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Identifying Metabolites as Markers of Fatigue in Athletes

By Christopher George

A Thesis Presented to the Department of Chemistry,
Colby College, Waterville, ME
In Partial Fulfillment of the Requirements for Graduation
With Honors in Chemistry

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Identifying Metabolites as Markers of Fatigue in Athletes

By Christopher George

Approved:

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(Julie T. Millard, Dorros Professor of Life Sciences)

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(Ronald F. Peck, Associate Professor of Biology)

_____Date

<u>VITAE</u>

Christopher George was born on October 21st, 1997. He grew up in North Kingstown, Rhode Island and graduated from North Kingstown High School in the Spring of 2016. Chris is currently a senior at Colby College and will graduate in May of 2020. He is a four year member of the Colby College Football Team and a Captain his senior year. After the football season, he received the team's Sportsmanship Award, and was later selected to the 2020 National Football Foundation Hampshire Honor Society. Chris is a chemistry major with a concentration in biochemistry. In his senior spring, Chris received the Colby College Chemistry Department Writing Award. Chris's first research experience began in Professor Julie Millard's lab where his thesis work began in the Fall of 2019 into the exploration of metabolomics. After graduating from Colby College, Chris plans to apply for and attend medical school.

ACKNOWLEDGEMENTS

I would like to acknowledge the numerous people who helped to make this study possible. First, a special thanks is owed to my mentor Professor Julie Millard. Without her guidance and inspiration this project would not have been conducted. She offered continual support throughout the entirety of the project. I had no laboratory experience before working in her lab, and she took a chance offering me the ability to do so. For that, I am and always will be grateful.

Professor Edmund Klinkerch was generous with his time and expertise helping me to analyze samples using the Colby College Chemistry Department gas chromatograph-mass spectrometer. He was especially helpful when the instrument would not respond, and worked tirelessly to fix it so I would be able to resume work. Without his help I would not have been able to analyze saliva samples from the subjects of the study.

I would also like to thank Professor Ron Peck, who helped as the reader of my final thesis, offering his time to assist in finalizing this submission.

A special thanks is owed to the training staff of the Colby College Athletic Department, especially Emily Vartabedian. She allowed me to collect data on subjects from the football team in the training room and helped collect blood pressure measurements.

In addition, I would like to thank Colby College Head Football Coach Jack Cosgrove for allowing players from his team to participate in my study.

Lastly, I would like to thank the 10 members of the Colby College Football Team who took the time to answer survey questions and let me collect fatigue data on them. Without them, there would have been no data to support the study.

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ABSTRACT

Fatigue in athletes caused by inadequate rest and other stressors can be severely detrimental to their health, and yet there is no reliable way to measure and track fatigue. Many classical measures of fatigue like body mass and resting heart rate are not reliable ways to track the physical fatigue of an athlete because they can change with many factors. In addition, it is not known how academic stress on top of physical stress affects fatigue. Metabolomics is a relatively new area of study and tracking metabolites offers the possibility to produce biomarkers to assess fatigue. This study collected saliva samples from 10 football players to determine salivary levels of the metabolites cortisol, alanine and glutamine to track the fatigue of the subjects. Due to the small subject pool, much of the changes in classical fatigue measures and metabolite concentrations were determined to be statistically insignificant. Even so, alanine exhibited relatively small changes in concentration, while glutamine exhibited a drop over the course of the football season. Cortisol did not seem to show any consistent trends.

<u>INTRODUCTION</u>

Introduction to Metabolomics

Metabolomics is a relatively new area of study, especially in relation to sports medicine and performance. A systematic review of metabolomics-based studies involving exercise showed that the metabolome, and the individual metabolites that constitute it, change drastically in response to acute and chronic exercise, although more work is needed to determine the effects of chronic exercise on the metabolome (1).

Previously, the metabolome has been studied using extremely invasive and inefficient methods. These include muscle and organ tissue biopsy as well as blood sampling to determine levels of metabolites in plasma and tissue. However, it has since been observed that saliva is a valuable biofluid in regards to the study of the metabolome (2). This means that while present in lower concentrations, metabolites in saliva are representative of metabolites in plasma. Collection of saliva samples is quick, easy and non-invasive for the athletes being tested.

