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Evaluating Salivary Alpha-Amylase as a Biomarker for Stress

Denise L. Alfano

Master's Thesis

Mentor: Dr. Roberta Moldow, Ph.D.

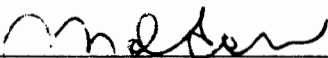
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July 31, 2008

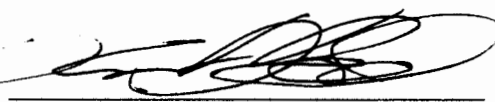
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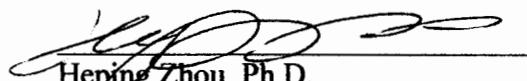
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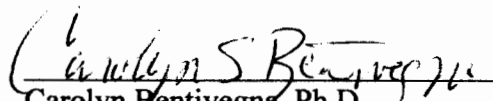

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All my love and gratitude goes to each of you for all you have done for me without ever asking anything in return.

1. Introduction:

1.1 Purpose

The goal of this research is to evaluate Salivary Alpha Amylase (sAA) as a reliable biomarker for activation of the Sympathetic Adrenal Medullary (SAM) System during the stress response. This research poses the questions, does sAA increase significantly during stressful situations; is this increase physiologically significant; if so, does this rise in sAA signal activation of the autonomic nervous system, and finally, does this activation correlate or interact with the activation of the Hypothalamic Pituitary Adrenal (HPA) axis and cortisol release? Cortisol, the quintessential stress biomarker, is measured in saliva as well to verify initiation of the stress response in the HPA axis. Thus, it is necessary to find a reliable biomarker for the SAM system activation that is as accurate and noninvasive as salivary cortisol is in relation to activation of the HPA axis (Chatterton et al., 1996; Van Stegeren et al., 2006).

Ultimately, the reason for this study is to learn more about the stress response in order to improve training procedures for military and first responders. Psychological stress has been implicated in cognitive function deficits and attentional processes impairment (Mackenzie et al., 2007), which may have drastic consequences for military personnel and first responders who are thrust into highly stressful situations but must maintain adequate cognition and attention in order to save the lives of others and themselves.

In this study, we examined salivary amylase and compared it to salivary cortisol as a biomarker for stress. Salivary amylase reflects activation of the sympathetic nervous whereas salivary cortisol reflects the HPA axis. The stressors examined were tasing, training drills and oral or written exams.

1.2 Stress Response: SAM system and HPA axis

Stress is defined as "...a mentally or emotionally disruptive or upsetting condition occurring in response to adverse external influences and capable of affecting physical health, usually characterized by increased heart rate, a rise in blood pressure, muscular tension, irritability, and depression" ("stress", 2004). It is "any strain or interference that disturbs the functioning of an organism" ("stress", 2008). The stress response is the result of two mechanisms working in concert: the SAM system and the HPA axis (Henry, 1992). Some studies have tried to separate the effects and reactivity of the two systems (Schommer et al., 2003) but according to Wetherell et al., "...HPA and SAM reactivity are rarely assessed simultaneously, either through omission, or inability of chosen stressor to elicit reactivity in both axes..." The stress response is characterized by activation and interaction of both axes. Measurement of one system only hinders investigation of other factors that modulate the stress response (Wetherell et al., 2006). Activation of the HPA axis releases cortisol into the blood. Reasonable increases in cortisol are healthy, but repeated exposure to stressors can lead to dysregulation of the HPA axis which can have adverse effects (Gupta et al., 2007; Armario et al., 2008). Activation of the SAM system causes a change in norepinephrine and epinephrine release from the adrenal medulla as well as increases in heart rate. Norepinephrine and epinephrine are hard to measure in plasma and virtually impossible in saliva. Thus, markers for the sympathetic nervous system have been sought.

1.3 Cortisol

Cortisol increases with stress due to activation of HPA axis and release of cortisol from the adrenal cortex. In the HPA Axis, cortisol has been widely studied as an indicator of the stress

response in part because it is easily measured in saliva, and most importantly, because salivary cortisol levels are representative of plasma cortisol levels in the body. HPA axis activation has been demonstrated in response to physical and psychosocial stressors such as cold-pressor tests (Schwabe et al., 2008), stressful video viewing (Takai et al., 2004; Pietrowsky and Schramm, 2006), and public speaking (Garcia-Leal et al., 2005; Bassett et al., 1987) among others.

1.4 Amylase

sAA may be an indicator of SAM system activation (Nater et al., 2004). A pharmacological study involving beta blockers supports the theory that elevation in sAA concentrations may be representative of adrenergic activity in response to psychological stressors (Van Stegeren, 2006). sAA is an enzyme found in saliva that sAA breaks down complex carbohydrates into simple sugars in the mouth at the beginning of the digestive process (Chiappelli, 2006). It is released via stimulation of the beta-receptor, and is produced and secreted by serous cells found in abundance in the parotid salivary glands. Both sympathetic and parasympathetic nervous fibers innervate the salivary glands and it has been reported by other publications that exercise and psychological stress may stimulate sAA production (Nater et al., 2004). Studies show that salivary amylase levels correlate with norepinephrine levels in the blood and thus may be indicative of SAM system activation (Chatterton, et al., 1996). sAA concentrations have been shown to rise with increases in psychological and psychosocial stressors (Noto et al. 2005) and pain (Shirasaki et al., 2006). Ehlert et al. proposed that since yohimbine hydrochloride caused a rise in adrenergic activity and activation of sAA and autonomic parameters, this supports the idea that rising amylase concentrations signals activation of central sympathetic systems and the autonomic nervous system (Ehlert et al., 2006).

Use of salivary biomarkers has become increasingly more popular due to advances in molecular technology allowing for easier, faster sample collection (Chiappelli et al., 2006) some requiring only 5uL of saliva (Yamaguchi et al., 2004). Due to its ease of measurement and noninvasive extraction, salivary biomarkers like amylase are an attractive candidate for biobehavioral research (Hofman, 2001; Rohleder et al., 2004).

2. Methods:

2.1 Subjects

2.1.1 Tasing Subjects

Tasing CH subjects [n=16 (10 male, 6 female), averaging 26 years of age, ranging from 20 to 48 years old], Tasing SF subjects [n=18 (17 male, 1 female), averaging 31 years of age, ranging from 22 to 50 years old], Tasing NB subjects [n=12, (7 male, 5 female), averaging 28 years of age, ranging from 23 to 44 years old], Tasing WH subjects [n=9 (8 males, 1 female), averaging 30 years of age, ranging from 22 to 40 years old], and Tasing FFX subjects [n=13 (9 males, 4 females) averaging 25 years of age, ranging from 22 to 28 years old] were all tased with an X-26 taser equipt with 50,000V peak open circuit voltage, 2.1mA average current (Taser®, 2007) for 5 seconds during a training exercise with the exception of Tasing WH subjects whose exposure time varied amongst individuals between 1 and 5 seconds.

Saliva samples were collected the morning before tasing [CH (0537-0730), SF(0614), NB (0754-0807), WH (0900- 1000), FFX (0628-0640)], 20 minutes after the tasing exposure [CH (0720-1119), SF (0754-1259), NB (1031-1511), WH tased approximately 9:40am(0922-0958),

FFX(0808-1237)] and the morning after tasing [CH (0540-0630), SF(0610), NB (0811-0817), FFX (0613-0630)].

In Tasing FC subject set, [n=15, (13 male, 2 female), averaging 25 years of age, ranging from 21 to 35 years old.] nine subjects' baseline samples were taken at 9:20am, tased between 9:45am and 9:55am, and twenty minutes after 5 second exposure to X-26 taser, a second saliva sample was collected. Eight subjects' baseline samples were taken at 6:40am, tased between 7:06am and 7:20, and twenty minutes after exposure a second saliva sample was collected. Third samples for all Tasing FC subjects were obtained the morning after tasing.

2.1.2 FIG Subjects

Subjects [n=17, (12 male, 5 female) averaging 20 years of age, ranging from 18 to 24 years of age.] were involved in a series of physical training exercises and volunteered saliva earlier in the morning, prior to the training at about 1:00pm, and a second time approximately ten minutes after the beginning of the exercise around 4:00pm. The training consisted of a series of stations in an obstacle course including a wall jump, low crawl, monkey bars, rope climbing, etc. Subjects were asked not to drink water prior to providing a sample.

2.1.3 PAD Subjects

Subjects [n=7, all males, average 46 years of age, ranging from 26 to 65 years old] coordinating the efforts of hundreds of individuals during a disaster drill for paramedics, first aid and first responders were asked to provide a sample before the drill at approximately 7:00am (time point 1) and after the drill at approximately 1:00pm (time point 2).

2.1.4 Exam Anxiety Subjects

E1 Subjects [n=6, all female, averaging 20 years of age, ranging from 19 to 20 years old], E2 Subjects [n=11 (2 male, 9 female), averaging 21 years of age, ranging from 19 to 21 years old], E3 Subjects [n=7, all female, averaging 19 years of age from 18 to 20 years old] and E4 Subjects [n=4 (2 male, 2 female), averaging 21 years of age, ranging from 20 to 22 years old] participated. Samples were obtained before and after the exam and at comparable times on a control day except for E1 where no control day samples were obtained.

2.1.5 Oral Presentation Anxiety Subjects

Subjects [n=2 (one male 35 years of age, one female 24 years of age)] performed a ten minute presentation in front of peers. Samples were collected immediately before and immediately after the presentation.

2.2 Saliva Sampling

Saliva samples were obtained in 1mL aliquots without the aid of chemical stimulants or cotton rolls which may interfere with assay results (Shirtcliff et al., 2001). Subjects were asked to supply saliva samples via passive drool into test tubes before and after the stress event. When possible, control samples were taken in the absence of the stressor either long before the event or days after. Samples were immediately placed on dry ice until they could be transported to a -70°C freezer for storage where they remained before being thawed on ice for assay purposes. Thawed samples were centrifuged at 1500 x g (3000rpm) for 15 minutes. Only clear sample was

assayed. Any particulate matter would interfere with results by hindering antibody binding. All samples were assayed in duplicate and the mean values were utilized for statistical analysis.

2.3 Biochemical Analysis

2.3.1 Amylase

Samples were analyzed using the Salimetrics™ Salivary Alpha-Amylase Assay Kit protocol. A chromagenic substrate, 2-chloro-p-nitrophenol linked with maltotriose, interacts with the enzyme amylase and can be detected via spectrometry at 405nm. The absorbance at 405nm is directly proportional to the amount of amylase activity and thus the concentration of amylase in the sample (Salimetrics™, State College, PA).

2.3.2 Cortisol

Samples were analyzed using Salimetrics™ Salivary Cortisol Enzyme Immunoassay Kit protocol (Salimetrics™, State College, PA).

2.4 Psychological Assessment

A series of psychological surveys were obtained from subjects (except those participating in the taser study and the oral presentations) before and after the stress event including the following self-rating tests; Borg Rating of Perceived Exertion Scale, Thayer Activation-Deactivation Adjective Check List (AD-ACL), and Spielberger State Trait Anxiety Inventory (STAI). For Borg, the subject rates how hard he or she believes he/she is exerting him or herself on a 16 point scale that ranges from 6 “no exertion at all” to 20 “maximal exertion.” AD-ACL is a list of

adjectives which subjects were asked to rate how much they feel like the particular adjective on a scale of 1-4, 1 being not at all and 4 being the greatest. The numbers were then entered into an equation whose result is a numerical value for energetic arousal and tense arousal among others. STAI is two sets of 20 items/adjectives where the subjects were asked to fill in answers for one, how they feel “at that moment” and the other how they “generally feel” on a scale of 1-4, 1 being not at all and 4 being the greatest.

