosen due to moderate past success in identifying correlations between perceived stress and cortisol concentrations (Wust et al. 2000). The acute scale was used to measure perceived acute stress, and was included due to literary evidence that show levels of acute and chronic perceived stress do not always correlate in parallel with cortisol levels (Murphy et al. 2010). Finally, the Owl-and-Lark-questionnaire was designed to determine a participant's chronotype, with lower scores indicating a preference towards an owl personality and higher scores indicating preference towards the lark personality.

*Salivary Cortisol Determination*

Saliva samples were used to determine each participant's free cortisol concentration levels, through analysis using the Enzyme-Linked Immunosorbent Assay (ELISA) technique. The procedure for the ELISA can be found in Appendix F, using the alternate, low range standard preparation procedure. Each of the four participant samples were analyzed in triplicate. A single participant's samples were analyzed during every ELISA reading in order to control for between plate reading discrepancies.

*Data Analysis*

Using the cortisol concentrations determined in each sample, each participant's cortisol awakening response reactivity could be determined by examining the concentration changes between each time point; 8-8:30 am, 8-8:45 am, 8-9 am, 8:30-8:45 am, 8:30-9 am, and 8:45-9 am. The concentration differences were determined as both absolute changes and percent changes. For each of these time point comparisons, the concentration changes were graphed against the complete set of TICS scores, acute stress scores and chronotype scores to determine possible individual correlation patterns.

In addition to comparing all of the cortisol concentration change data to perceived chronic stress, perceived acute stress and chronotype score, the same comparisons were made after cortisol concentration change results was subdivided into low and high changes. It was then determined whether possible correlations were expressed within these two cortisol subdivisions.

The TICS, acute stress and chronotype scores were also broken down into low, medium and high subdivisions. Each of these subdivisions were then graphed against each cortisol concentration, in the same manner as the complete data pool set, to determine any possible individual correlation patterns within each subdivided group. The chronotype score subdivision of low, medium and high represented characteristics of a moderate owl, neutral and moderate lark respectively.

Finally, the mean scores of each subdivided category was determined, and used to complete a factorial ANOVA analysis between two independent variables, perceived stress and chronotype, and the dependent variable of cortisol concentration changes. Two separate factorial ANOVA analyses were used to determine whether there was an effect between either of the independent variables and the dependent variable. The first using perceived stress scores for chronic stress, and the second using perceived stress scores for acute stress. This statistical test also determined whether there was an interaction between the two independent variables, perceived stress and chronotype.

**Results**
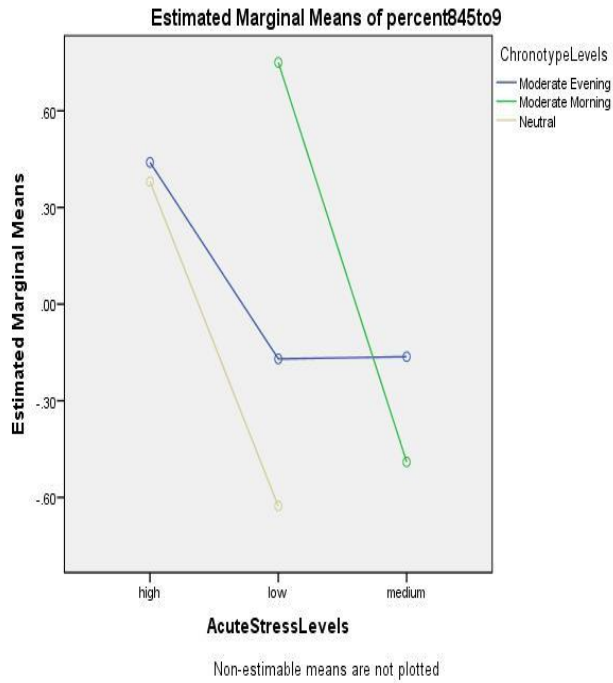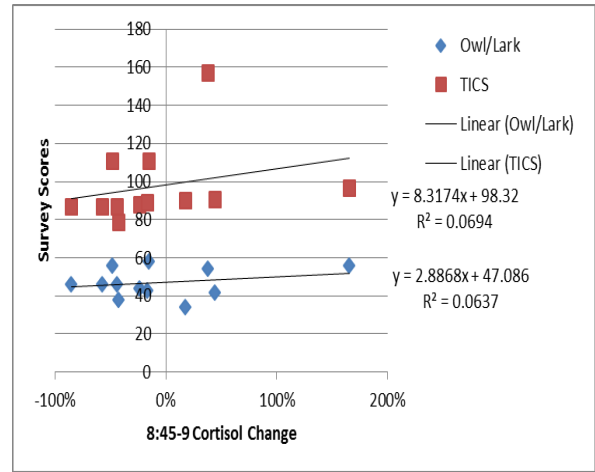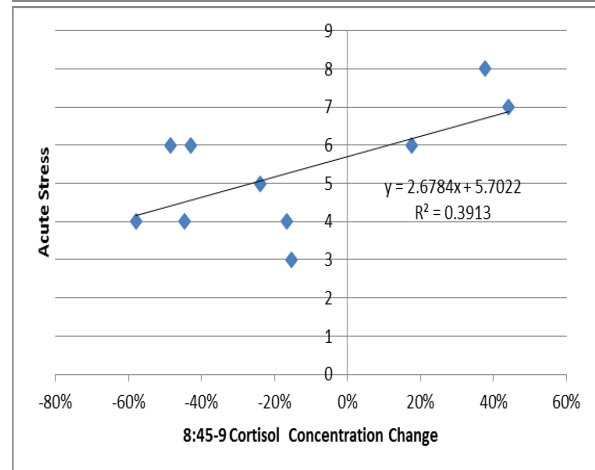