While sample collection is very easy, analysis and quantification of the metabolites in each saliva sample is more complicated. Different research groups have used different analytical techniques, depending on the metabolite. Some researchers have used competitive enzyme linked immunosorbent assay (ELISA) plates, while others have used proton NMR. Many studies have effectively used gas chromatograph-mass spectrometry (GC-MS) or liquid chromatograph-mass spectrometry (LC-MS).

A number of studies set a precedent for the experiments done in this study. Ra et al. examined the metabolome of soccer players over the course of three consecutive games (3). Saliva samples were collected from subjects and examined using capillary electrophoresis-time of flight mass spectrometry. After analysis of the salivary metabolome, it was found that all salivary

metabolites of all players categorized as fatigued (by classical measurements) were increased after the third soccer game. In particular, alanine, valine and taurine were found to be significantly increased. This study correlated classical measurements of fatigue to changes in the metabolome to track the condition of soccer players during consecutive days of exercise. One of the main benefits of this study is that instead of attempting to simulate competition conditions, the researchers studied athletes involved in a competition. One restriction to this study is that it was conducted over the short term. Fatigued athletes were found after three days. Our investigation fills this gap by monitoring the metabolome of athletes over multiple months of in-season play or intense training.

Goals of This Study

This study aims to determine the change in concentration of specific metabolites in the saliva of athletes over the course of their season or training period. This will be done in an effort to find a reliable biomarker of fatigue in athletes. In addition, the study will investigate how an increased academic workload and a lack of sleep combine to affect the affect salivary metabolites, and thus the level of fatigue of each subject. Many studies have only examined the effect of physical exercise or sleep deprivation and physical exercise on fatigue and the metabolome. Our study will correlate the effects of physical exercise, sleep deprivation and academic stress on fatigue and the metabolome. We predict that all three will combine to increase how fatigued each athlete is more than any single factor can do on its own. To accomplish this, two groups of athletes were tested. One group was composed of 10 members of the Colby College Football Team, and the other group was composed of recreational long distance runners training for and ultimately competing in marathons.

Over the course of the 2019 football season, data were collected on ten Colby College football players who have been split into two groups: those more likely to be fatigued and those less

likely to be fatigued. The "more likely to be fatigued" group contained six players with the following positions: defensive back, running back, offensive lineman, two defensive linemen, and one linebacker. Players from these positions have been put into this group because they are larger, run more than players in other groups, and sustain more contact, both in force and quantity.

The "less likely to be fatigued" group was composed of three kickers and one quarterback. Kickers almost never sustain contact or run a meaningful amount in games or practices, and are active in a fraction of the plays that those in the "more likely to be fatigued" group are. While quarterbacks are active for the entire game, they sustain far less contact than players in the other group, and run far less than players in the other group over the course of a game or practice.

Data collection for the football players was done at four times over the course of the season. Ideally, the first collection would be before preseason started, but due to limitations, the first collection was done after 3 days of preseason practice. The second data collection was done the week prior to midterm exams. The third collection was done following the week midterms had concluded. The final data collection was done three weeks after the end of the season, during final exams. These collection times were selected to determine how academic stress and lack of sleep affected the physical fatigue of the players.

The second study was a pilot study involving marathon runners. Data collection was to be done every other week of training leading up to the marathon, directly after the marathon, and approximately one month after the marathon. This group did not contain college students so we were not able to examine the impact of academic stress on the metabolome. Nonetheless, this group of athletes will still give us insight into how an increased training load affects classical markers of fatigue and key metabolites in saliva. This information may be helpful to avoid over-training before it happens.

During data collection times, multiple parameters were measured to assess fatigue, and saliva samples were taken for further analysis in the lab. Heart rate, body mass, blood pressure, and blood lactate were taken. Subjects also completed a "Profile of Mood States" assessment. Saliva was worked up in the laboratory to determine the levels of cortisol, alanine and glutamine.

Implications of Fatigue in Athletes

Identifying fatigued athletes is of the utmost importance. Fatigue is more than just feeling tired after an athletic performance, but occurs over the duration of a lengthy athletic season or training period. Fatigue can be defined as a decrease in the baseline psychological and physiological condition of an athlete prior to beginning a match (4). In general, fatigue occurs when an athlete is not given adequate time to rest and recover after a match is played or training is done (2). There is not consensus among the sports medicine community on the amount of time needed for an athlete to recover after training, and part of this is due to differences between athletes and the work they perform, as well as individual external stressors.

