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Changes in Salivary and Plasma Markers during and Following Short-Term Maximal Aerobic Exercise Assessed during Cognitive Assessment

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Abstract

This study assessed multiple salivary and plasma markers before and after incremental short-term maximal aerobic exercise and in a non-exercising control in conjunction with cognitive testing. Subjects: Apparently healthy 18 - 30 years old low CVD risk females participated (n = 19). Methods: Subjects completed two conditions: 1) exercise: short maximal treadmill exercise and cognitive assessment pre- and post-exercise and, 2) non-exercise: with cognitive assessment timed to match testing in the exercising condition. Non-stimulated, timed salivary samples and venous blood were collected before and after exercise and after recovery. Results: Saliva: Over time α-amylase increased in both exercise and non-exercising conditions. Exercise had increases in α-amylase at time matched control points up to 36% greater than the non-exercising conditions. Following exercise and recovery from exercise α-amylase increased compared to baseline (ranging from 47% to 290%). Baseline cortisol was 33% higher than post-exercise and 59% higher than recovery irrespective of exercise. Plasma: NEFA was 50% higher at post-exercise and recovery compared to baseline without exercise and 36% higher at post-exercise and recovery compared to baseline with exercise. Glucose and lactate were, 18% and 50% higher respectively, after exercise compared to baseline and recovery with exercise. Post-exercise glycerol was 11% higher than recovery. Differences between Conditions: Post-exercise glucose and lactate were 20% and 40% higher respectively with exercise. Glycerol was 11% lower after exercise. Conclusions: We demonstrated that acute exercise coupled with cognitive task increased α-amylase levels, but not cortisol, potentially due to a differential stress response, but most likely due to the timing of sample collection.

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Keywords

***α*-Amylase, NEFA, Cortisol, Exercise, Salivary Biomarker**

1. Introduction

Aerobic exercise affects every system in the human body. Blood flow, metabolism, and multiple chemical levels all change acutely as a result of aerobic exercise; some changes have fleeting effects, and others are long lasting. Chronic adaptations also occur to numerous systems as a result of repetitive aerobic training over time. Authors have demonstrated that gene expression in the brain is up-regulated and that performance on cognitive tasks is altered by chronic aerobic exercise [1]. The mechanisms responsible for these changes in the brain however have yet to be identified.

Changes occurring in the periphery impact the brain, sometimes directly, sometimes indirectly. Assessing changes in hormones, metabolites, and other chemicals in the body during varying exercising conditions may help us to identify potential chemical mechanisms involved in altering brain structure and function. Aerobic exercise has the potential to affect brain function in many ways. Over time aerobic training results in increased blood volume and the ability to increase cardiac output [2] [3]. Increased cardiac output allows for increased delivery of fuel and oxygen and removal of substrates in the brain. If chemical changes observed during and following acute short-term maximal aerobic exercise bouts alter how the brain works, via changes in fuel availability and delivery, these changes could be part of the mechanism(s) responsible for changes in cognitive function during and following exercise and as such need to be assessed.

Saliva is an easily collected, non-invasive, biological sample that is capable of providing valuable information on acute changes in hormones and metabolites, without some of the potentially negative consequences of other biological samples such as venous and arterial blood or tissue biopsies. Salivary Alpha-amylase [4]-[8] and cortisol [9]-[11] have both been assessed in numerous studies assessing the impact of both physical and psychological stressors on the levels of these hormones. Changes in glucose, lactate, glycerol, and NEFA levels during and following exercise are all potential indicators of altered fuel source availability [12]-[26]. Changes in fuels sources could play an important role in fatigue. The purpose of the current study was to assess changes in multiple salivary and plasma factors as assessed during cognitive testing before and after incremental short-term maximal aerobic exercise in young healthy women.

2. Methods

2.1. Subjects

Apparently healthy 18 - 30 years old females, currently using pharmaceutical birth control were recruited to participate in this experiment (no minors were allowed to participate). Subjects were deemed “apparently healthy” when it was determined that they were free of signs and symptoms of cardiovascular and pulmonary disease and met the criteria for the American College of Sports Medicine (ACSM) low risk stratification for coronary artery disease [27]. Additional exclusionary criteria included: diagnosed learning disability, a concussion in the preceding six months (since both learning disabilities and concussion could be a confounding factor when assessing cognitive function), and lastly, the use of medication that could influence cognitive performance, including painkillers and antidepressants. Nineteen subjects completed the study.