Figure 3. The data recorded during the 8:45-9 am time point comparison, represented through (A) the graphical representation of the factorial ANOVA analysis between acute stress, chronotype and percent cortisol change. (B) The relationship between chronic stress (TICS), chronotype (owl/lark) and percent cortisol concentration change. (C) The relationship between acute stress and percent cortisol concentration change.
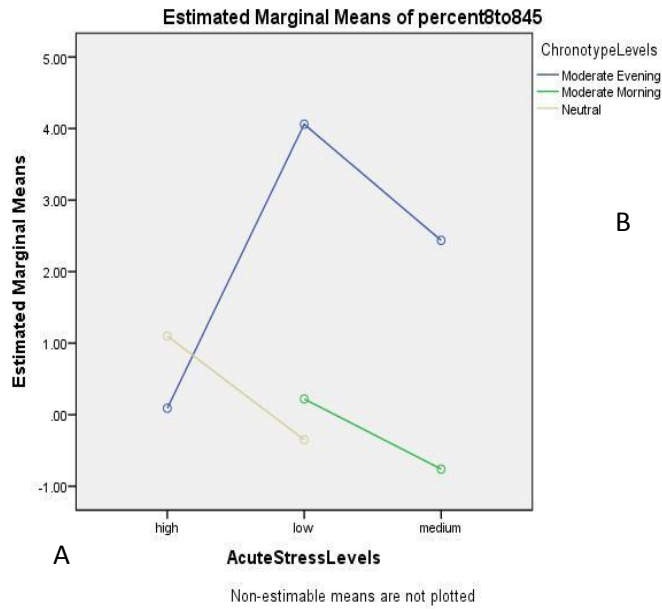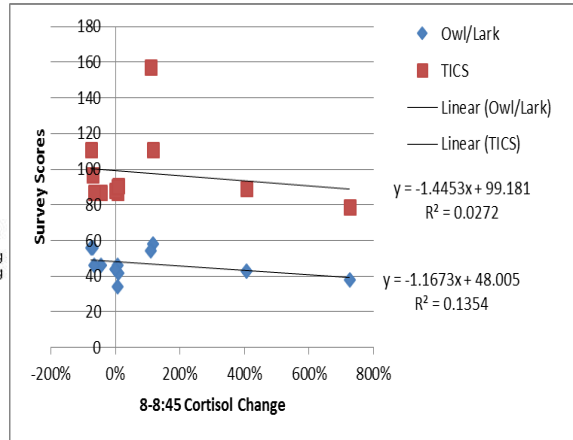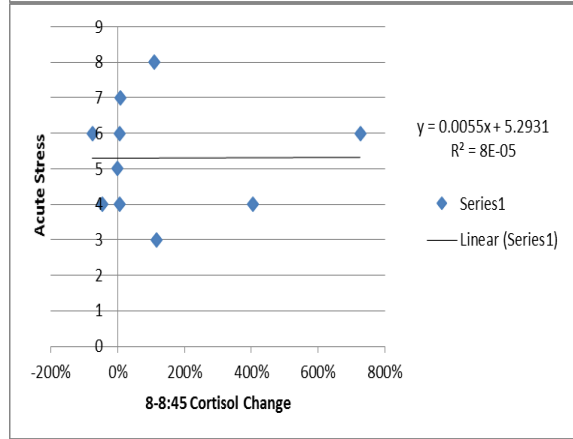
Figure 4. The data recorded during the 8-8:45 am time point comparison, represented through (A) the graphical representation of the factorial ANOVA analysis between acute stress, chronotype and percent cortisol change. (B) The relationship between chronic stress (TICS), chronotype (owl/lark) and percent cortisol concentration change. (C) The relationship between acute stress and percent cortisol concentration change.