The effects of fatigue on an athlete are far reaching. Normally, fatigue is first noticed because of a decrease in sports performance by an athlete (4). But there are other, more serious outcomes as well. Fatigue has been shown to increase the risk of injury and illness (4). One study following soccer players who made it to the World Cup found that the extension of the playing season brought with it a drop in performance of 32% of the athletes and injury to 29% percent of the athletes. In addition, the risk of injury was greater for those athletes who played more games per week leading up to the World Cup (4). Fatigue can also cause an increased risk of illness, as a rapid increase in training volume leads to immunosuppression, and an increased risk of illness if the athlete is not allowed to recover (4).

When the burden of fatigue becomes overbearing, it can lead to a more serious problem called overtraining syndrome (OTS). Under this condition, athletes do not recover from fatigued conditions even following 2 weeks of rest. OTS is often associated with "prolonged underperformance" in addition to depression and frequent minor illnesses, particularly those of the respiratory tract (5). It is also noted that other stressors, including academic stress for student athletes, contribute to the overall fatigue of an athlete. It is critical for coaches and support staff to prevent an athlete from reaching OTS by supplying them with sufficient rest during their season. Being able to quantify and measure how fatigued an athlete is can help prevent fatigue from turning into OTS. If the importance of rest is not already understood, Koutedakis et al. researched the effect of 3-5 weeks of rest on underperforming Olympic athletes. They found that after rest, VO_2 max and body weight both increased, as well as the athlete's overall mood as assessed with a Profile of Mood States (POMS) questionnaire (6). This study shows that fatigue and overtraining plague athletes at all ability levels, and adequate rest is needed to combat fatigue.

While fatigue is easy to observe in an athlete, it can often be difficult to quantify. Classically, fatigue has been measured by tracking resting heart rate, body mass and by conducting mood assessments like the POMS test (7, 3). In particular, a rise in resting heart rate accompanied by a decline in body mass over the course of an athletic season are indicative of a fatigued athlete. However, these measurements of fatigue are not always reliable, and can fluctuate depending on the time of day data is collected, and thus do not always accurately characterize the fatigue state of an athlete. For this reason, a universal, accurate biomarker of fatigue must be identified in order to maintain the health and safety of student athletes, and athletes in general. In recent years, the study of the metabolome has opened up new doors to measure the fatigue of an athlete.

Sleep Deprivation and Fatigue

The Colby College Football Team practices at 6 am on Tuesdays and Wednesdays and at 7 am on Thursdays and Fridays. This practice schedule remained the same throughout the course of the season. It does not change during exam weeks, when players are under the additional burden of an increased academic load. For the majority of players, this results in an inadequate amount of sleep during exam periods, because they stay awake late to study and wake up early to practice. A lack of sleep has been shown to have serious negative impacts on student athletes.

A study done on male weight lifters found that athletes underperformed on maximal and submaximal effort lifts after suffering three nights of sleep deprivation (8). Just as the main side effect of fatigue on an athlete is underperformance, we see from this study that sleep deprivation has the same effect. This leads us to hypothesize that the combination of physical fatigue and lack of sleep will lead to serious consequences like underperformance that should be detectable in the metabolome.

A similar study found that after one night of total sleep deprivation, subjects were severely limited in their ability to expend energy. In addition to this, total sleep deprivation caused a significant rise in daytime cortisol levels of the subjects (9). If players who are sleep deprived are not able to recover, then after physical exercise their cortisol levels will continue to rise, and this chronic stress response may contribute to an increase in the overall fatigue that the athlete experiences. To show how important adequate sleep is, Stanford varsity basketball players with normal sleep schedules were allowed to extend their sleep time about two hours to ten hours nightly. After the six-week period of sleep extension, the players had faster sprint times, as well as higher shooting percentages, indicating an overall increase in their athletic performance (10).