2.5 Statistical Analysis:

The numerical values obtained from psychological surveys as well as the concentrations of steroid/enzyme were analyzed using Number Cruncher Statistical Software (NCSS) to perform the following tests/analyses: Error bar charts, Repeated Measures ANOVA (RM-ANOVA) Tukey Kramer Multiple Comparison Test (with the exception of Tasing NB, Tasing WH, and E4 subject sets in which the less stringent Newman-Keuls Multiple Comparison Test was used due to lack of significance with Tukey Kramer Multiple Comparison Test), paired t-tests, linear regressions, and correlation matrices.

2.6 Consent

Sample collection was completely anonymous and completely voluntary except for the taser study. Subjects read the consent forms but did not provide a signature, as it would negate anonymity. Any questions they had regarding sample collection and testing were answered prior to participation. Volunteers were not pressured to participate and were reminded that they could stop at any time. All recruitment procedures and sample collection and analysis protocols detailed here were approved by the Institutional Review Boards (IRB) of SHU, USAMRMC,

and/or UMDNJ where appropriate. Once consent was provided, they were asked to complete a survey to gather demographic information (e.g., gender, age, medications, and food and beverage consumption).

3. Results:

3.1 Tasting

3.1.1 Tasting CH

Repeated Measures ANOVA reveals that there is a significant increase in salivary amylase concentrations at 20 minutes after tasting (time point 2) compared to the mornings before (time point 1) and after (time point 3) tasting (**Figure 1A**) [$F(2,47)=8.640$; $p=0.001$]. There is a significant increase in salivary cortisol concentrations at time point 2 [$F(2,47)=12.470$; $p < 0.001$] (**Figure 1B**). If one looks at the individual responses for amylase as seen in **Figure 1C**, a large variation in the response is evident as well as with the initial levels. Linear regression analysis between cortisol and amylase here is not significant (**Figure 1D**) [$R^2 = 0.043$; $F(2,47) = 2.073$; $p \geq 0.050$].

3.1.2 Tasting SF

In **Figure 2 A and B**, there is a significant increase in salivary amylase concentrations [$F(2,53) = 13.230$; $p < 0.001$] and salivary cortisol concentrations [$F(2,53) = 7.160$; $p=0.003$] at 20 minutes after tasting (time point 2) as compared to the morning before (time point 1) and after (time point

3). If one looks at the individual responses for amylase as seen in **Figure 2C**, a large variation in the response is evident as well as with the initial levels. Linear regression reveals that there is no significant correlation between cortisol and amylase (**Figure 2D**) [$R^2 = 0.002$; $F(2,53) = 0.071$; $p \geq 0.050$].

3.1.3 Tasting FFX

In **Figure 3**, Repeated Measures ANOVA for salivary cortisol concentrations reveals a significant increase 20 minutes after tasting (time point 2) when compared to the morning before (time point 1) and after (time point 3) tasting [$F(2,38) = 6.830$; $p = 0.005$] (**Figure 3B**). Amylase however does not show similar trends [$F(2,38) = 1.190$; $p \geq 0.050$] (**Figure 3A**); tasting had no significant effect on salivary amylase concentrations, especially considering the broad range of overlapping. The difference in the salivary amylase and cortisol responses can be seen by comparing the individual responses to salivary amylase (**Figure 3C**) and salivary cortisol (**Figure 3D**). Thus, it is no surprise that linear regression analysis between salivary cortisol and amylase levels shows no relationship [$R^2 = 0.037$; $F(2,38) = 1.297$; $p \geq 0.050$] (**Figure 4**).

3.1.4 Tasting NB

Salivary amylase concentrations [$F(2,35) = 5.810$; $p = 0.009$] (**Figure 5A**) and salivary cortisol concentrations [$F(2,35) = 15.400$, $p < 0.001$] (**Figure 5B**) are significantly higher 20 minutes post tasting (time point 2) based on Repeated Measures ANOVA. When looking at individual responders, Subject 18 showed cortisol levels as well as amylase levels increasing at 20 minutes after tasting, time point 2 (**Figure 5 C and D**). Linear regression analysis between cortisol and amylase showed no trends [$R^2 = 0.022$; $F(2,35) = 0.753$; $p \geq 0.050$] (**Figure 6**).

3.1.5 Tasting WH

Twenty minutes post tasting (time point 2), salivary cortisol concentrations were elevated, approximately 900pg/100uL as compared to the morning before (time point 1) and the morning after tasting (time point 3) [F(2,26)=24.400; $p < 0.001$] which were also significantly different from one another. The morning before tasting (time point 1), salivary alpha amylase levels were significantly lower than twenty minutes after tasting (time point 2) as well as the morning following the tasting (time point 3) F(2,26)=6.080; $p = 0.011$] (**Figure 7A-C**). Linear regression showed no correlation between salivary amylase and salivary cortisol concentrations [$R^2 = 0.028$; F(2,26) = 0.712; $p \geq 0.050$] (**Figure 7D**).

3.1.6 Tasting FC

Repeated measures ANOVA [F(2,44)= 0.590; $p \geq 0.050$] found no differences in salivary amylase levels amongst three time points, the morning before tasting, twenty minutes after, and the morning after, as a result of a great deal of overlap between groups and large variation amongst individuals (**Figure 8A and 8C**). Salivary cortisol concentrations demonstrated a significant increase twenty minutes after tasting [F(2,44)= 21.060; $p \ll 0.001$] (**Figure 8B and 8D**). Linear regression, amylase vs. cortisol [$R^2 = 0.017$; F(2,44)= 0.743; $p \geq 0.050$] was not significant (**Figure 9**). It should be noted that the morning before tasting sample time for this group was right before tasting.

3.2 FIG Training Exercise

Figures 10A and 10B clearly indicate that, ten minutes following the training exercise (time point 2), both salivary amylase ($p < 0.001$) and salivary cortisol ($p = 0.005$) concentrations were elevated as compared to earlier in the day (time point 1). A linear relationship was present between amylase and cortisol in this case [$R^2 = 0.419$; $F(1,33) = 23.081$; $p < 0.001$] (**Figure 10C**). Cortisol at this time of day should be decreasing to around 150pg/uL. After the training exercise, salivary cortisol concentrations more than doubled this value, indicating a stress response, though it was not as intense as what was seen during the tasing event. Cortisol did not increase with physical exertion [$R^2=0.052$; $F(1,33)= 1.150$; $p \geq 0.050$] (**Figure 11A**). Cortisol levels tended to increase with Tension (**Figure 11C**) in the linear regression model [$R^2 = 0.236$; $F(1,33) = 9.596$; $p = 0.004$], but amylase does not show a similar relationship [$R^2=0.085$; $F(1,33)= 2.879$; $p \geq 0.050$] (**Figure 11D**). Although it was predicted that rising amylase levels would coincide with an increase in tension, amylase levels tended to increase as physical exertion increased [$R^2= 0.296$; $F(1,33) = 8.817$; $p = 0.007$] (**Figure 11B**), suggesting that a rise in physical stress may relate to the increase in amylase.

3.3 PAD Drill

Early morning concentrations of salivary amylase before the disaster drill (time point 1) were not significantly higher than those after the drill (time point 2) ($p \geq 0.050$) but salivary cortisol levels before the drill were higher than after ($p = 0.001$) based on a paired t test (**Figure 12A-B**). There is no trend present between amylase and cortisol [$R^2= 0.111$; $F(1,13)= 1.498$; $p = 0.245$] and no relationship exists between amylase and Borg [$R^2=0.011$; $F(1,13)= 0.075$; $p \geq 0.050$] perhaps because this was a psychological as opposed to a physical stressor as seen with the FIG Training

Exercise. However, it is interesting that amylase showed no statistical relationship to Tension in this case [$R^2= 0.004$; $F(1,13)= 0.044$; $p \geq 0.050$] (**Figure 13A-C**).

3.4 Exam Anxiety

To determine the ability of exam anxiety to elicit a stress response, cortisol and amylase levels were used as biomarkers for activation of the HPA axis and SAM system respectively.

3.4.1 E1

Repeated Measures ANOVA showed no significant difference in salivary cortisol [$F(1,11)= 0.010$; $p \geq 0.050$] or salivary amylase [$F(1,11)= 0.020$; $p \geq 0.050$] (**Figure 14A-B**) concentrations before the exam as compared to after the exam. No correlation exists between amylase and cortisol [$R^2=0.000$; $F(1,11) = 0.000$; $p \geq 0.050$].

3.4.2 E2

In **Figure 15B**, before the exam, salivary cortisol levels were higher than those after the exam as well as the after control [$F(3,29)= 5.280$; $p= 0.005$] (**Figure 15B**). Although there is a statistical difference between levels before the exam and after, there is no difference between pre-exam versus pre-control levels of amylase (**Figure 15A**) [$F(3,29)= 1.750$; $p \geq 0.050$]. Thus, little physiological significance exists because the average numbers are about normal for that time of day. Amylase levels and tension [$R^2= 0.026$; $F(3,29) = 0.992$; $p \geq 0.050$] and tense arousal [$R^2= 0.074$; $F(3,29) = 3.051$; $p \geq 0.050$] did not correlate in this case. There was a large amount of variation amongst individuals for amylase with only one subject, Subject 3, exhibiting any

significant changes in pre-exam and pre-control values (**Figure 15C**). Linear regression analysis amylase vs. cortisol did not correlate [$R^2 = 0.028$; $F(3,29) = 1.209$; $p \geq 0.050$] (**Figure 15D**).

3.4.3 E3

Repeated Measures ANOVA reveals the pre-control group is significantly higher than the post-exam group for amylase [$F(3,27) = 3.630$; $p = 0.038$] (**Figure 16A and B**). There are no significant differences between any of the groups for cortisol as reflected in Repeated Measures ANOVA [$F(3,27) = 0.230$; $p \geq 0.050$] (**Figure 16C and D**). Salivary amylase and salivary cortisol do not correlate [$R^2 = 0.000$; $F(3, 27) = 0.009$; $p \geq 0.050$] (**Figure 17**).

3.4.4 E4

There were no significant differences in the salivary amylase concentrations [$F(3,15) = 0.260$; $p \geq 0.050$] (**Figure 18 A and C**). Salivary cortisol levels were statistically significant [$F(3,15) = 7.190$; $p = 0.009$], with the pre-control group appreciably higher than the rest of the time points, but the range of cortisol levels were normal for time of day (**Figure 18B and D**). Linear regression between amylase and cortisol was significant [$R^2 = 0.479$; $F(3,15) = 12.886$; $p = 0.003$].

3.5 Oral Presentation Anxiety

Time point 1 is just before the presentation. Time point 2 is just after the presentation, approximately ten minutes after time point 1. Both amylase and cortisol levels were significantly higher after the presentation, roughly four times as great (**Figures 19A-B**).

Amylase versus cortisol linear regression analysis was not significant [$R^2 = 0.004$; $F(1,3) = 0.007$; $p \geq 0.050$] (**Figure 19C**).