Figure 5. The cortisol subdivided data recorded during the 8-8:45 am time point comparison, represented through (A) the relationship between chronic stress (TICS), chronotype (owl/lark) and low percent cortisol concentration change. (B) The relationship between chronic stress (TICS), chronotype (owl/lark) and high percent cortisol concentration change.
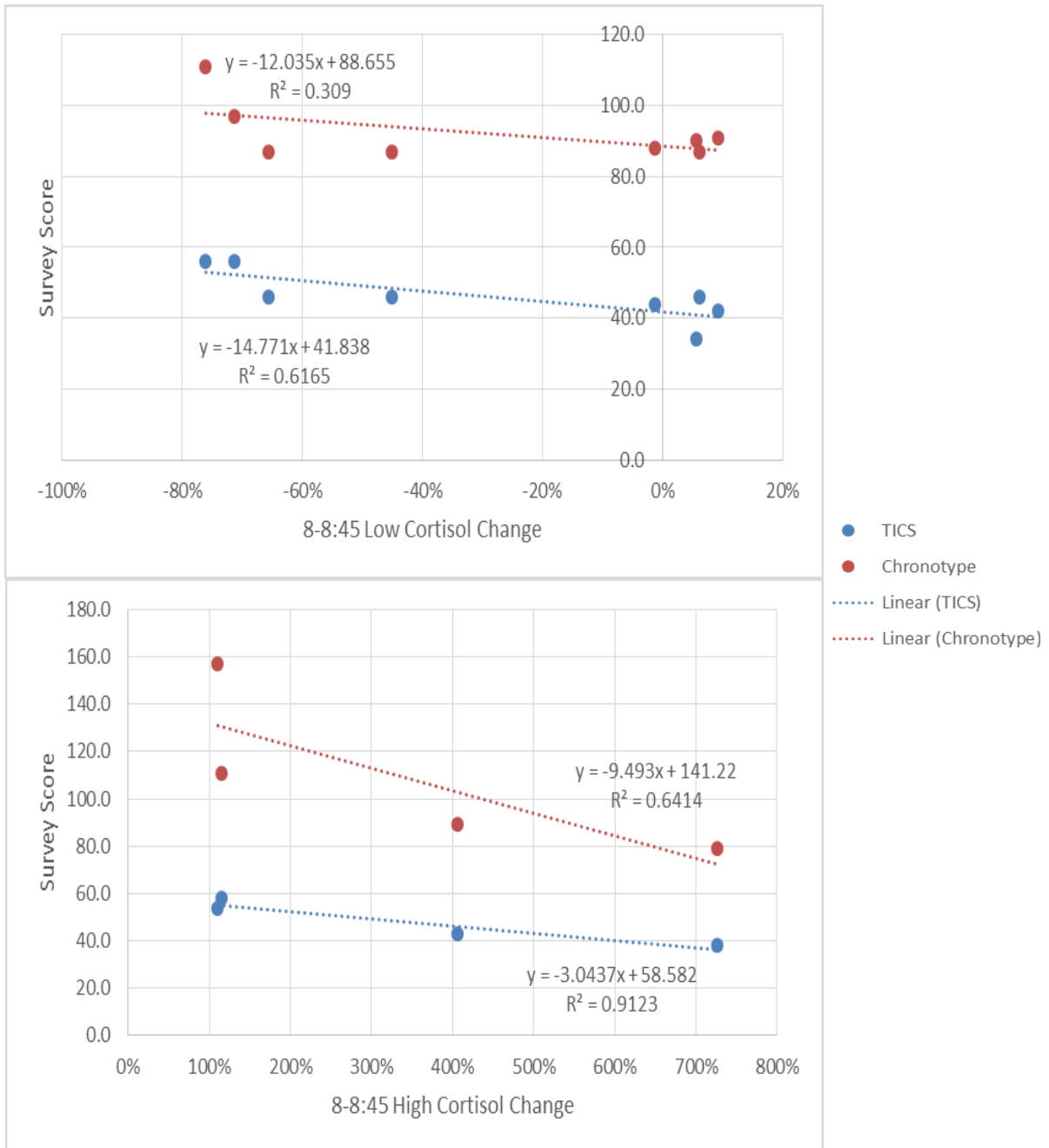
After the factorial ANOVA analyses it was determined that chronic perceived stress, acute perceived stress and chronotype had no statistically significant effect on the cortisol awakening response reactivity. This held true when the cortisol changes were analyzed as percent or as absolute changes. When analyzing correlative data trends between different time point comparisons, contradictory patterns emerged. The data gathered during the 8:45-9 am timespan is represented in Figure 3. This data shows a positive correlation between chronic stress and cortisol percent change, and chronotype score and cortisol percent change, with low $R^2$ values of .07 and .06 respectively. The positive correlative trend is seen again when acute stress scores are compared to cortisol percent changes, which has a moderate $R^2$ value of .4. However, when the data gathered during the 8-8:45 am timespan in Figure 4 is examined, it shows a negative correlation between chronic stress and cortisol percent change, and chronotype score and cortisol percent change, with $R^2$ values of .03 and .1 respectively. When acute stress is examined as a whole, there are no correlation trends. Each of the other time span comparisons lacked correlative trends, having very low $R^2$ values. This general lack of statistical significance, and the contradictory correlative trends across different time point comparisons, continued to hold true when each of the chronic stress, acute stress and chronotype different subgroupings were analyzed.