A recent study (2019) conducted by Akazawa et al. examined the effect of sleep quality (not duration) on the metabolome, athletic performance and cognitive function on male volleyball

players. Similar to the study done by Ra et al., capillary electrophoresis and time of flight mass spectrometry was done to identify metabolites. In the study, there were two groups: better and lesser sleep quality. The better sleep quality group also averaged 20 more minutes of sleep per night than the lesser sleep quality group, but this was not the focus of the researchers. Not surprisingly, it was found that subjects in the better sleep quality group responded faster during the Stroop test, indicating higher levels of cognitive function with sufficient and high quality sleep. This is important to the current study, showing that not only does sleep deprivation affect athletic performance, but it also affects academic performance. In addition to cognitive function, the better sleep group had higher salivary concentrations of urea cycle intermediates accompanied by lower concentrations of urea (11). This is relevant to the study because glutamine plays an active role in the transport of ammonia to the liver for metabolism and excretion by the urea cycle.

Biomarkers of Interest

Many of the ways that fatigue has been characterized, quantified and tracked in the past is not as accurate as necessary to predict and stop the onset of fatigue before it is too late. Resting heart rate has often been tracked, with an increase over time signaling a state of fatigue. This method is not always accurate, because heart rate can fluctuate depending on the time of day it is measured, or if a subject is under stress from external factors. Body weight also has been tracked in athletes, with a decrease over time indicating fatigue. This can be more reliable, but also varies on the time of day, hydration status and when the subject last ate. Blood lactate levels can also indicate how fatigued an athlete is, but this can also vary. Different people are able to clear lactate from their blood faster than others, and will not show an increase over time.

Due to the limitations of classical measures of fatigue, the goal of this study is to measure three metabolites present in saliva to create a dependable method for predicting, quantifying, and stopping the onset of fatigue. The three metabolites of interest are cortisol, alanine and glutamine.

1. Cortisol

Cortisol is one of the three biomarkers for fatigue that this study will be quantifying in the saliva of the athletes. During a stress response, cortisol is released by the adrenal glands to help the body in a "fight or flight" situation (Figure 1). Cortisol is a steroid hormone that has multiple effects during a stress response with the main goal of mobilizing and making energy stores available for the body to use. One way it does this is by triggering muscle break down to make amino acids available for gluconeogenesis. Gluconeogenesis, the reverse process of glycolysis, uses amino acids to construct glucose in the liver for the brain to use as fuel during a stress response. In addition, cortisol activates lipolysis. This releases fatty acids for beta-oxidation, which generates ATP for the body to use. Moreover, cortisol decreases release of insulin by the pancreas and increases release of glucagon. Glucagon signals for the increased activity of processes like glycogenolysis, gluconeogenesis and lipolysis which all aid the body in energy mobilization. Cortisol also acts to increase the effect of other stress hormones like glucagon and epinephrine (12).

In addition to mobilizing energy, cortisol acts as an anti-inflammatory signal. It does this by downregulating specific proteins that regulate inflammation in the body. This can help by reducing pain at the site of an injury or helping the body stay at homeostasis while pro-inflammatory cytokines are being released. Prolonged activation of cortisol has been shown to have immunosuppressive activity in the body (13). This is a possible reason that a fatigued athlete is more likely to experience frequent infection. In previous work, chronic exercise has been shown to

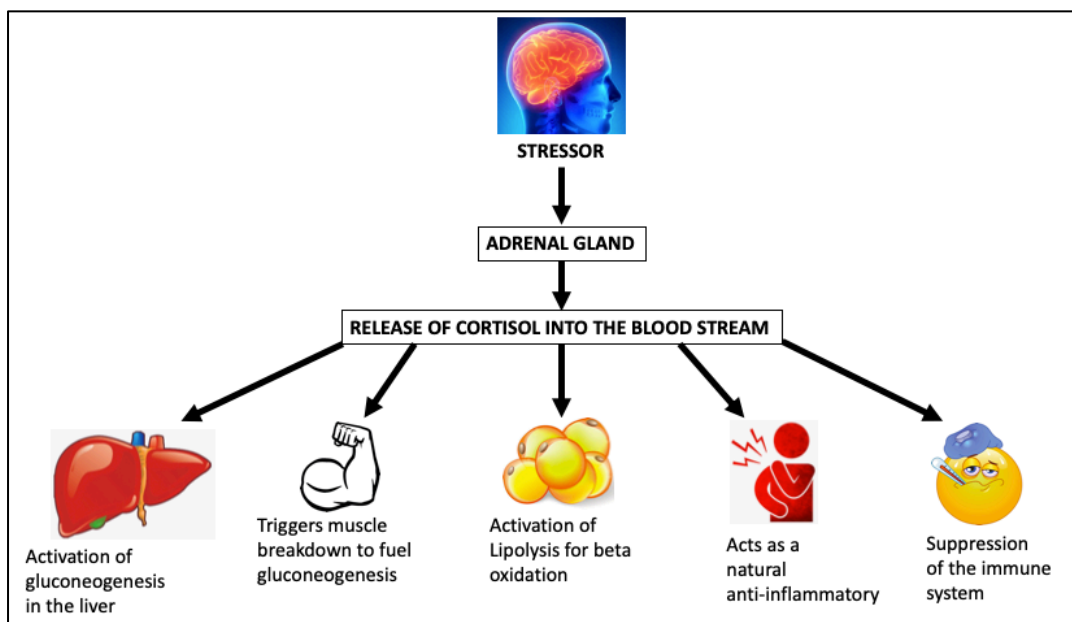increase levels of cortisol, until the athlete experiences OTS, at which point cortisol levels will drop (14).