4. Discussion:

In this study, we examined the effect of a variety of different potential stressors on salivary amylase and cortisol. Tasing subjects were exposed to an x-26 taser for up to 5 minutes. FIG subjects completed an obstacle course that consisted of various physical activities while being observed by their peers and superiors. PAD drill subjects were in charge of coordinating a massive disaster drill involving hundreds of volunteers and first responders. Exam Anxiety subjects were exposed to the psychological stress of taking an exam. Oral Presentation subjects in the classical psychological stress paradigm performed a ten minute speech before peers and superiors.

The circadian rhythm of amylase proposed by other publications (Nater et al., 2007; Rohleder et al., 2004), suggests levels should be lower in the morning and rise throughout the day. Cortisol has a circadian pattern peaking at 400pg/100uL around 6:00AM and then slowly decreasing as the day progresses, averaging around 150-200pg/uL around noon and dropping to about 100pg/uL in the evening. When amylase levels roughly doubled in response to stress conditions, this was considered significant in accordance with other studies. For instance, Rohleder et al. found amylase to increase from 175U/mL to about 300U/mL, nearly doubling ten minutes after stress was applied (2004), Nater et al. found amylase to increase from ~120U/mL to 225U/mL in the stress condition (2005), and again in another article in 2006 Nater et al. showed amylase more than doubling during the stress condition, peaking at just five minutes after initiation of

stressor. The significance of individual responses has been utilized by other studies (Wetherell, 2006; Quirin et al., 2008) to point out how variation amongst specific subjects can cause fluctuations in results due to differences in HPA axes or SAM systems to react to a given stressor. Findings reported here were congruent with other studies (Van Stegeren et al., 2008) showing little or no correlation between salivary cortisol and salivary amylase concentrations.

4.1 Tasing

The circadian rhythm of amylase proposed by other publications (Nater et al., 2007; Rohleder et al., 2004), suggests levels should be rising at the time the post-tasing samples were collected; this may be a confounding factor for the 20 minute post-tasing time point. Thus, these results cannot be used as positive controls. Still, the amount of increase in some of the subject sets (**Figures 1A, 2A, and 5A**) were roughly double the morning before (time point 1) and the morning after (time point 3) tasing, suggesting a stress response. At 10 minutes after tasing, one would expect amylase to peak and at 20 minutes, one would expect the levels to be coming down from the stress response. This might suggest that the 10 minute levels were higher, but the half life of amylase remains to be elucidated (**Figure 1 A and B**).

Since the tasing data set was part of a study supported by the National Institute of Justice to determine if tasing elicited the stress response and not for the development of biomarkers, it was more important to obtain a sample at the peak of the cortisol response (20 minutes) since this is the quintessential stress indicator. Increasing salivary cortisol concentrations in the twenty minutes following taser exposure (time point 2) are highly suggestive of a stress response because normal salivary cortisol concentrations, as suggested by diurnal course studies (Nater et

al., 2007; Ferguson, 2008), will peak in the morning and taper off as the day progresses but will never reach levels as high as 900pg/100uL (as seen in **Figure 7B**) unless a stressor is introduced. Subjects whose post tasing levels more than doubled their control concentrations were considered responders, and those who showed little or no response were classified as non-responders. All post-tasing salivary cortisol concentrations were elevated with respect to controls, though subjects exhibited different levels of reactivity. For instance, in **Figure 2A** and **2C** with regard to amylase, Subject 12 is an example of a responder and subject 18 is an example of a nonresponder. In **Figure 5C**, and **5D** Subjects 17 and 18 were responders based on significant elevation in salivary cortisol and salivary amylase concentrations twenty minutes following taser exposure (time point 2), whereas Subject 3 was a non-responder, exhibiting no similar increases in reference to controls (time points 1 and 3).

For Tasing FC, because salivary cortisol was elevated at twenty minutes after tasing (time point 2), this suggests the stress response was initiated due to the effects of tasing only, not anticipatory stress. The levels prior to tasing were not elevated and this suggests that anticipation was not a factor. A study done by Stefano et al. suggests the existence of this anticipatory stress response which maintains arousal in the form of “initial sympathetic activation” and elevated norepinephrine levels upon expectation of stressful events (Stefano et al., 2008). There seems to be no anticipatory stress increases in pre-tasing baselines for either salivary amylase or salivary cortisol concentrations when compared to the morning after tasing (time point 3) with the possible exception of Tasing WH (**Figure 7**) where the pre-tasing baseline and the twenty minute post tasing cortisol levels are both significantly higher than the morning after tasing.

4.2 FIG Training Exercise

Studies analyzing the effects of psychological stress with respect to skydiving (Chatterton et al., 1997) simulated job interviews (Nater et al., 2005), and investigating the effects of anxiety in a psychosocial stress paradigm (Nater et al., 2004; Grillon et al., 2007) have shown that amylase levels should increase under psychological stress situations. This training exercise is a combination of physical and psychological stress. However, here the data suggest that physical stress may also lead to a rise in amylase levels and perhaps sympathetic activation since the amylase data correlates with the Borg rating of perceived exertion. This is an ideal exercise because amylase levels in the body peak at ten minutes following initiation of the stress response and we were able to collect samples at this time point. Once again, amylase levels nearly double, suggesting a stress-dependent increase rather than a diurnal effect. This may contradict one study which found physical activity to have no effect on salivary amylase levels (Nater et al., 2007), because here, rather than amylase correlating with tension, it tended to correlate with Borg rating of perceived exertion, suggesting an increase in amylase with physical activity. However, it must be noted that correlation does not indicate causality. Based on linear regression, cortisol levels rose with tension and psychological stress was observed, but amylase levels showed no relationship to this increase in tension. In this case, it was difficult to segregate psychological and physical stress because although it was a physical training exercise, subjects were performing under the watchful eyes of their peers and their superiors. This exercise was not only a physical training exercise, but subjects were in groups which competed for best times on the obstacle course. According to a study done by Kivlighan and Granger, concentrations in salivary alpha amylase tend to change, increasing significantly in response to competitive psychological atmospheres as well as with previous exposure to the given situation (Kivlighan

and Granger, 2006). This may be another explanation for the rise in salivary amylase concentrations. Another study involving army recruits demonstrates salivary cortisol levels increasing much higher in socially dominant subjects than in other less dominant individuals (Hellhammer et al., 1997). Perhaps this too may account for individual differences.

4.3 PAD Drill

Cortisol circadian rhythms make it difficult to interpret a stress response increase from such early morning samples regardless of the presence of a stressor because diurnal patterns suggest salivary cortisol levels should peak in the morning and taper off as the day progresses. However, one study has shown that more stressed individuals have a steeper morning cortisol peak (Ferguson, 2008). The salivary cortisol levels reported here are appropriate for the times of day the samples were taken. Since definite circadian rhythms of amylase have yet to be established, it is unclear what is happening with amylase. Perhaps enzyme levels spike upon waking, drop low in the morning and rise steadily throughout the day as proposed by other literature (Nater et al., 2007). If this is the case, Time point 1 samples were taken well after waking around 7:00 AM, and Time point 2 samples were taken around 1:00 PM after the drill had ended. Anticipatory stress was predicted to raise the levels of amylase but the data is inconclusive.

The fact that there is no trend present between amylase and no relationship exists between amylase and Borg may be because this was a psychological as opposed to a physical stressor which was seen with the FIG Training Exercise. Subjects here were in charge of organizing the disaster event and coordinating ten different disaster scenarios involving hundreds of volunteers, first aid personnel, search dogs, and first responders, among others. Therefore, it

is interesting that amylase showed no statistical relationship to Tension in this case (**Figure 13A-C**).

4.4 Exam Anxiety

The exam anxiety model did not elicit the hypothesized increase in salivary cortisol and salivary amylase levels required to assess HPA and SAM system reactivity. Many factors contribute to HPA reactivity. Although many of the exam groups exhibited normal levels or non-significant increases in salivary cortisol and/or salivary amylase, it has been reported that repeated exposure to the same stressor results in HPA and SAM system habituation, causing a decrease in response and hormone release (Schommer et al., 2003). This may account for the lack of response.

It is difficult to determine why amylase levels go down for controls or after the exam itself in E2, but, taking a closer look at the data, one can consider the importance of individual responders and non-responders to account for the deviation (**Figure 15C**). For example, Subject 3 exhibited high levels before the exam suggesting anticipatory anxiety, which was approximately five times greater than levels after the exam and both sets of control levels. A similar trend is seen for the same subject for salivary cortisol levels. Clearly, exam anxiety elicited a stress response in this subject. In the same way, amylase levels of Subject 11 before the exam and after the exam were more than three times as great as control levels, suggesting the response continued throughout the course of the exam itself. Cortisol levels for Subject 11 prior to the exam are also relatively high for the time of day at approximately 450pg/100uL (should be ~175pg/100uL) but drop to about 250pg/100uL after the exam. Controls for this set are about normal for the time of day (150-200pg/100uL) significantly lower than before the exam. Although this spike is significant, it is not as intense as the increase seen in the tasing positive

control which peaked around 900pg/100uL (**Figure 7**). In E3, Subject 2 demonstrated a slight elevation in cortisol levels before the exam and dropped more than 100pg/100uL after the exam, as seen in **Figure 16 D**. Subject D in **Figure 18C** exhibited extremely high salivary amylase levels before the exam, which nearly tripled the post-exam and control levels, perhaps suggesting sympathetic activation due to psychological anxiety.

With the exception of these responders, this particular subject set did not exhibit appropriate levels of biomarkers to indicate activation of the stress response. Although significant differences amongst certain time points exist, only those mentioned above are physiologically significant. For instance, for E4 amylase concentrations, the fact that the post-exam group and the pre-control group are significantly different is not noteworthy due to increasing amounts of overlap; thus, no physiological significance exists.

4.5 Oral Presentation Anxiety

Performing an oral presentation in front of a group of peers is the classical psychological stress model. Again, as with FIG, the timing is optimal for alpha-amylase analysis. Since amylase peaks at ten minutes, this second time point in **Figure 19A** represents the amount of stress at the start of the oral presentation when one would expect anxiety to be greatest. Salivary amylase tends to increase with psychological stress (Chatterton et al., 1997; Grillon et al., 2007; Nater et al., 2004; 2005; 2006) and with post presentation levels more than doubling pre-presentation concentrations this is highly suggestive of a stress response.

Cortisol peaks at twenty minutes and, had a sample been taken twenty minutes following the start of the presentation, perhaps it would have demonstrated a similar peak. Cortisol was significantly elevated for the time of day anyway, even just ten minutes after the start of the

presentation (**Figure 19B**). These trends for cortisol support other findings (Kirschbaum et al., 1992) that public speaking elicits a stress response and raises salivary cortisol levels significantly, yet contradicts simulated public speaking models which failed to elicit HPA axis activity and salivary cortisol elevation immediately after or in the 10, 15, 30 or 60 minutes following the on-camera speech (Garcia-Leal et al., 2005).

5. Conclusion

Tasing elicited a stress response with salivary cortisol levels rising significantly higher than normal circadian levels in the twenty minutes following taser exposure. Salivary amylase concentrations varied among individuals but as a whole, tasing resulted in significant increases in amylase as well. A combination event of physical exertion and psychological stress as demonstrated by the FIG training exercise caused an increase in salivary cortisol which correlated with increased Tension, and an increase in salivary amylase which correlated with rising Borg levels. PAD drill salivary cortisol levels followed the decrease associated with the circadian rhythm. Amylase levels were not significant and showed no relationship to tension. As for exam anxiety, the majority of the groups exhibited no stress responses based on salivary amylase and cortisol concentrations which were either normal for the time of day or demonstrated non-significant changes. Oral presentation anxiety on the other hand was extremely successful in inducing a stress response increase in both salivary amylase and cortisol concentrations.