Despite the low correlation seen in Figure 4, where the entire 8-8:45 am time frame data pool is considered as a whole, a drastic change is seen when the cortisol concentrations changes were divided into low and high cortisol subgroupings. This subgroup examination can be seen in Figure 5, which shows a negative correlation for chronic stress and low percent cortisol change, as well as for chronotype and low percent cortisol change. These correlations show a higher $R^2$ value of .6 and .3 respectively. Figure 3 also shows a negative correlation for chronic stress and high percent cortisol change, as well as for chronotype and high percent cortisol change. These correlations have a high $R^2$ value of .9 and .6 respectively.

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**Discussion**

The purpose of this study was to determine whether stronger correlations between perceived stress and cortisol concentration changes could be determined, if the primary HPA axis regulatory forces could be controlled for, by accounting for circadian rhythm and chronotype. The hypothesis suggested that if these interacting factors were accounted for, then a positive correlation between perceived stress and cortisol concentration change, during the cortisol awakening response, would be observed. The consistently statistically insignificant differences for cortisol concentration changes, when chronotype and perceived stress were considered, are evidence against this hypothesis. Additional opposing evidence is the fact that there seems to be conflicting correlative trends when the complete data pool is examined at different time span comparisons.

As seen in Figure 3, the general trend was towards weak positive correlations between stress scores, chronotype and percent cortisol changes during the 8:45-9 am period. As perceived stress increased, both chronic and acute, participants had a larger cortisol concentration change. This correlative trend also indicated that people who were classified as evening chronotypes experienced lower cortisol changes. Yet, in Figure 4, the general trend was towards weak negative correlations between stress scores, chronotype and percent cortisol changes during the 8:-8:45 am period. As chronic perceived stress increased, participants had lower cortisol concentration changes. This correlative trend also indicated that people who were classified as evening chronotypes experienced higher cortisol changes.

When compared to 15 minute intervals during the CAR, literature has indicated that cortisol awakening response measurements, taken across trials, have higher consistent validity during the first $30 - 45$ minutes of the response. This increased validity suggests that the results expressed in Figure 4 may have a greater weight than the results expressed in Figure 3. If this is true, then this study supports

that there may be a negative correlation between perceived stress and cortisol changes during the CAR. This negative correlative trend is supported by the sharply increased $R^2$ values seen in Figure 5, when the cortisol concentrations are divided into the low and high change subgroups. Another interesting trend that can be seen is the parallel correlative trends when perceived stress, both chronic and acute, and chronotype are examined. Whether the correlation with cortisol concentration change is positive, or negative, both perceived stress and chronotype follow the same pattern. This can be seen when the data is analyzed as a whole, and when it is divided into the cortisol change subgroups.

Although this data is technically statistically insignificant, the low completion rate for participants in this study led to a very small data pool that could be analyzed, decreasing the possibility for statistically significant data. In light of this, it is important to consider the data trends that appear. When these are considered, it appears that there is evidence that a correlation between cortisol change and perceived stress can be established, even if this correlation is a negative one and is contrary to what is expected. These data trends also point towards a possible interaction between chronotype and perceived stress, giving supportive evidence to the importance of controlling chronotype and circadian rhythm when stress is being compared to cortisol concentration changes. This trend does support the hypothesis.

The emerging trends found through this research should be examined more closely in future research, during which the limitations of this study should be corrected. The largest limitation experienced was that the combination of participant anonymity, combined with participant resistance to waking up early in the morning, led to the inability to encourage full research completion by each of the participants. This severely limited the ability to analyze the substantial amounts of data that is needed to determine valid correlations. Future research will have to find a way to encourage a higher participant completion rate, possibly by adding rewards for higher completion percentages within

smaller anonymous groupings. This would have stronger encouragement possibilities than trying to

work with one large group of anonymous students, as was done in this study. Another major limitation

was that the early sampling, necessary to control for the CAR, required full time students to collect

samples individually, outside of a lab setting. This led to decreased control over confounding variables,

and created too large of an unknown, which was how precisely participants followed the instructions

and what degree they correctly noted any deviations. Future research may need to require supervised

collection periods in order to overcome this limitation. Finally, most college students identified

themselves as evening chronotypes, further limiting the comparative data available. Future research

may need to increase the age range of participants, as studies have shown that people tend to identify

with earlier chronotypes as they age (Straub, 2014).