Only females actively taking birth control were included to avoid potentially confounding results based on phase of the menstrual cycle since hormone levels vary based on the phase of the menstrual cycle (even though phase of menstrual cycle has been shown to have insignificant impact on cognitive performance) [28]. Testing was done after the first 3 days in a pill or birth control patch cycle and before the last three days of the birth control cycle. Prior to any participation in this study, including screening, a copy of the informed consent approved by the University at Buffalo’s Institutional Review Board, had to be signed. Subjects completed three laboratory visits; all visits were completed at the same time of day, in the morning to avoid changes based on circadian variations. The day before each lab visit subjects: 1) refrained from strenuous exercise, 2) did not drink alcoholic beverages for the entire day, 3) and did not eat or drink anything after midnight.

2.2. Screening Lab Visit

After subjects read and signed the approved consent form, a questionnaire documenting the subject's demoFigureics, medical history, and other pertinent information was completed. Fasting cholesterol (TC and HDL) and blood glucose were measured using a Cholestech LDX (Hayward, CA) via a finger stick blood sample. Resting heart rate, blood pressure, height and weight were measured. Body composition was assessed via 4-site skin caliper measurements, using Lange® skin calipers (Beta Technology Inc., Santa Cruz, CA), and then calculated using equations derived by Jackson and Pollock [29]. These results were used to determine whether a subject met the ACSM low risk stratification for coronary artery disease. Subjects also provided a urine sample, collected immediately prior to testing, that was used for pregnancy screening, hCG ACON laboratories Inc. (San Diego, CA) to assure that only non-pregnant women participated.

Following the successful completion of ACSM low CVD risk screening, each subject completed a discontinuous Modified Bruce Treadmill VO_2 max treadmill test on a Landice L7 treadmill using a Vacumed CPX Mini system (Ventura, California), which was used to perform breath by breath gas analysis. This served to determine each subject's level of aerobic fitness and obtain an objective measure of exercise to exhaustion for each subject. Subject's heart rate was continually monitored throughout testing with a Polar© heart rate monitor (Woodbury, NY). Rate of perceived exertion was measured at the end of each exercise interval, using the Borg Scale 6 - 20.

2.3. Second & Third Lab Visits

The second and third visits were assigned in counterbalanced order to prevent an effect of learning. The "**Exercising Visit**" involved computerized neuropsychological assessment before, and after short-term aerobic exercise to exhaustion. The "**Non-Exercising Visit**" involved computerized neuropsychological assessment over time without any exercise, where cognitive assessments occurred at time points that mirrored the cognitive testing in the exercise protocol.

Cognitive Testing: During each of these two visits subjects were instructed how to take Automated Neuropsychological Assessment Metrics (ANAM), the computerized software program used to test cognitive variables for this study. ANAM is a windows/PC based, mouse operated software program designed to assess various aspects of cognitive performance; it has strong correlations to traditional neuropsychological tests and was created by the US Department of Defense as a rapid, reliable, easily repeatable neuropsychological test [30] [31].

The full ANAM full battery takes approximately 12 to 15 minutes to complete and has been used to evaluate simple reaction time and both the speed and accuracy of other cognitive functions, including information processing, visual spatial memory, continual processing (attention), code substitution (short term memory), and working memory [30] [31]. We used the 2001 version of ANAM which consisted of seven modules: 1) simple reaction time, 2) code substitution, 3) procedural reaction time, 4) spatial processing, 5) visual spatial memory, 6) working memory, and, 7) code substitution delayed [30] [31]. For a more in depth review of these subtests please consult (Lo Bue-Estes *et al.*, 2008). Each module is preceded by written on screen instructions that explain the specific sub-test, and all but the delayed memory test are followed by several practice problems.