Salivary amylase is a biomarker that is easily obtained in the field and simple to assay in the laboratory, but it is not without drawbacks (Granger et al., 2007). The wide range of variability reported here and by other publications echoes the need for standardization of salivary

components and collection in order to obtain consistently reproducible results (Amerongen et al., 2007). In summary, FIG training exercise and psychological stress of oral presentation stimulates amylase release. Salivary amylase responses due to tasing, PAD drill, and exam anxiety were inconclusive. This may be accounted for by any number of factors which require expounding. The consequence of gender on salivary amylase and cortisol concentrations has shown equal incidence of stress response amongst males and females, but variety in intensity (Lovallo et al., 2006; Van Stegeren et al., 2008). In addition, possible confounding factors of amylase such as gender, circadian patterns and individual variation require further investigation. Therefore, although salivary amylase has been reported here and elsewhere as increasing significantly during moments of psychological stress (Takai et al., 2004), results are inconsistent and more studies must be done to declare sAA a reliable biomarker for SAM system activation.

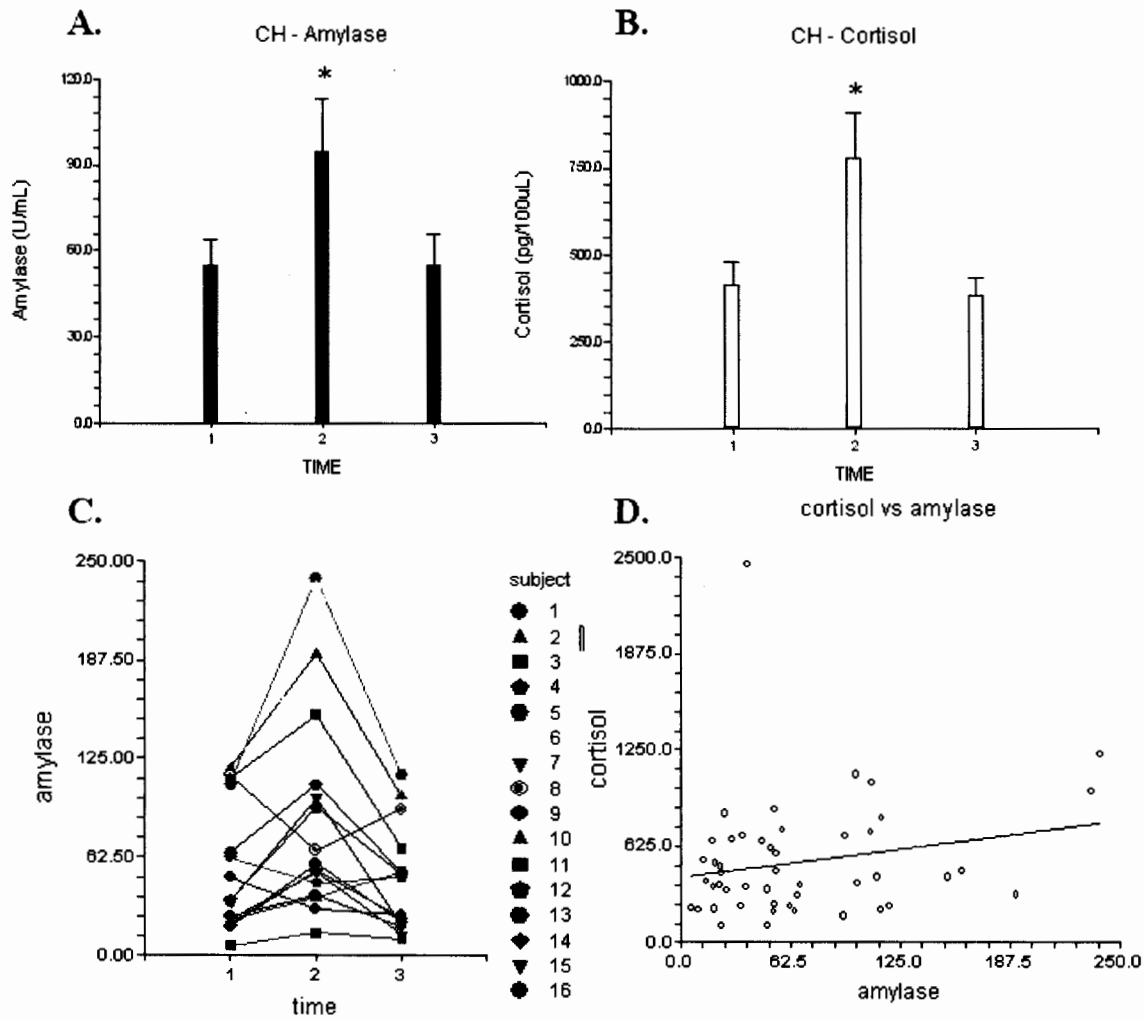


Figure 1 A. Salivary amylase levels. B. Salivary cortisol levels. C. Individual responses amongst the subjects for amylase. D. Linear regression between cortisol and amylase.

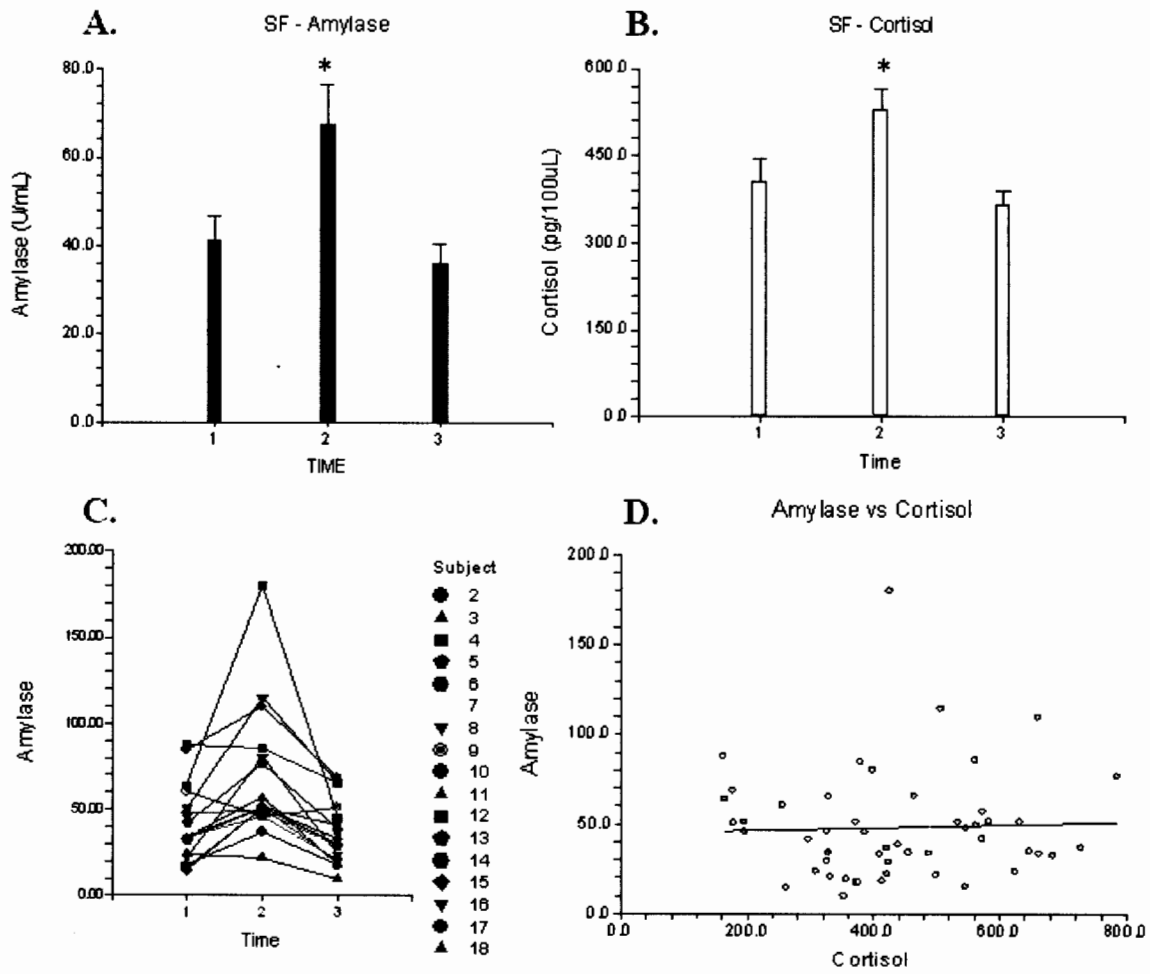


Figure 2 A. Salivary amylase levels. B. Salivary cortisol levels. C. Individual subject responses for amylase. D. Linear regression, amylase versus cortisol.

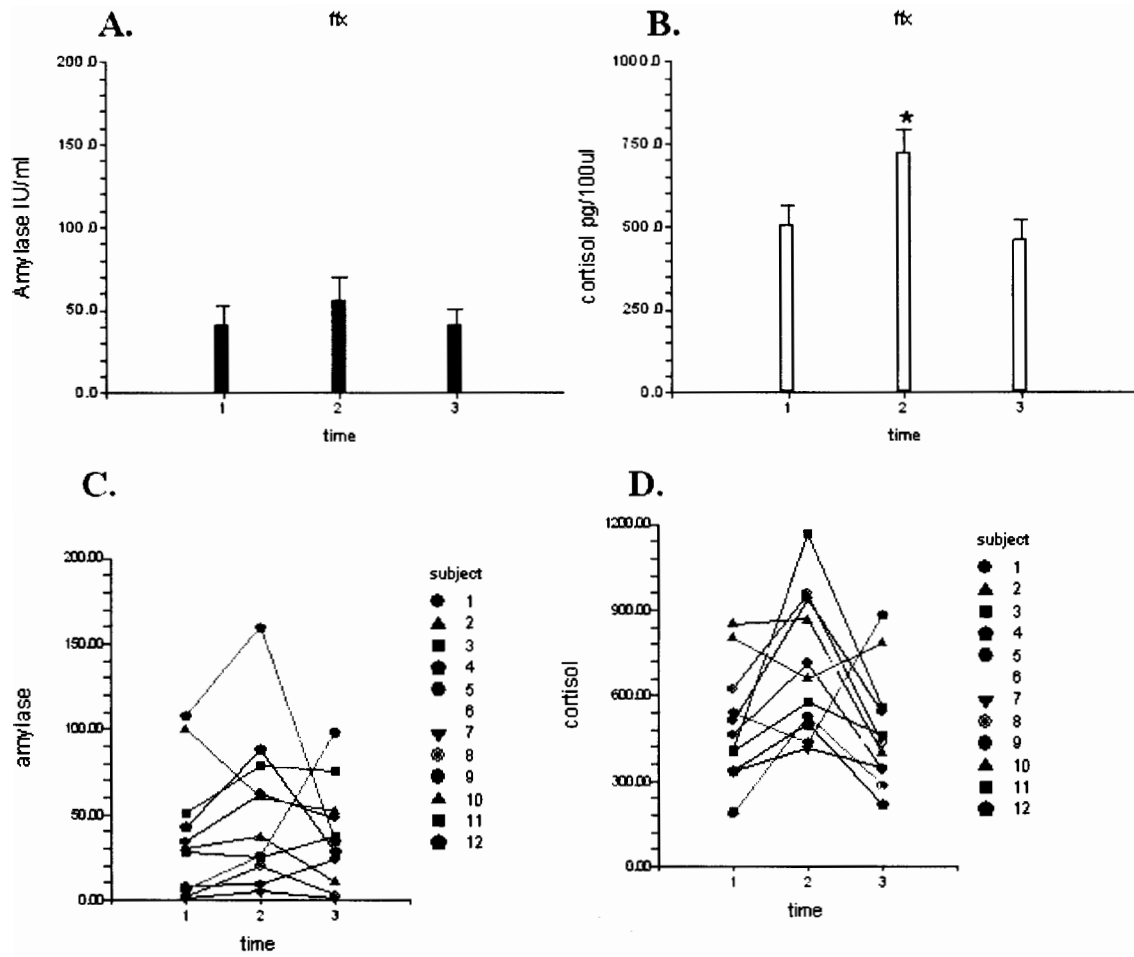


Figure 3 A. Salivary amylase levels. B. Salivary cortisol levels. C. Individual subject responses for amylase. D. Individual subject responses for cortisol.