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INFORMED CONSENT FORM

My name is Adam Bender, and I am a student at Union College in Schenectady, NY.  I am inviting you to participate in a research study.  Involvement in the study is voluntary, so you may choose to participate or not.  A description of the study is written below.

The purpose of this research is to determine if perceived stress correlates with salivary cortisol levels, when the effects of circadian rhythm and chronotype, whether an individual is an evening or morning person, are taken into account. If you consent to participate in this study, you will be provided with sterile collection tubes, asked to submit saliva samples collected at 0, 30, 45 and 60 minutes after waking during one morning beginning at 8 am, and include a timestamp for each collection. These samples can be stored in a personal freezer until they can be turned in later that day or can be brought to Prof. Cohen in Wold 220 or Wold 205. You will also be asked to complete the Horne and Ostberg's Owl-and-Lark-Questionnaire, in order to determine your chronotype, the Trier Inventory for Chronic Stress (TICS), which will measure chronic stress, and one question asking you to rate current stress levels from 1 through 10. Agreeing to participate in this study means that the results of the measured cortisol levels and the perceived stress scores will be compared in order to determine if there is a correlation between perceived stress and cortisol concentrations during the awakening cortisol response. You will not receive information about your individual results from this study. The entire study is done blindly and no names will be used on the samples or surveys.  Your decision about whether or not to participate will not jeopardize your future relations with Union College, your professors, or the investigators, and you can withdraw from this study at any time without penalty. This will take approximately twenty minutes over the course of 1 hour. You will also be asked to sign this informed consent form.

If you decide to participate in this study, fill out the following consent form, surveys, and submit the saliva samples. Your saliva samples will be kept in the Biochemistry Laboratory at Union College and will only be accessible by the researchers listed at the beginning of this information packet. We will be using the enzyme-linked immunosorbent assay (ELISA) technique in order to determine the cortisol concentration of the saliva sample.

If at any time you have concerns about your emotional or psychological health, Union College provides counseling services for students.  The Counseling Center can be contacted at:
Hours:  M - F 8:30 a.m. - 5 p.m.
Location: Wicker Wellness Center
Phone: (518) 388-6161
Email: uchealthcenter@union.edu
Web: www.union.edu/counseling
If you have any questions, please ask.  If you have additional questions, Prof. Brian Cohen: cohenb@union.edu, or I: bendera@union.edu, will be happy to answer them.

By signing below, you indicate that you understand the information above, and that you wish to participate in this research study.

_____        _____        _____
Participant Signature                                    Printed Name                                       Date

Appendix B

## Sample Collection Instructions

For this research study, we ask that each participant wakes up at 8 am to begin the saliva collection. Please prepare one sample at 0, 30, 45 and 60 minutes after waking in the provided tubes labeled A, B, C and D respectively. Each collection tube should be ½ to ¾ filled. On the sheet provided record the exact time that the collection occurred for each sample and rate your current stress level out of ten during the hour of sampling.  Additionally, please complete the TICS and the Owl and Lark questionnaires on the day of sample collection. Store the saliva samples within a freezer until they can be returned, with the completed surveys, to Adam Bender or Professor Cohen in Wold 205 or Wold 220. Within the lab, samples should be stored in the freezer and the completed surveys can be left in the green folder by the printer. During the hour of collection sampling, do not eat or brush your teeth, as both will affect cortisol concentrations.

| Collection Tube | Actual Time of Sample Collection |
|---|---|
| A – 0 Minutes | |
| B – 30 Minutes | |
| C – 45 Minutes | |
| D – 60 Minutes | |

On a scale from 1 – 10, with 10 being the highest, how would you rate your current level of stress?

1        2        3        4        5        6        7        8        9        10