2.4. Exercising Visit

Once the subject was instructed how to take ANAM, the subject took one practice test to familiarize herself with the test. The subject then had a five-minute rest between the **practice ANAM** and her **baseline ANAM**. Then each subject participated in a treadmill VO_2 max test that was customized for her based on her performance on the Modified Bruce test in the first lab visit. By customizing the workloads based on each person's fitness level the amount of time each subject spent on the treadmill was very consistent between subjects. **Non-Exercise Visit:** Once the subject was instructed how to take ANAM, the subject took one practice tests to become familiar with the test. The subjects had a five-minute rest between her practice and baseline ANAM test. Then there was a break that mirrored the time a subject would spend exercising during the "Exercising Visit" followed by another ANAM, a 30-minute break following the completion of that ANAM and then the last full ANAM.

2.5. Blood & Saliva Collection

At three of the data collection time points, **baseline**, **post maximal exercise**, and **recovery**, a non-stimulated, timed salivary sample and a 15 mL venous blood sample were taken. The salivary sample was collected into a 5

mL polypropylene cryovial (to avoid hormone binding) via passive drool collected through a short section of common drinking straw. The venous blood sample was collected using 21G 3/4 Vacutainer® brand Safety-Lok™ blood collection set into in a BD Vacutainer EDTA K_2 sterile tube and a BD Vacutainer Serum tube). (*Note*: *Midway through the study, after completion of the* 1st *seven subjects, BD the maker of our vacutainers switched from glass vials to plastic.*) Blood samples were spun for 30 minutes using a Sorvall RT6000B centrifuge, Wilmington, DE (at 3000 rev/min (approximately 6 1/3 on the speed dial)) at 4 degrees Celsius. Salivary samples were weighed and then vortexed using a Vortex-Genie prior to aliquotting, while still in the cryovial used for sample collection. Salivary samples were then aliquoted using glass Fisher Brand Pasteur into 2 mL polypropylene cryovials.

2.6. Serum Sample Processing

Serum samples for all times (1 = Baseline, 2 = Post, and 3 = Recovery) and conditions (1 = Non-exercise Visit, 2 = Exercise Visit) were processed using a COBAS FARA II (Basel, Switzerland). Chemicals from Wako Chemicals USA Inc. (Richmond, VA) were used in all serum and plasma testing. None of the samples had undergone any freeze-thaw cycles prior to this round of analysis. Samples were allowed to thaw for 60 minutes at room temperature in the morning, and then placed into a refrigerator to thaw the rest of the way overnight. The following morning 300 µL samples were pipetted using Gilson Pipetman pipettes into Fara Cups and loaded into numbered racks. Racks were placed in the refrigerator until processing began the following morning. Samples were refrigerated when not actively being assessed. Twenty-nine plate wells were used to run samples. Total Cholesterol, Glucose, Glycerol, and Non-esterified fatty acids (NEFA) were all measured in duplicate.

2.7. Saliva Sample Processing

Saliva samples for all times (1 = Baseline, 2 = Post and 3 = Recovery) and conditions (1 = Non-exercise, 2 = Exercise) were packaged and sent for analysis to: Salimetrics, LLC (State College, PA). Salivary estradiol was assessed in duplicate using a high-sensitivity enzyme immunoassay (Cat. No. 1-3702/1-3712, Salimetrics LLC, State College PA). The test used 100 ul of saliva per determination, has a lower limit of sensitivity of 1.0 pg/mL, standard curve range from 1.0 pg/mL to 32.0 pg/mL, an average intra-assay coefficient of variation of 7.1% and an average inter-assay coefficient of variation 7.5%. α-Amylase assay was completed using a commercially available kinetic reaction assay (Salimetrics LLC, State College PA) following Granger *et al.* [32]. Intra-assay variation (CV) computed for the mean of 30 replicate tests was less than 7.5%. Inter-assay variation computed for the mean of average duplicates for 16 separate runs was less than 6%. Salivary cortisol in assays was performed in duplicate using a highly sensitive enzyme immunoassay (Salimetrics, PA). The test uses 25 ul of saliva per determination, has a lower limit of sensitivity of 0.003 ug/dl, standard curve range from 0.012 to 3.0 ug/dl, and average intra- and inter-assay coefficients of variation 3.5% and 5.1% respectively. Method accuracy, determine by spike and recovery, and linearity, determined by serial dilution are 100.8% and 91.7%.

2.8. Statistics

All statistical calculations for salivary and plasma variables were assessed using Sigma Stat 3.5 (Jandel). Two Way Repeated Measure ANOVAs, were run to assess the effects of time, condition, and time by condition. All subject data are presented as mean ± SD, plasma and salivary samples are presented as mean ± SEM.