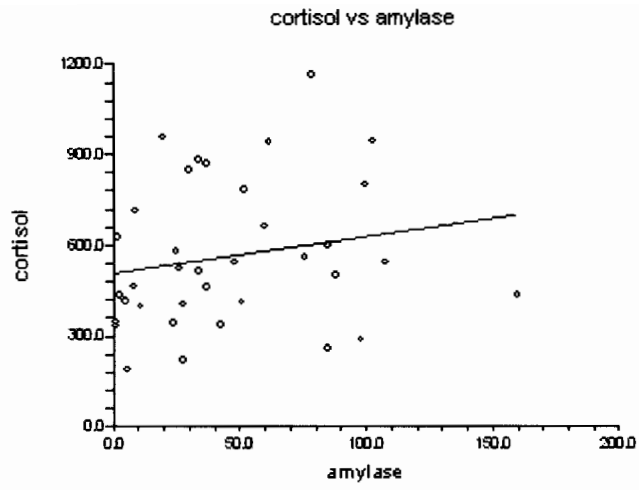


Figure 4 FFX, Linear Regression, cortisol versus amylase.

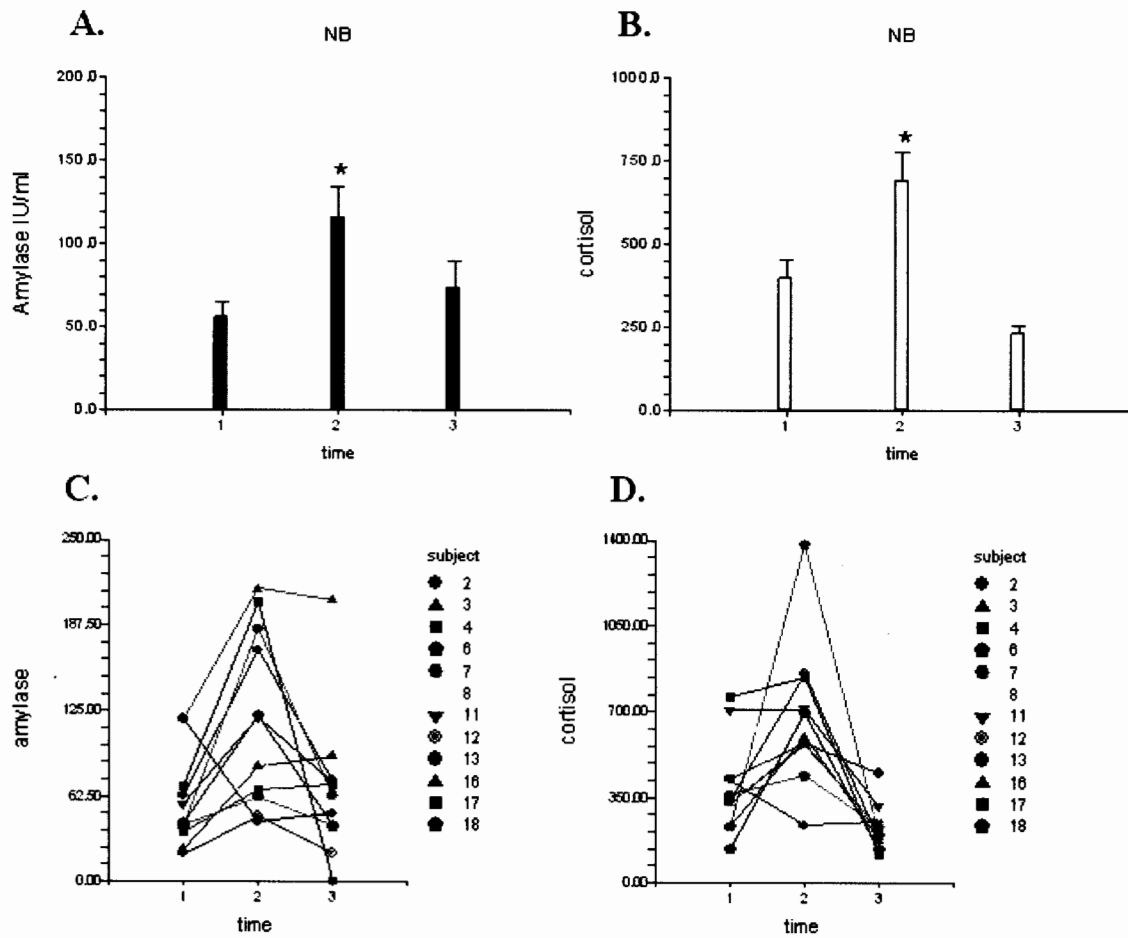


Figure 5 A. Salivary amylase levels. B. Salivary cortisol levels. C. Individual subject responses for amylase. D. Individual subject responses for cortisol.

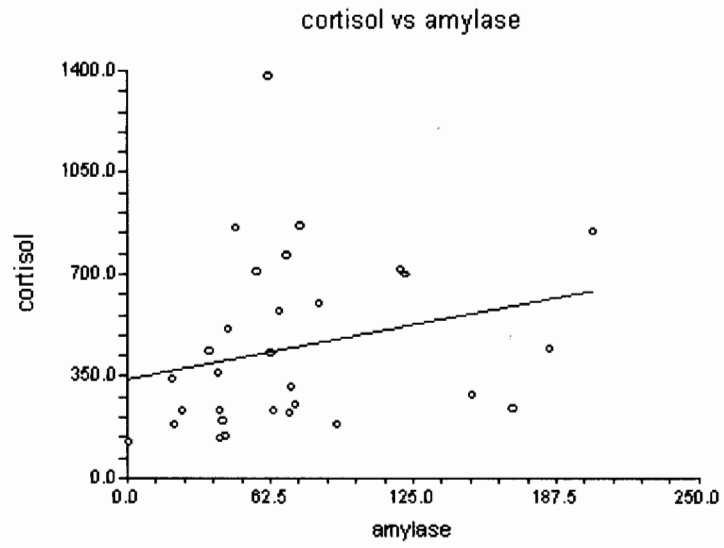


Figure 6 NB, Linear Regression between cortisol and amylase.

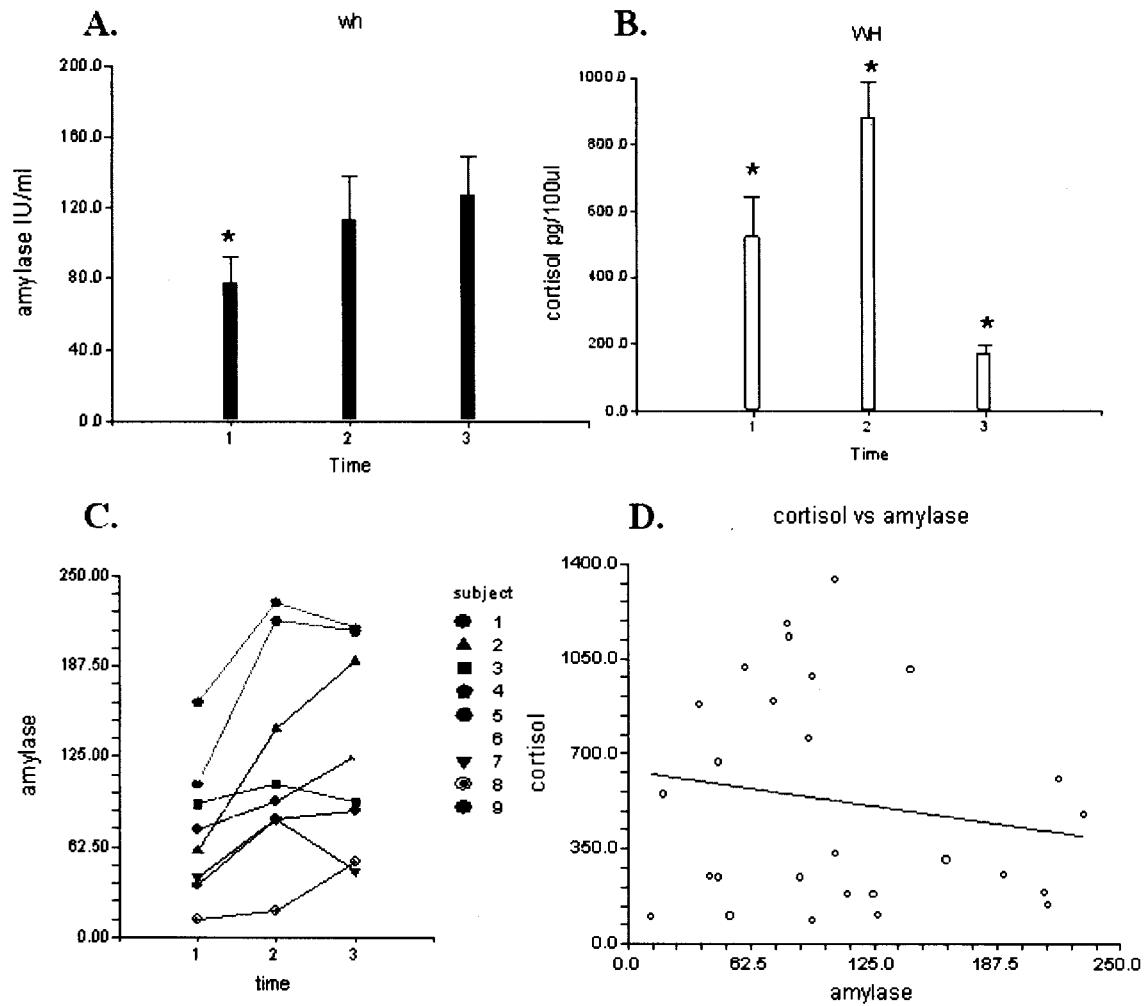


Figure 7 A. Salivary amylase levels. B. Salivary cortisol levels. C. Individual subject responses for amylase. D. Linear regression, cortisol versus amylase.