Figure 1 shows a schematic of the systems affected by the release of cortisol during a stress response.

2. Alanine

Of particular interest to the present study is the increase in alanine that occurs during muscular work. Using principle analysis, Ra et al. determined that changes in alanine indicated changes in larger metabolites, and that was determined to be most indicative of fatigue. Alanine by itself may also represent how fatigued an athlete is throughout a season. Alanine is active in the glucose-alanine cycle. Alanine acts as a shuttle for both pyruvate carbon skeletons and ammonia groups from skeletal muscle to the liver. Pyruvate is generated in skeletal muscles from the breakdown of glucose during muscular activity. It is taken to the liver to be used in gluconeogenesis. Ammonia is generated from muscle breakdown, and alanine also shuttles it through the blood to the liver where it will be excreted from the body by the urea cycle (Figure 2).

Alanine must transport ammonia through the blood because free ammonia in the blood stream is toxic (15). It is expected that salivary levels of alanine will increase over the course of a season as an athlete becomes fatigued and more stress is placed on the glucose-alanine cycle.

There are ways to simulate game conditions in the laboratory. One of these methods is the "yo-yo" test, which evaluates an athlete's ability to perform repeated high intensity exercise. One such study used this test to induce a change in the metabolome that was measured using proton NMR (2). Of particular significance is that this study agreed with Ra et al., finding an increase in alanine over the course of the test. In addition to alanine, this study determined that lactate concentrations and glutamine levels changed significantly before and after the yo-yo test.
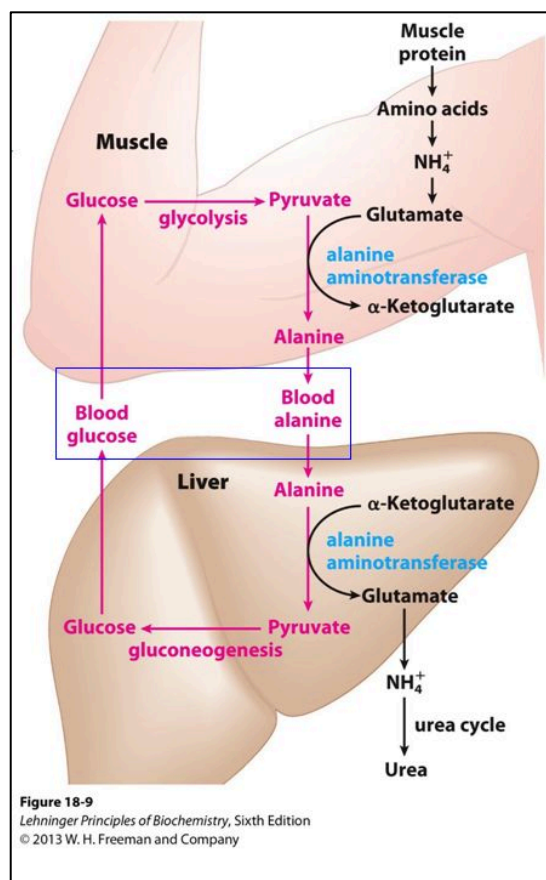

Figure 18-9
Lehninger Principles of Biochemistry, Sixth Edition
© 2013 W. H. Freeman and Company

Figure 2 shows how alanine is involved in muscle breakdown by shuttling ammonia and carbon skeletons from skeletal muscle to the liver (15).