3. Results

Subjects were 21.8 ± 2.7 years old, 23.5 ± 4.9 percent body fat, 176.3 ± 8.9 cm tall, 61.6 ± 8.7 kg, with an average VO_2 max of 51.3 ± 6.8 ml/kg/min.

3.1. Salivary Results

Estradiol was assessed only at the baseline time point in both conditions to determine if there was a difference in hormone levels between the experimental conditions. No difference in salivary estradiol was present (Non-Exercise = 2.4 ± 0.4 pg/mL, Exercise = 2.5 ± 0.3 pg/mL), with both conditions having estradiol levels that most closely reflect the follicular phase. α-Amylase was higher post and recovery than baseline in both conditions

(ranging from 47% to 290%) and was also 35.5% higher at the post-exercise assessment in the Exercise condition compared to the Non-Exercise condition (see **Figure 1**). Cortisol at baseline was 33% higher than post-exercise and 59% higher than recovery (see **Figure 2**) but was not different at any time point between conditions.

3.2. Plasma Results

Non-Exercising condition: Glucose (**Figure 3**) and Glycerol (**Figure 4**) levels were not different at any time point. Lactate was 61% (**Figure 5**) higher at post compared to baseline and recovery. NEFA (**Figure 6**) was 50% higher at post and recovery compared to baseline. *Exercising condition*: Glucose and lactate were, 18% and 50%

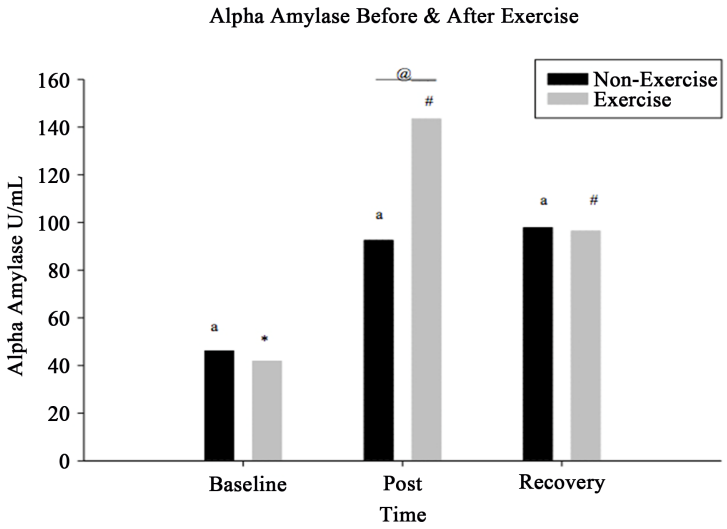

Figure 1. Values are Mean ± SEM, non-exercising time points not sharing a common letter different $P \leq 0.05$, exercise time points not sharing a common symbol different $P \leq 0.05$, time points with symbol over both non-exercise & exercise data different between conditions $P \leq 0.05$.