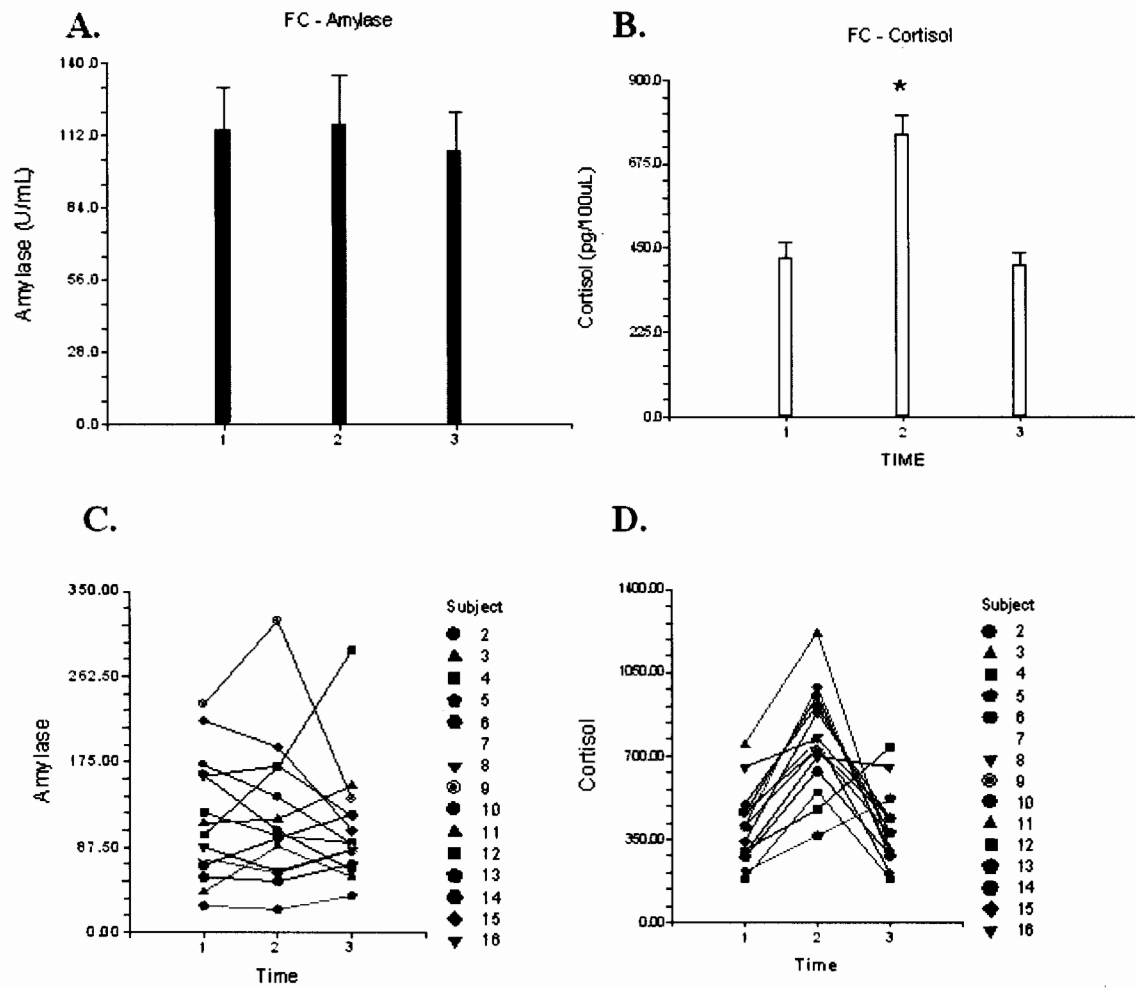


Figure 8 A. Salivary amylase levels. B. Salivary cortisol levels. C. Individual responses for amylase. D. Individual subject responses for cortisol.

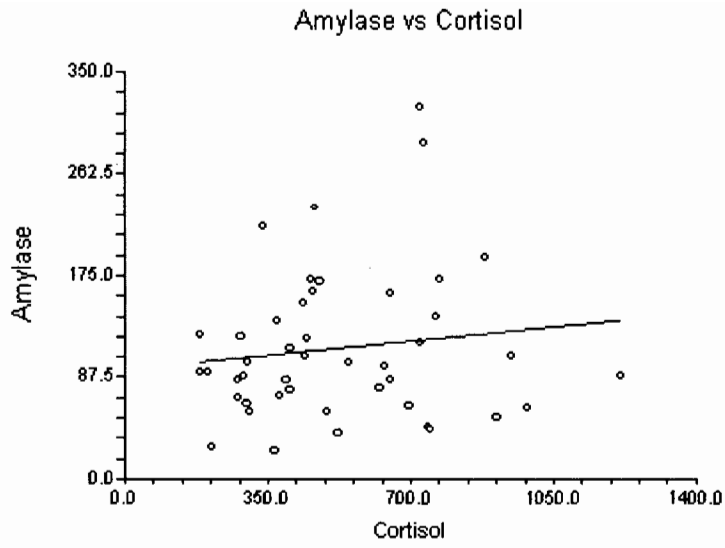


Figure 9 Tasing FC, Linear Regression, amylase versus cortisol.

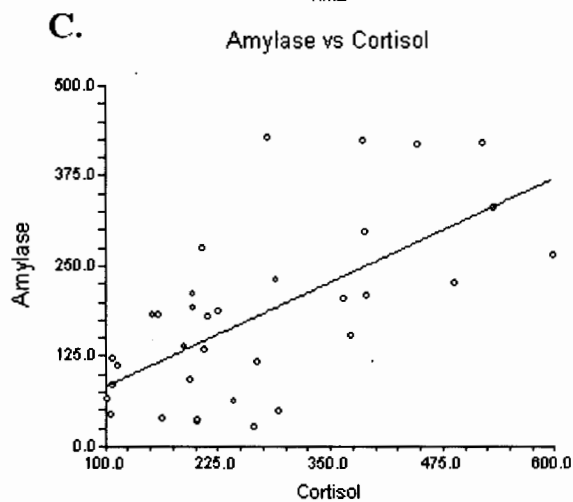
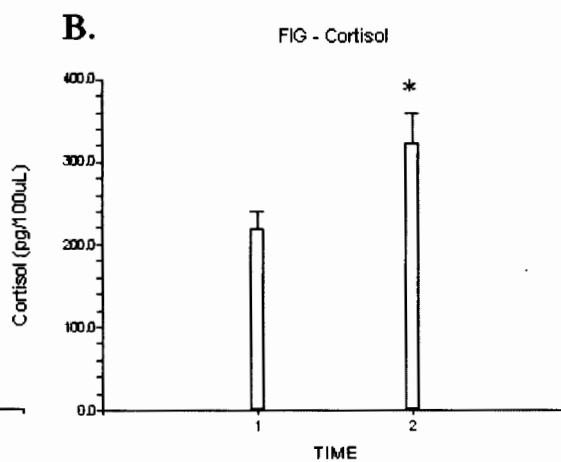
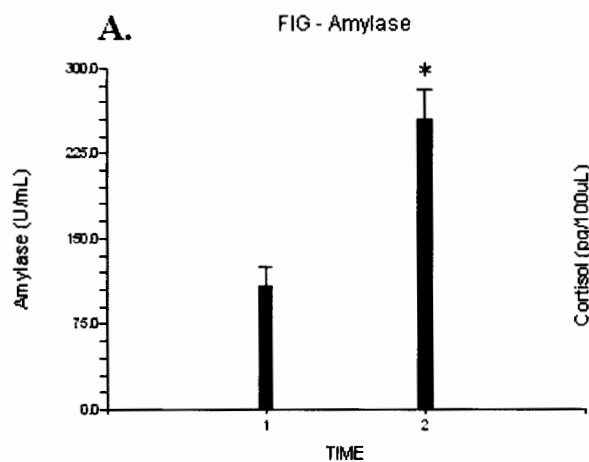


Figure 10 A. Salivary amylase levels. B. Salivary cortisol levels. C. Salivary amylase vs. Salivary cortisol.

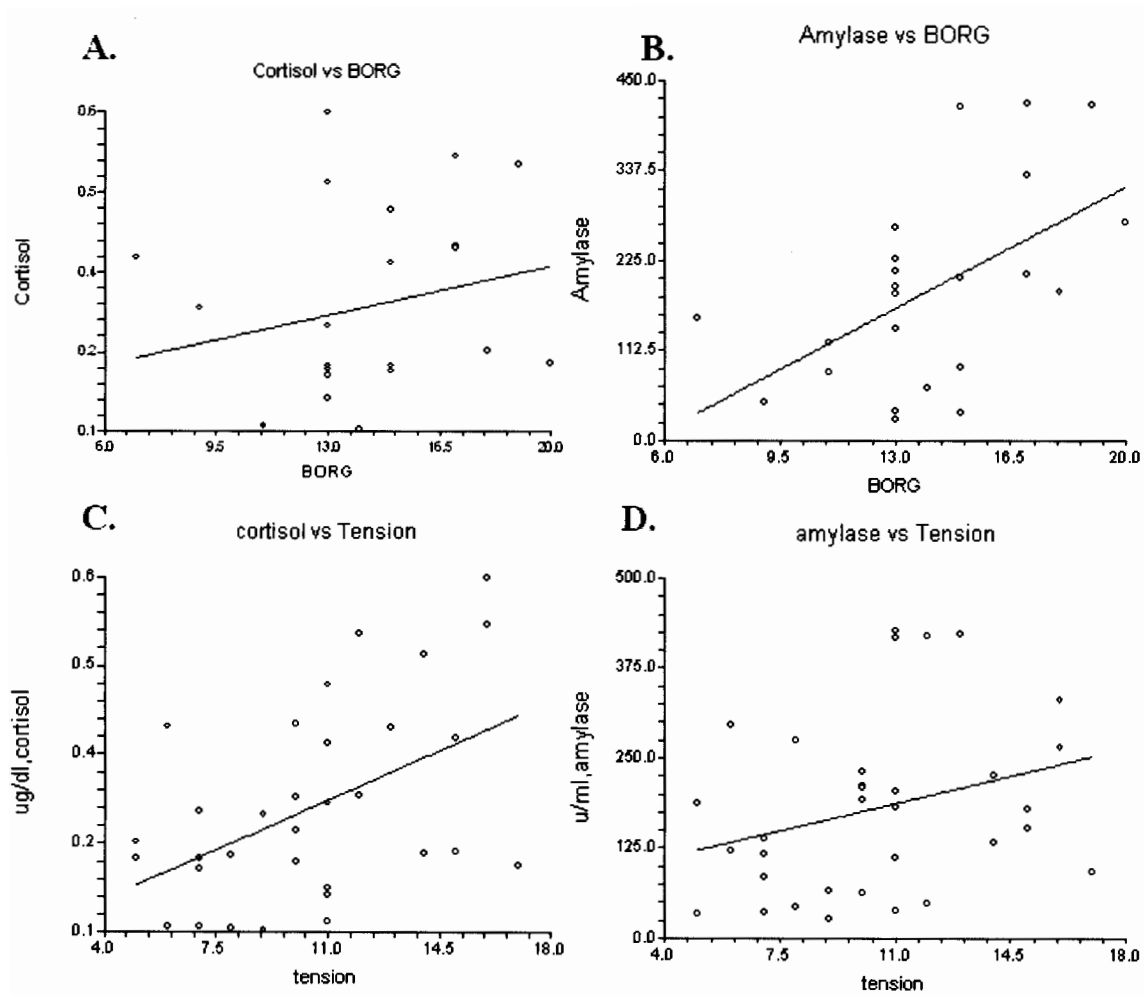


Figure 11 FIG Training Exercise, Linear Regression Analyses: **A.** Amylase vs. Cortisol. **B.** Amylase vs. Borg Rating of Perceived Physical Exertion. **C.** Cortisol vs. Tension. **D.** Amylase vs. Tension.