3. Glutamine

The third metabolite of interest is glutamine. Keast et al. had subjects perform twice daily strenuous exercise for ten days. They showed that acute, as well as chronic, physical activity decreased plasma levels of glutamine. The study also found that glutamine levels may take as many as six days to recover to levels prior to the training period (16). Glutamine plays an active role in the urea cycle. Much like alanine, it acts as a transport for free ammonia produced by skeletal muscle breakdown. In the muscles, an extra amine group is added to glutamate, transforming it into glutamine. Glutamine shuttles the ammonia to the mitochondria of the liver, where it is changed to urea and excreted (Figure 3) (15). The mechanisms that cause physical stress to depress glutamine levels instead of raise them are not yet understood.

Figure 18-10
Lehninger Principles of Biochemistry, Fifth Edition
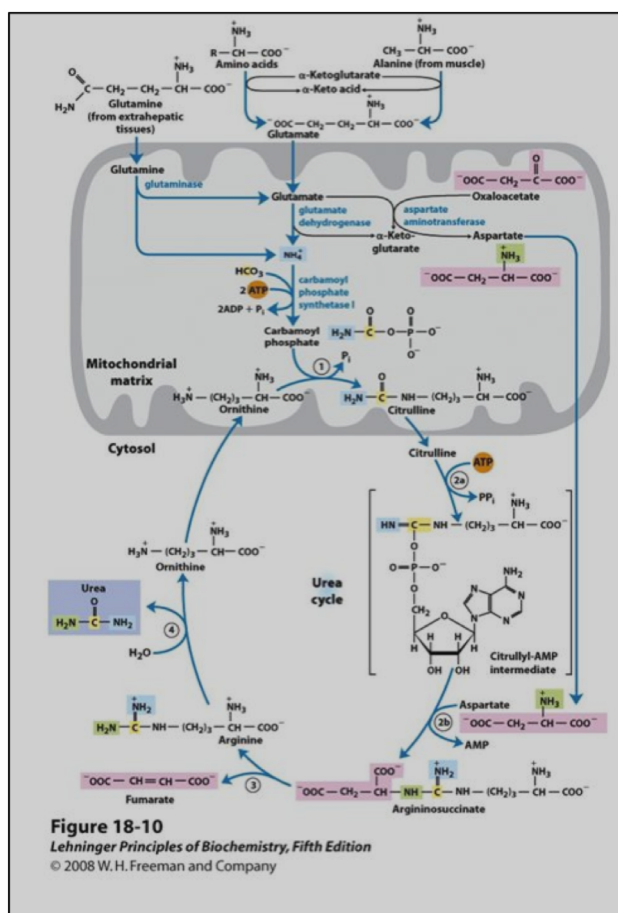© 2008 W. H. Freeman and Company

Figure 3 shows how glutamine plays a role in muscle breakdown by delivering free ammonia from skeletal muscle to the liver to be excreted from the urea cycle (15).

Impact of This Study

      While many studies have examined the effect of fatigue and insufficient sleep on athletic and cognitive performance, as well as the human metabolome, no study has attempted to determine the combined effects of physical fatigue, academic stress and sleep deprivation on the performance of student athletes through examination of the metabolome. This study aims to fill in the gaps in previous work, and determine if fatigue is increased (as predicted) by a combination of stressors. Classical measurements of fatigue along with targeted metabolomics were the primary tools used to accomplish the goals of this study.

MATERIALS AND METHODS

Ethical Statement

This study involving human subjects was approved by the Colby College Institutional Review Board before being started. Each participant was aware of the procedure of the study and the purpose for which it was being conducted. All subjects signed an informed consent form prior to any testing being done. All subject data was kept confidential and the findings of the study were reported so as not to breach this confidentiality.

Athlete Groups

Two groups of athletes were tested throughout the course of the study. Ten Colby College football players were tested in the fall of 2019 and two marathon runners were tested in the spring of 2020. The subjects from the football team were further divided into two categories based on the positions they played. The categories were those more likely to be fatigued (six members) and those less likely to be fatigued (four members). The more likely to be fatigued group was made up of one running back, one strong safety, one linebacker, two defensive linemen and one offensive lineman. The less likely to be fatigued group was made up of three kickers and one quarterback. The two distance runners were not split into separate groups, but both were in the midst of training for upcoming marathons.