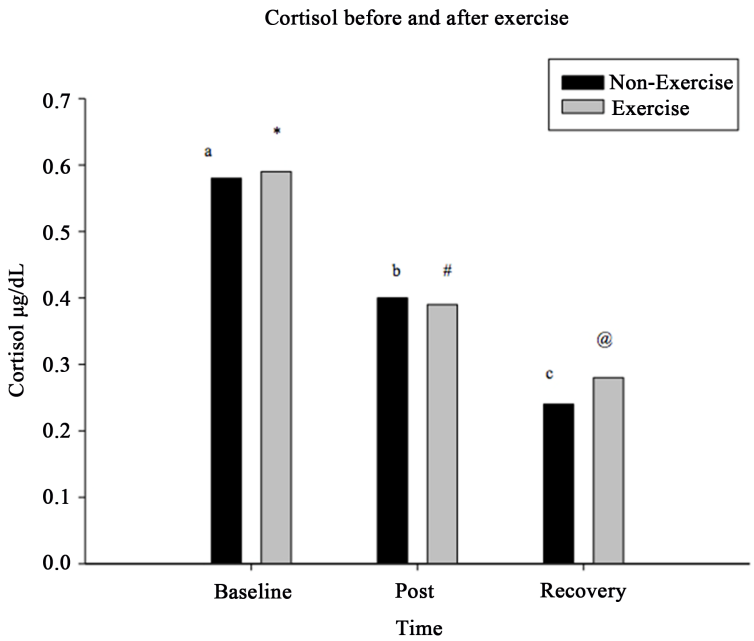

Figure 2. Values are Mean ± SEM, non-exercising time points not sharing a common letter different $P \leq 0.05$, exercise time points not sharing a common symbol different $P \leq 0.05$, time points with symbol over both non-exercise & exercise data different between conditions $P \leq 0.05$.

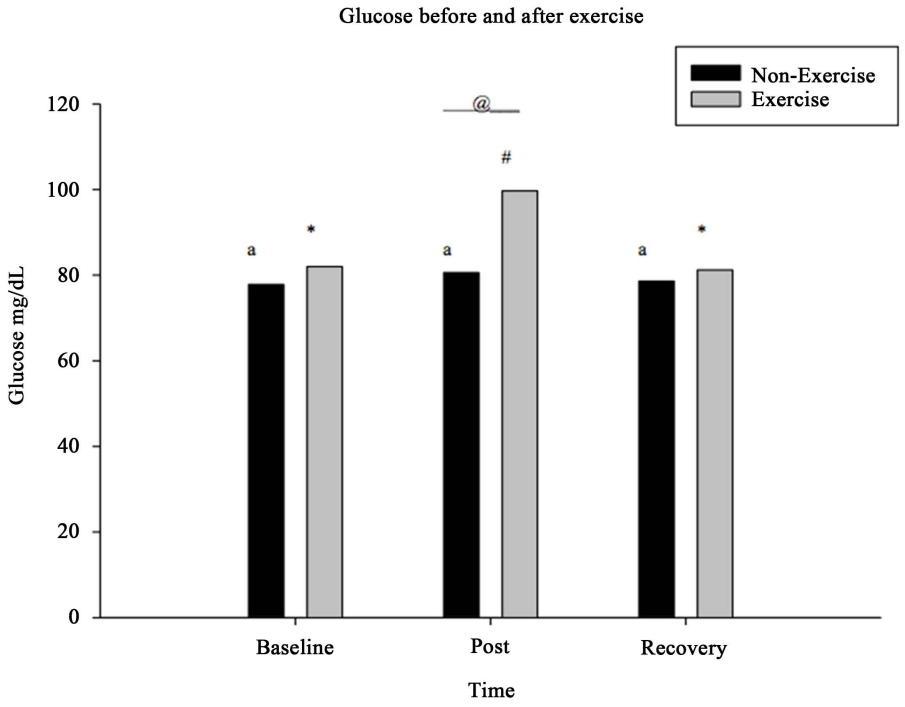

Figure 3. Values are Mean ± SEM, non-exercising time points not sharing a common letter different $P \leq 0.05$, exercise time points not sharing a common symbol different $P \leq 0.05$, time points with symbol over both non-exercise & exercise data different between conditions $P \leq 0.05$.