Appendix C

| | Experience | In the past 3 months, I experienced it… | | | | |
|---|---|---|---|---|---|---|
| | | never | rarely | some-times | often | very often |
| 01 | I have to postpone much needed rest. | ⓪ | ① | ② | ③ | ④ |
| 02 | I receive too little appreciation for my accomplishments. | ⓪ | ① | ② | ③ | ④ |
| 03 | I make too many mistakes because what I have to do demands too much of me. | ⓪ | ① | ② | ③ | ④ |
| 04 | I do not have enough time to perform my daily tasks. | ⓪ | ① | ② | ③ | ④ |
| 05 | I must perform tasks that seem nonsensical to me. | ⓪ | ① | ② | ③ | ④ |
| 06 | I have differences of opinion with other that lead to tension. | ⓪ | ① | ② | ③ | ④ |
| 07 | I have work to do that involves carrying a lot of responsibility for other people. | ⓪ | ① | ② | ③ | ④ |
| 08 | Situations in which I must make an effort to win others' trust. | ⓪ | ① | ② | ③ | ④ |
| 09 | I worry that something unpleasant will happen. | ⓪ | ① | ② | ③ | ④ |
| 10 | My daily tasks are not interesting. | ⓪ | ① | ② | ③ | ④ |
| 11 | Times when I am lonely. | ⓪ | ① | ② | ③ | ④ |
| 12 | Situations when I must take pains to have a good relationship with others. | ⓪ | ① | ② | ③ | ④ |
| 13 | I have to perform tasks that I do not enjoy. | ⓪ | ① | ② | ③ | ④ |
| 14 | I have tasks to perform during which I am being critically observed. | ⓪ | ① | ② | ③ | ④ |
| 15 | I have conflicts with others because they have different goals. | ⓪ | ① | ② | ③ | ④ |
| 16 | Times when I cannot suppress worrisome thoughts. | ⓪ | ① | ② | ③ | ④ |
| 17 | Times when so many business appointments accumulate that I can barely get caught up. | ⓪ | ① | ② | ③ | ④ |
| 18 | I try in vain to gain recognition for my good work. | ⓪ | ① | ② | ③ | ④ |
| 19 | I spend a lot of time dealing with other peoples' problems. | ⓪ | ① | ② | ③ | ④ |

| | Experience | In the past 3 months, I experienced it… | | | | |
|---|---|---|---|---|---|---|
| | | never | rarely | some-times | often | very often |
| 20 | I perform my tasks inadequately, despite trying my best. | ⓪ | ① | ② | ③ | ④ |
| 21 | Times when none of my tasks seem meaningful to me. | ⓪ | ① | ② | ③ | ④ |
| 22 | I have work to do that must not disappoint others. | ⓪ | ① | ② | ③ | ④ |
| 23 | I have to try to make a good impression with people. | ⓪ | ① | ② | ③ | ④ |
| 24 | Times when I can no longer cope with the demands of my work. | ⓪ | ① | ② | ③ | ④ |
| 25 | Times when my worries overwhelm me. | ⓪ | ① | ② | ③ | ④ |
| 26 | I have conflicts with other because I do not act the way they expect me to. | ⓪ | ① | ② | ③ | ④ |
| 27 | Times when I must work under strict deadlines. | ⓪ | ① | ② | ③ | ④ |
| 28 | I have to deal with other peoples' problems too much. | ⓪ | ① | ② | ③ | ④ |
| 29 | Times when I do not have the opportunity to share my thoughts and feelings with others. | ⓪ | ① | ② | ③ | ④ |
| 30 | Situations in which it depends entirely on me if a relationship with another person develops satisfactorily. | ⓪ | ① | ② | ③ | ④ |
| 31 | Although I do my best, my work is not appreciated. | ⓪ | ① | ② | ③ | ④ |
| 32 | I have tasks to fulfill that pressure me to prove myself. | ⓪ | ① | ② | ③ | ④ |
| 33 | I have conflicts with others because they meddle too much in my affairs. | ⓪ | ① | ② | ③ | ④ |
| 34 | Times when I am isolated from other people. | ⓪ | ① | ② | ③ | ④ |
| 35 | Times when I am not able to perform as well as expected. | ⓪ | ① | ② | ③ | ④ |
| 36 | Times when I worry a lot and cannot stop. | ⓪ | ① | ② | ③ | ④ |
| 37 | I object to duties that I must fulfill. | ⓪ | ① | ② | ③ | ④ |
| 38 | Times when I have too many duties to fulfill. | ⓪ | ① | ② | ③ | ④ |

| | Experience | In the past 3 months, I experienced it... | | | | |
|---|---|---|---|---|---|---|
| | | never | rarely | some-times | often | very often |
| 39 | I must frequently care for the well-being of others. | ⓪ | ① | ② | ③ | ④ |
| 40 | Situations in which I must make an effort to please others. | ⓪ | ① | ② | ③ | ④ |
| 41 | Times when I have nothing meaningful to do. | ⓪ | ① | ② | ③ | ④ |
| 42 | Times when I have too little contact with other people. | ⓪ | ① | ② | ③ | ④ |
| 43 | People have high expectations for tasks that I must fulfill. | ⓪ | ① | ② | ③ | ④ |
| 44 | Times that my work overwhelms me. | ⓪ | ① | ② | ③ | ④ |
| 45 | I have arguments with people that lead to long-lasting conflicts. | ⓪ | ① | ② | ③ | ④ |
| 46 | I am not adequately rewarded for my efforts. | ⓪ | ① | ② | ③ | ④ |
| 47 | I worry that I will not be able to fulfill my tasks. | ⓪ | ① | ② | ③ | ④ |
| 48 | I must do work that does not take advantage of my abilities. | ⓪ | ① | ② | ③ | ④ |
| 49 | Situations in which the well-being of others depends on how well I work. | ⓪ | ① | ② | ③ | ④ |
| 50 | I have too many tasks to perform. | ⓪ | ① | ② | ③ | ④ |
| 51 | Times when I miss having contact with others. | ⓪ | ① | ② | ③ | ④ |
| 52 | I have unnecessary conflicts with others. | ⓪ | ① | ② | ③ | ④ |
| 53 | Times when I have no tasks that make me happy. | ⓪ | ① | ② | ③ | ④ |
| 54 | I experience having too much to do. | ⓪ | ① | ② | ③ | ④ |
| 55 | Although I try, I do not fulfill my duties as I should. | ⓪ | ① | ② | ③ | ④ |
| 56 | Times when I have no friends to do things with. | ⓪ | ① | ② | ③ | ④ |
| 57 | Times when my responsibility for others becomes a burden to me. | ⓪ | ① | ② | ③ | ④ |