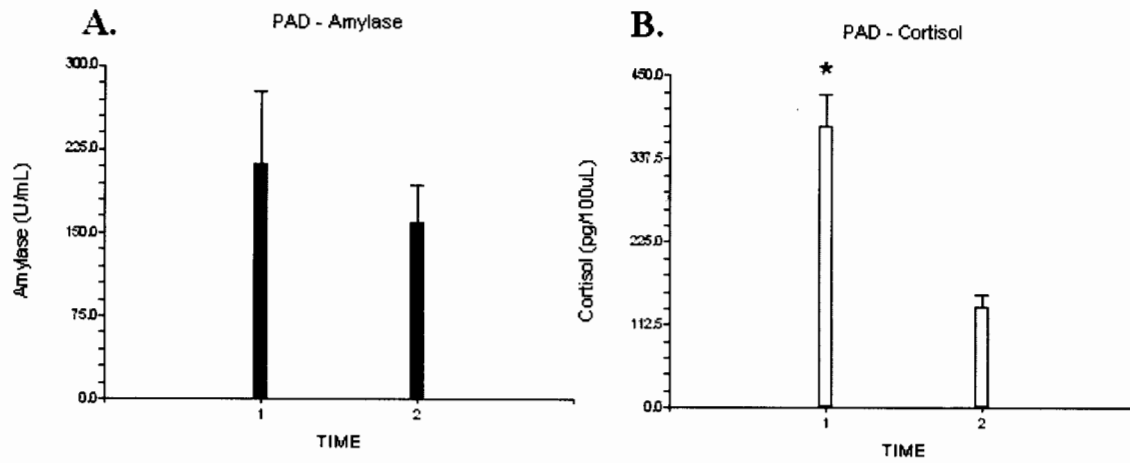


Figure 12 A. Salivary amylase levels. B. Salivary cortisol levels.

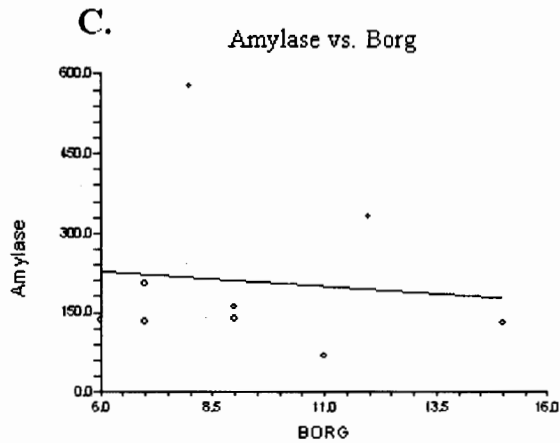
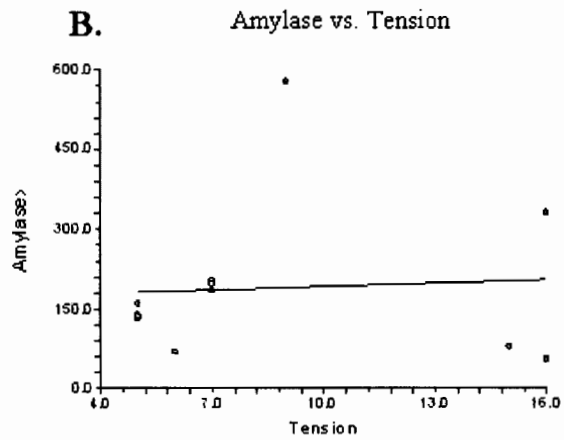
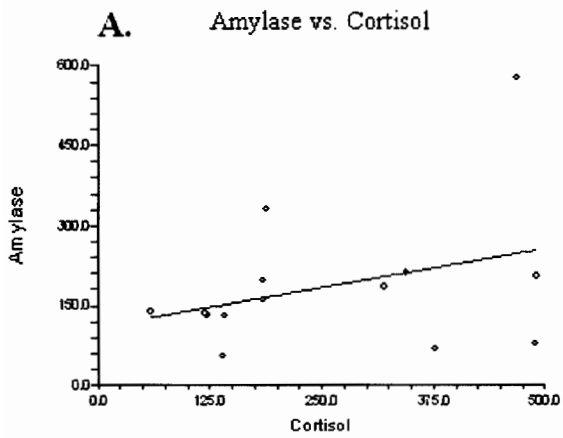


Figure 13 PAD Drill, Linear Regression Analyses A. Amylase vs. Cortisol. B. Amylase vs. Tension, not significant. C. Amylase vs. Borg, Rating of Perceived Physical Exertion.

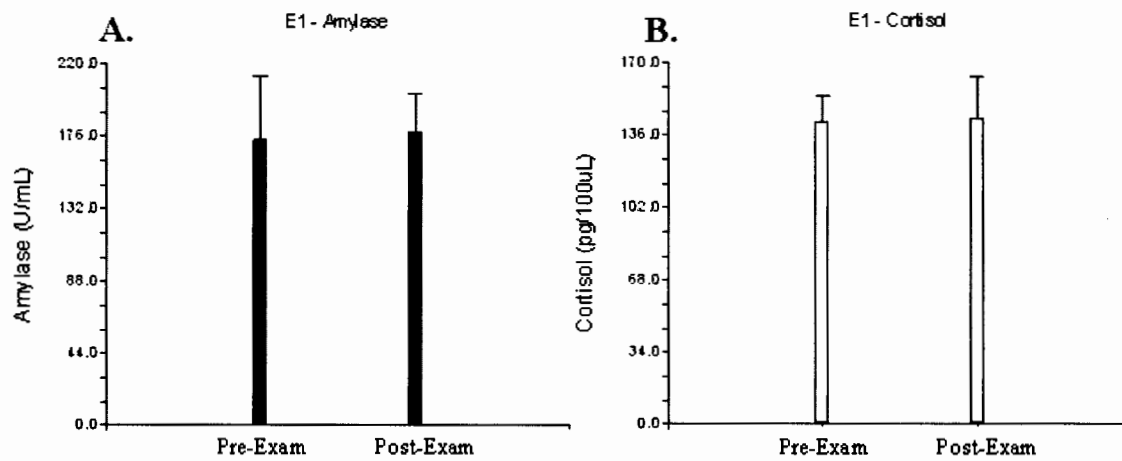


Figure 14 A. Salivary amylase levels. B. Salivary cortisol levels.

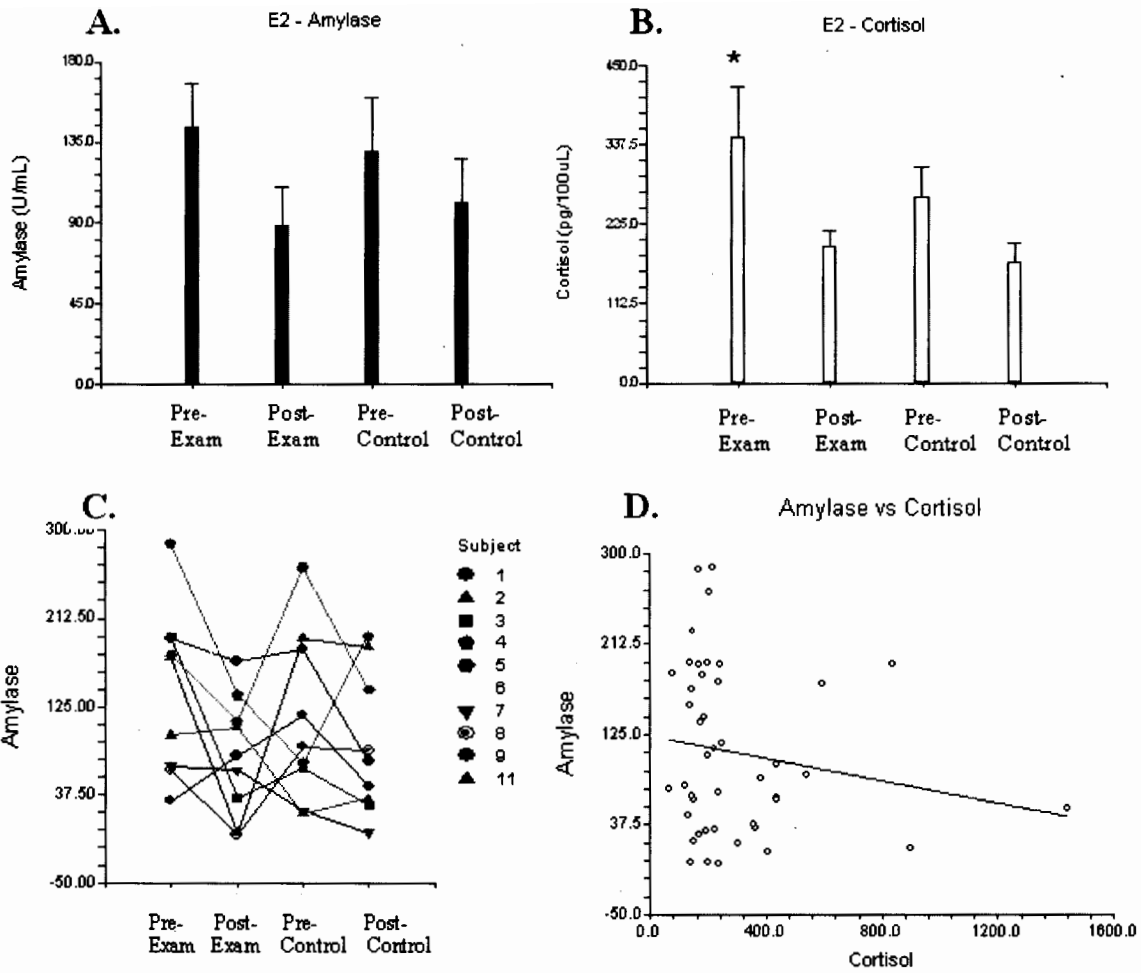


Figure 15 A. Salivary amylase levels. B. Salivary cortisol levels. These elevated levels suggest anticipatory stress prior to the exam task. C. Individual responses for amylase. D. Linear regression between amylase and cortisol.