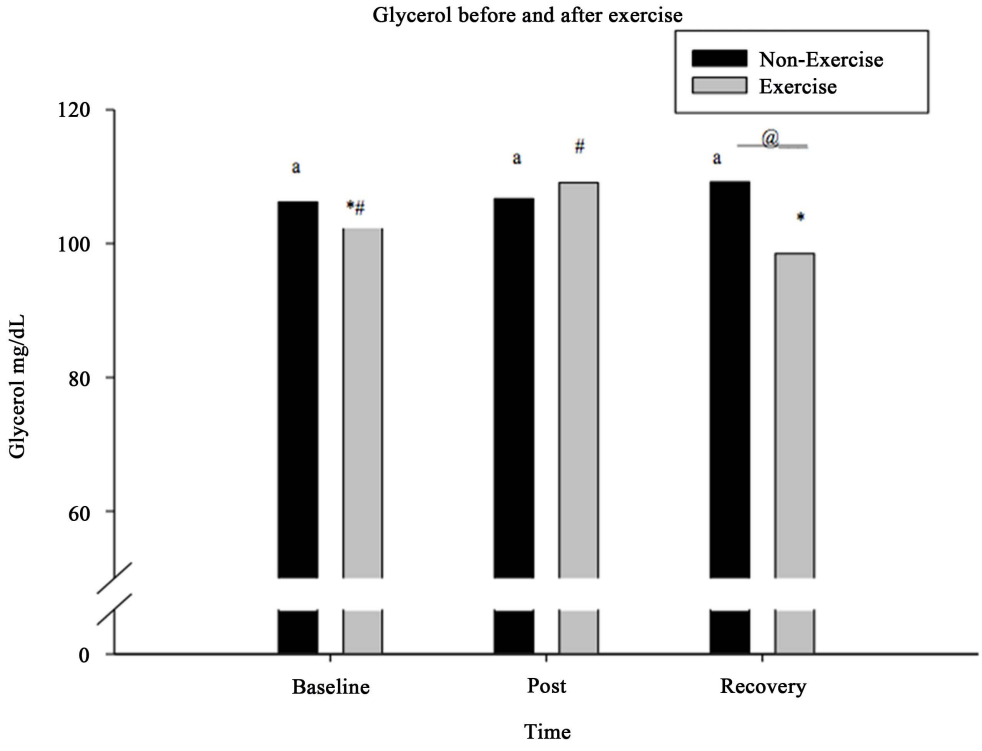

Figure 4. Values are Mean ± SEM, non-exercising time points not sharing a common letter different $P \leq 0.05$, exercise time points not sharing a common symbol different $P \leq 0.05$, Time points with symbol over both non-exercise & exercise data different between conditions $P \leq 0.05$.

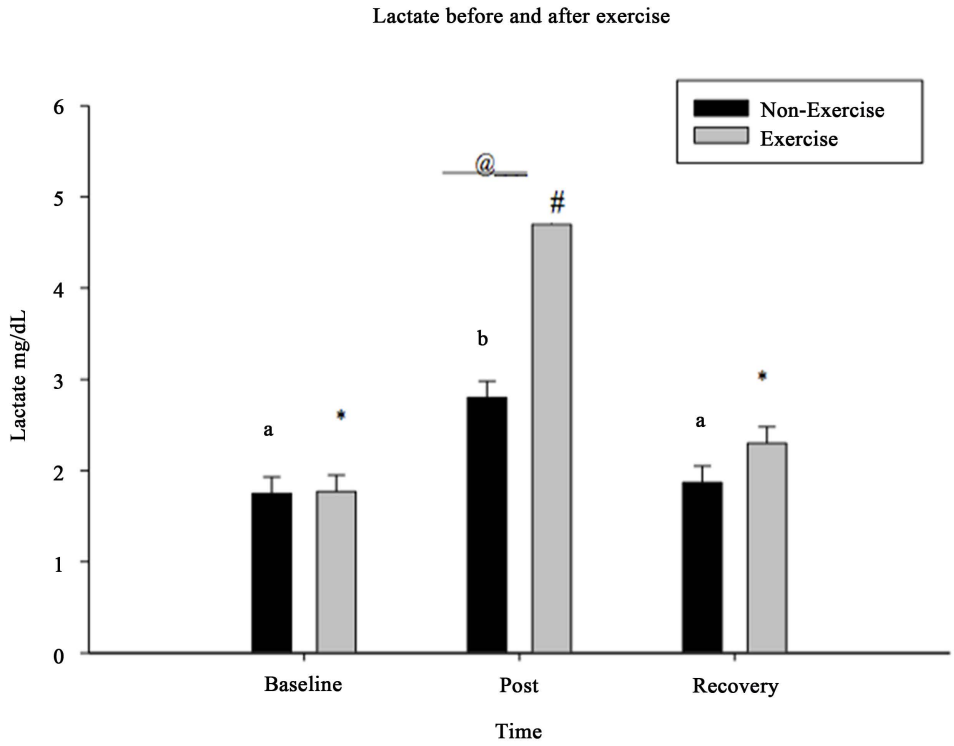

Figure 5. Values are Mean ± SEM, non-exercising time points not sharing a common letter different $P \leq 0.05$, exercise time points not sharing a common symbol different $P \leq 0.05$, Time points with symbol over both non-exercise & exercise data different between conditions $P \leq 0.05$.