## Lark or Owl Questionnaire

- Make sure that you read each question carefully before answering.
- Although each question has a selection of answers, choose one only.
- Answer every question.
- Trust your initial response and answer honestly.
- There is no right or wrong answer; it is your personal experience that counts.
- When you have finished, add up your score to determine your 'best time' performance.

1. Taking into account only your own 'feeling best' rhythm, at what time would you get up if you were entirely free to choose?

   | | |
   |---|---|
   | Before 6 am | 5 |
   | 6 -7 am | 4 |
   | 7 – 9 am | 3 |
   | 9 – 10 am | 2 |
   | After 10 am | 1 |

2. Taking into account only your own 'feeling best' rhythm, at what time would you go to bed if you were entirely free to choose?

   | | |
   |---|---|
   | Before 9 pm | 5 |
   | 9 – 10 pm | 4 |
   | 10 pm – 12 am | 3 |
   | 12 – 1 am | 2 |
   | After 1 am | 1 |

3. If there is a particular time by which you must get up in the morning, to what extent do you rely on being woken up by an alarm of some kind?

   | | |
   |---|---|
   | Not at all dependent | 4 |
   | Somewhat dependent | 3 |
   | Fairly dependent | 2 |
   | Completely dependent | 1 |

4. After a usual night's sleep, do you find it easy to get up in the mornings?

   | | |
   |---|---|
   | Not at all easy | 4 |
   | Not very easy | 3 |
   | Fairly easy | 2 |
   | Very easy | 1 |

5. Within 30 minutes of getting up in the morning, how alert do you feel?

   | | |
   |---|---|
   | Not at all easy | 4 |
   | Not very easy | 3 |
   | Fairly easy | 2 |
   | Very easy | 1 |

6. Within 30 minutes of getting up in the morning, how stimulated is your appetite?

   | | |
   |---|---|
   | Not at all easy | 4 |
   | Not very easy | 3 |
   | Fairly easy | 2 |
   | Very easy | 1 |

7. Within 30 minutes of getting up in the morning, how tired or otherwise do you feel?

| | |
|---|---|
| Very tired | 4 |
| Fairly tired | 3 |
| Fairly refreshed | 2 |
| Very refreshed | 1 |

8. If you have no special plans the next day, at what time would you go to bed compared with your normal routine?

| | |
|---|---|
| Seldom or never later | 4 |
| Less than 1 hour later | 3 |
| 1-2 hours later | 2 |
| More than 2 hours later | 1 |

9. You and a friend commit to doing some regular exercise together for 1 hour twice a week, and it suits your friend to do it between 7 and 8 am.  Taking into account only your own 'feeling best' rhythm, would you...?

| | |
|---|---|
| Be in good form | 4 |
| Be in reasonable form | 3 |
| Find it difficult | 2 |
| Find it very difficult | 1 |

10. You have given an undertaken to do 2 continuous hours of hard physical work, but you can choose any time at all to do it. Taking into account your own 'feeling best' time, which one of the following slots would you choose?

| | |
|---|---|
| 8-10 am | 4 |
| 11 am – 1 pm | 3 |
| 3-5 pm | 2 |
| 7-9 am | 1 |

11. At home in the evening, at what time would you feel tired and make preparations for bed?

| | |
|---|---|
| Before 9 pm | 5 |
| 9-10 pm | 4 |
| 10 pm – 12 am | 3 |
| 12-1 am | 2 |
| After 1 am | 1 |

12. You have got a 2-hour, mentally exhausting test coming up.  Taking into account your 'feeling best' time, which of the following time slots would you choose to do the test in?

| | |
|---|---|
| 8-10 am | 4 |
| 11 am – 1 pm | 3 |
| 3-5 pm | 2 |
| 7-9 pm | 1 |

13. Would you be tired if you went to bed at 11 pm?

| | |
|---|---|
| Not at all | 4 |
| A little | 3 |
| Fairly | 2 |
| Very | 1 |

14. If you have gone to bed several hours later than usual but are free to do whatever you like the following morning, wi you...?