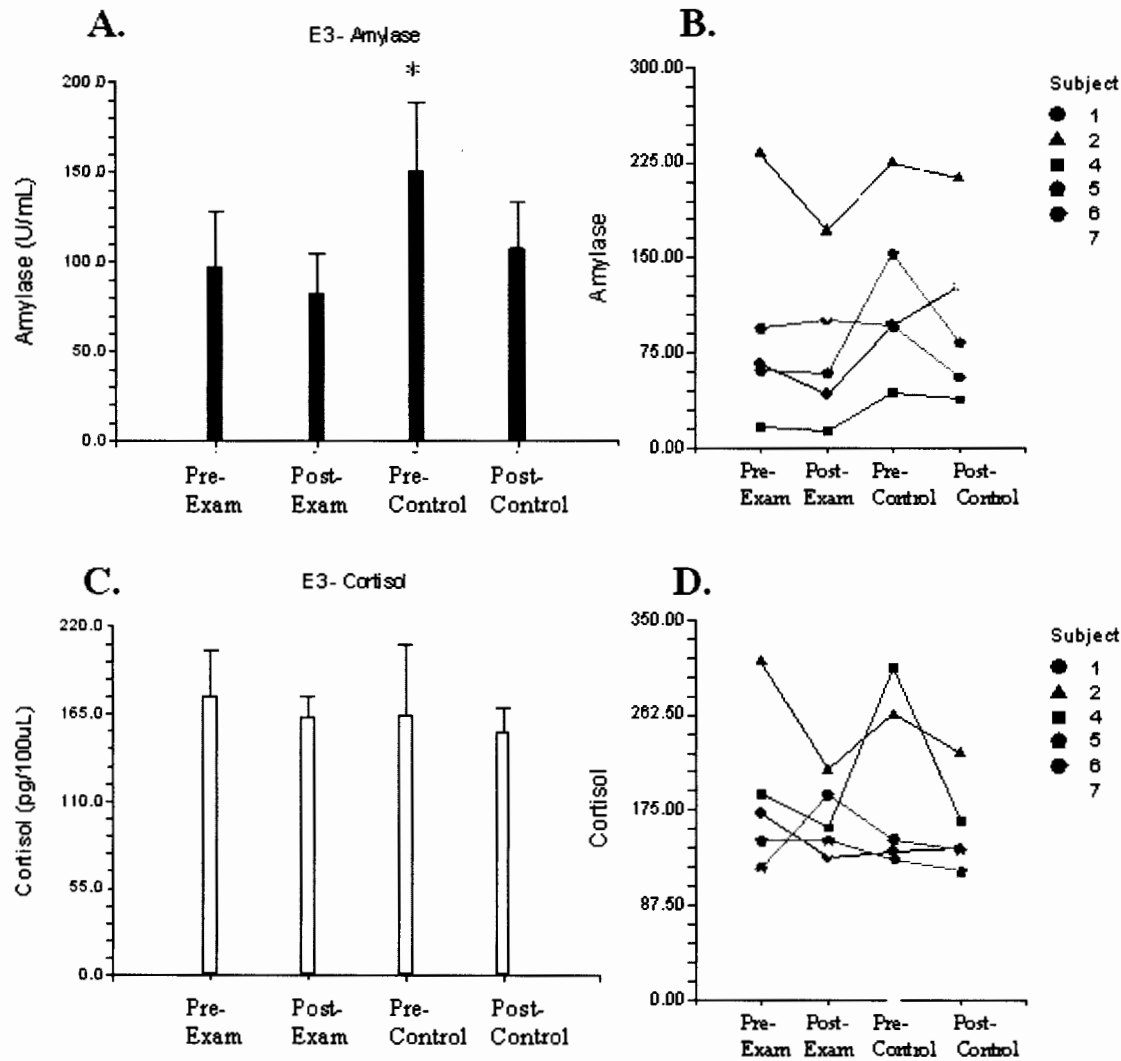


Figure 16 A. Salivary amylase levels. B. Individual subject responses for amylase. C. Salivary Cortisol levels. D. Individual subject responses for cortisol.

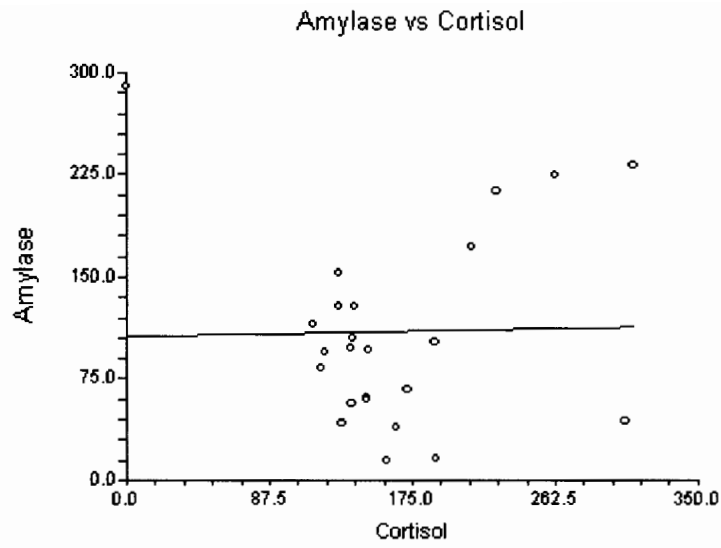


Figure 17 E3, Linear Regression between amylase and cortisol.

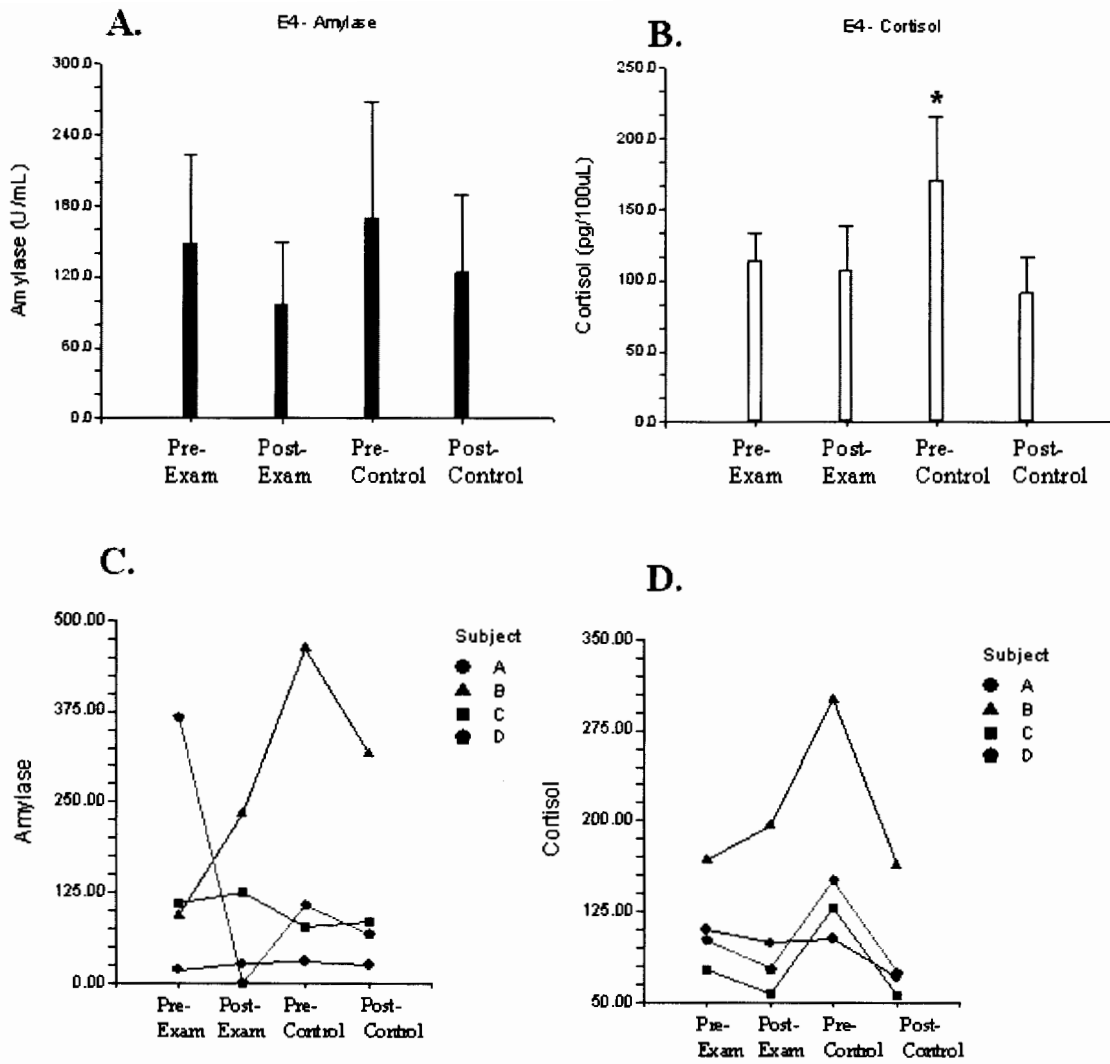


Figure 18 A. Salivary amylase levels. B. Salivary cortisol levels. C. Individual subject responses for amylase. D. Individual subject responses for cortisol.

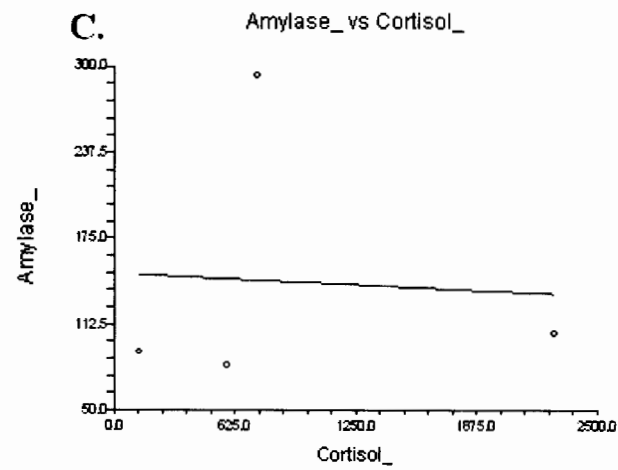
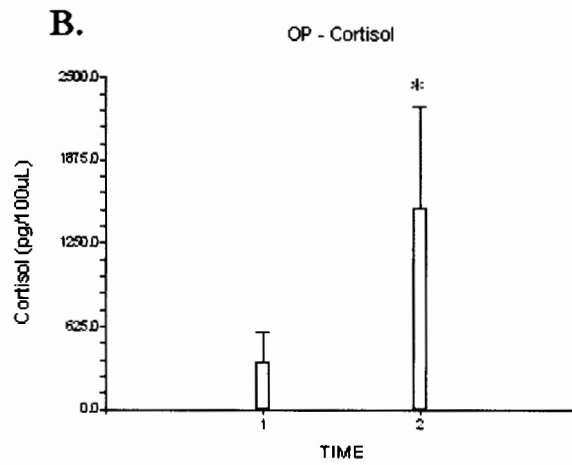
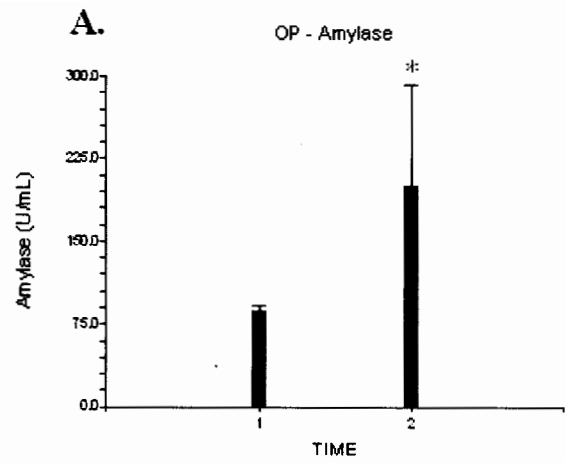


Figure 19 A. Salivary amylase levels. B. Salivary cortisol levels. C. Linear regression, amylase versus cortisol.

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