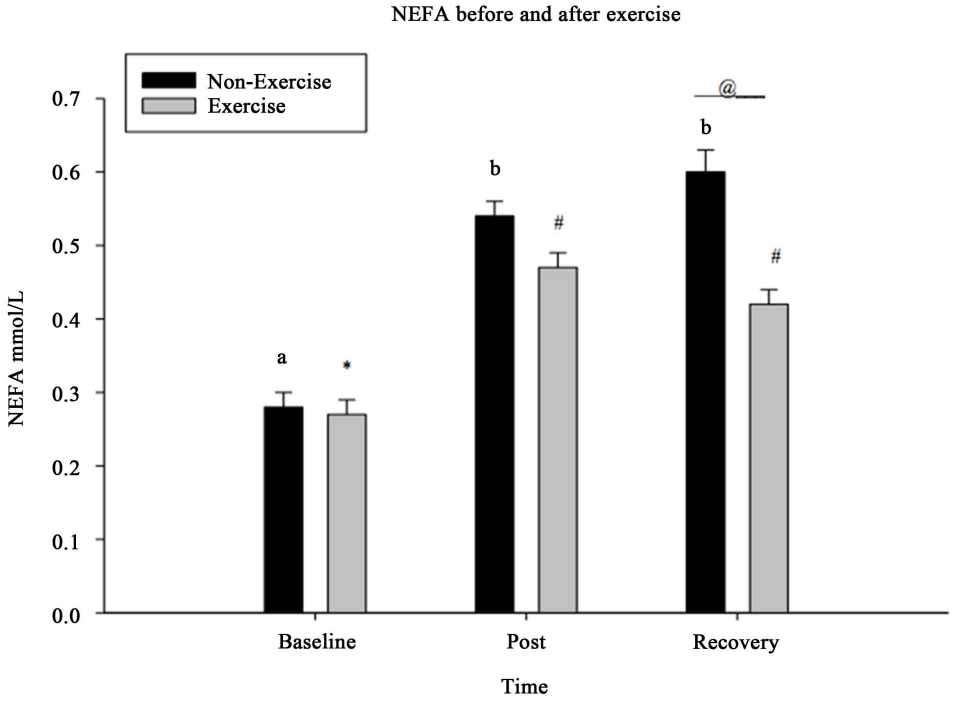

Figure 6. Values are Mean ± SEM, non-exercising time points not sharing a common letter different $P \leq 0.05$, exercise time points not sharing a common symbol different $P \leq 0.05$, time points with symbol over both non-exercise & exercise data different between conditions $P \leq 0.05$.

higher respectively, post exercise compared to baseline and recovery. Post exercise glycerol is 11% higher than recovery. NEFA was 36% higher at post and recovery compared to baseline. *Differences between conditions*: post exercise glucose and lactate were, 20% and 40% higher respectively in the Exercise condition. NEFA was 15% higher in the Non-Exercise condition at both post exercise and recovery. Glycerol was 11% higher in the Non-Exercise Condition.

4. Discussion

The methodological restriction of using only females actively taking birth control during certain phases of their medication cycle proved useful as the levels of estradiol did not vary between conditions, thus removing that hormone as a potential covariate. Salivary α-amylase is considered an indicator of stress induced changes in the sympathic adrenomedullary system [4]-[6] and salivary cortisol is an indicator of hypothalamus pituitary adrenocortical system [5]. In addition to being indicative of stresses within different systems these salivary markers also exhibit different time courses of appearance/disappearance. The time course of appearance and disappearance of salivary α-amylase is approximately 10 minutes, whereas cortisol's peak takes approximately 20 min as cortisol diffuses into the saliva from other tissues [4]. It is also important to understand that these markers are affected by circadian variations (which is why controlling for time of day and taking into account time of day in interpreting results and having an accurate control condition are key).

Salivary α-amylase has been shown to be sensitive to both physical and psychological stressors and is secreted directly by salivary glands [4] [5]. We observed no change over time in the levels of α-amylase during the non-exercise condition, showing that level of psychological stress did not change in our control (non-exercise) condition. This finding is different than much of what is currently in press [5] [7] however it may be due to the fact that much of the previous research used psychological tasks that involved a socially stressful component as well. Our findings that the exercise intervention significantly increased α-amylase levels compared to baseline levels and increased the α-amylase levels between conditions with the exercise condition being higher than the non-exercising condition are consistent with much of what is in press [33] [34].