| | |
|---|---|
| Wake up at the usual time and not fall asleep again | 4 |
| Wake up at the usual time and then doze for a while | 3 |
| Wake up at the usual time and then go back to sleep | 2 |
| Not wake up until later than usual | 1 |

15. You're involved in a night-watch exercise, which means you have to stay awake between 4 and 6 am.  Assuming n commitments the following day, which one of the following options would best suit you?

| | |
|---|---|
| Don't go to bed until the watch is over | 4 |
| Take a nap before and sleep properly afterwards | 3 |
| Take a good sleep before and nap afterwards | 2 |
| Go to bed in time to have a full sleep before the watch | 1 |

16. You and a friend commit to doing some regular exercise together for 1 hour twice a week and it suits your friend to do i between 10 and 11 pm.  Taking into account only your own 'feeling best' rhythm, would you?

| | |
|---|---|
| Be in good form | 4 |
| Be in reasonable form | 3 |
| Find it difficult | 2 |
| Find it very difficult | 1 |

17. You were offered a really interesting job that paid by results and involved working for 5 consecutive hours each day, whic 5-hour time slot would you choose?

| | |
|---|---|
| 4-9 am | 5 |
| 7 am – 12 pm | 4 |
| 10 am – 3 pm | 3 |
| 4-9 pm | 2 |
| 9 pm – 2 am | 1 |

18. On any given day, at what time would you achieve your 'feeling best' peak?

| | |
|---|---|
| 5-7 am | 5 |
| 8-9 am | 4 |
| 10 am – 4 pm | 3 |
| 5-9 pm | 2 |
| 10 pm – 4 am | 1 |

19. If you had to describe yourself as a morning or evening type of person, which one of the following would come closet?

| | |
|---|---|
| Absolutely a morning type | 4 |
| Tend more towards a morning type then an evening type | 3 |
| Probably more an evening type than a morning type | 2 |
| Absolutely an evening type | 1 |

Appendix E

Gender?                    M        F

Academic Year?        First Year        Sophomore        Junior   Senior

re you currently receiving counseling from a mental health professional?Y        N

Are you currently taking any prescription medication related to your mental health?        Y        N

Appendix F

**Cortisol-EIA: Last Modified 10/1/2008**

**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:<mark>30,000</mark> 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:<mark>6000</mark> 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.

- 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_2$0; Store at 4$^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 4ºC):

| | | |
|---|---|---|
| 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 |
| dd$H_2O$ | 50 ml | 100 ml |

Adjust pH to 7.4; Store at 4ºC.

- 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$_2$PO$_4$ | 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 37ºC** (NOTE: Plates can also be incubated overnight at 4ºC)

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
- 
14.) Other plate reader notes:
- For the standards, fit a "4 Parameter Curve" – this setting is good for situations such as ours where we have a non-linear/sigmoidal relationship within the data set.

## IV. References

Carey, J.B. and McCormick, S.D. 1998. Atlantic salmon smolts are more responsive to handling and confinement stress than parr. Aquaculture 168:237-253.

Munro, C. and Stabenfeldt, G. 1984. Development of a microtiter plate enzyme immunoassay for the determination of progesterone. J. Endocr. 101, 41-49.

## V. Summary of Steps for Cortisol EIA

### DAY 1:
1.) **ANTI-BODY COATING**: at 1:30,000 final dilution in Coating Buffer, 150 µl/well. Seal plate, then **incubate for 3 Hours at 37ºC**

2.) **WASH THE PLATE 5X** (Prime Washer, then Protocol "P1")

3.) **BLOCKING**: 250 µl/well EIA Buffer; 30 minutes at room temperature, then aspirate the wells ("P2" on washer; Rinse washer when done)

4.) **ADD HRP, BUFFER, SAMPLES AND STANDARDS**:
- 100 µl/well of cortisol-HRP conjugate at 1:6000 in EIA Buffer to all except Blanks, which get 100 µl/well EIA Buffer only
- Add 2.5 µl/well of Standard or Sample.
- 150µl/well EIA Buffer to the plate (all wells).

5.) **OVERNIGHT INCUBATION –** Seal the plate and incubate overnight at 25ºC.

*DAY 2:*
6.) Measure out **TMB Peroxidase substrate** and warm to room temperature (~1.5 hr).

7.) **SET UP MICROPLATE READER**

8.) **WASH THE PLATE 5X** (Prime Washer, then Protocol "P1")

9.) **ADD TMB REAGENT:** 150µl/well

10.) **KINETIC RUN:** ~10 minutes. The desired range is $E_0$=0.6-0.9.

11.) **ENDPOINT RUN #1**

12.) **STOP THE REACTION** by adding **100 µl of 0.5 M HCl** to each well

13.) **ENDPOINT RUN #2** The desired range is $E_0$=1.8-2.0