The timing of our saliva collection occurred during the optimal window to detect changes in alpha amylase, but not in the optimal window to detect cortisol changes. Some studies show correlations between α-amylase and cortisol and others do not [35], this disparity may be 2 fold, partly because of the difference in the timing of appearance/disappearance for these markers and or because they are indicative of the stress responses in different systems. Some interventions may affect one stress system and not the other or affect the systems to varying degrees. A recent meta analysis by Dickerson *et al.* [11] reviewed 208 articles and found that while in general that cortisol increases in response to psychological stress, but that the type of stress matters and that not all stressors elicit a cortisol response. Stressor that involve uncontrollable aspects especially when coupled with social stresses elicit cortisol responses more so than those stressors that are not uncontrolled, or are not coupled with social stresses. We showed changes in cortisol levels consistent with circadian changes over the course of a morning, with subjects' cortisol levels decreasing as the morning progresses (prior to noon); but contrary to many other authors' findings we did not show a difference between our exercise and non-exercise conditions. As previously mentioned, timing of sample collection is most likely responsible for a lack of detected changes both between conditions (exercise vs. non-exercise) and times within the conditions.

Glucose and lactate were both higher at the post time points compared to all others, but significantly more so in the Exercise than non-exercise condition. So while the availability of both of these fuel sources changed over time in the non-exercising condition, they changed to a greater degree during the exercising condition. This change in fuel availability during the Exercise condition may lead to increased ability to deliver more fuel to metabolically active tissues. If more fuel is available to drive metabolism an individual may be able to "do more" than they would without this increased fuel availability. A spike in glucose following short-term maximal exercise is consistent with gluconeogenesis occurring as a direct result of the increased fuel demand occurring peripherally [3]. The post exercise spike in lactate is also consistent with the normal alterations in lactate following a maximal aerobic exercise bout [3]. Also in the case specifically of lactate, since there is an approximate time lag of about 30 minutes from when lactate appears in the plasma to when it crosses the blood brain barrier [36], changes in brain function/performance may experience a time delay as the lactate takes time to be delivered. Increased availability of lactate has also been shown to increase lactate transport across the blood brain barrier [36].

Increases in NEFA levels in the non-exercise condition are consistent with lypolysis and changes in LPL activity in healthy fasted individuals over time [22]. The increased amount of NEFA present at the recovery time point in the non-exercising compared to Exercising condition is most likely due to increased uptake of fatty acids post-exercise to replenish energy stores used during the exercise bout. Our NEFA findings are consistent with Marion-Latard *et al*. showing increased oxidation of NEFA during recovery from exercise [24]. Glycerol, which is a measure of triglyceride breakdown and mobilization from both adipose and muscle tissues, similarly to NEFA showed increased levels at the recovery time point in the non-exercise compared to the exercise conditions. It was also however increased during the exercising condition post exercise compared to recovery. This disparity between the non-exercising and exercising conditions again similarly to NEFA is most likely a result of increase peripheral uptake post-exercise to replenish energy stores. The increase in glycerol levels post exercise compared to recovery is also most likely a result of two phenomena, 1) increased fuel mobilization during exercise to fuel the increased demands of intense exercise and, 2) the previously mentioned peripheral increased fuel uptake post exercise to replenish energy stores used during exercise. Wee *et al*. [37] showed an increase in glycerol following a 20 minute bout of submaximal exercise (cycling at 70% of VO_2 max) followed by a sharp decline in the initial recovery phase, hours into recovery from exercise they observe additional increases in glycerol levels. The magnitude of increase that they observed is much greater than ours, but this is most likely due to the fact that our initial post exercise assessment occurs significantly later than theirs.

5. Conclusion

We demonstrated that acute exercise coupled with cognitive task in our protocol increases α-amylase levels, but not cortisol, potentially due to a differential stress response, but most likely due to the timing of sample collection. Additional research with sample collection occurring at time points that would maximize the ability to detect changes in both salivary α-amylase and cortisol is warranted to see if this combination of physical and psychological stresses taxes both of the representative stress systems equally or in a similar fashion.

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