Data Collections

For the football players, data was collected at four different times. The first collection date occurred on 8/28/19 approximately 30 minutes after practice ended and was three days into the start of preseason. Due to limitations, data could not be collected prior to preseason practice. The

second data collection was done over 10/1– 10/2 approximately 30 minutes after practice had ended each day. This data collection was done the week before midterm exams took place. Midterms took place from 10/7 to 10/11, and data was collected on Sunday, 10/13/19. The final data collection took place from 12/4 to 12/13, over the course of final exam week, when no football practice was held. The final data collection was extended compared to previous collection times due to scheduling accommodations for subjects studying for and taking final exams. These four data collections are depicted in the data as A, B, C, and D, respectively.

For the two marathon runners, one subjected was tested weekly starting 1/27/20 until the date of the subject's marathon, 5 weeks from the first collection, and one time approximately one week after the race. The second marathon runner was tested bi-weekly starting 1/29/20 until mid-March. Unfortunately, the second runner's marathon was cancelled due to Covid-19. In addition, saliva was not able to be worked up for either marathon runner due to Colby's cancellation of on-campus learning in response to the Covid-19 pandemic.

Heart Rate

For subjects on the Colby College football team, resting heart rate was taken using fingertip Pulse Oximeter from Beijing Choice electronic. For the marathon runners, average resting heart rate was determined by the subjects' own FitBits or smart watches.

Body Weight

Body mass was measured to the nearest 1 pound using the scale in the Colby College training room for all subjects on the football team. For the marathon runners, their respective personal home scales were used.

Blood Lactate

Blood lactate was measured using the Lactate Plus meter and matching lactate test strips from Nova Biomedical. The finger of each subject was first cleaned with 70% isopropyl alcohol dabbed on a cotton ball before being pricked with the SurgiLance safety lancet manufactured by MediPurpose.

Blood Pressure

Blood pressure of subjects on the football team was measured using a manual blood pressure collar operated by one of the Colby College athletic trainers. The blood pressure of the marathon runners was measured using an automated blood pressure monitor manufactured and sold by Walgreens.

POMS and Weekly Hours

Each subject on the Colby College football team filled out a mood and wellness survey where they recorded their average nightly sleep for the past week, as well as the average amount of time they spent per day for the previous week on football related activities, and the average amount of time they spent on academic work per day for the past week. In place of average football time and class time, subjects who are marathon runners recorded the number of miles they ran throughout the previous week.

In addition, all subjects performed a Profile of Mood States (POMS) assessment at the time of each collection to assess how they were feeling in that moment. This study used an abbreviated version of the POMS assessment previously used to successfully measure mood disturbance in athletes (17). The questionnaire is made up of 40 items where the subjects selected 0, 1, 2, 3, or 4 for each item. 0 means "not at all" and 4 means "extremely". The questionnaire assesses five

negative categories (tension, anger, fatigue, depression, and confusion) and two positive categories (esteem-related affect and vigor). The POMS is scored by subtracting the sum of the positive subscales from the sum of the negative subscales (17).

Saliva Collection

Saliva was collected during each collection by having subjects spit into a 15 mL plastic test tube which was subsequently stored at -20°C until further analysis was done.

Cortisol Detection by ELISA

Prior to beginning the cortisol ELISA protocol provided in the kit manufactured by NEOGEN, all saliva samples were transferred from the collection tubes to 2 mL microcentrifuge tubes. All saliva samples were spun down in an Eppendorf Centrifuge 5402 at 6000 x g for 8 minutes with the temperature set at 4°C. The supernatant was transferred to clean microcentrifuge tubes and the pellet was discarded.

Each saliva sample was diluted by a factor of 10. 50 μL of each saliva sample was diluted with 500 μL of EIA buffer in clean microcentrifuge tubes. In addition, following the protocol provided by NEOGEN, eight cortisol standards were made to be run on the ELISA plate alongside the saliva samples. The standards had concentrations of 0 ng/mL, 0.04 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.4 ng/mL, 1.0 ng/mL, 2.0 ng/mL and 10 ng/mL.

Following the NEOGEN protocol, the enzyme conjugate was diluted where 1 μL of enzyme was mixed with 50 μL EIA buffer for each well being used. 50 μL of either standard or saliva sample was added to the appropriate well. Then 50 μL of diluted enzyme conjugate was added to each well in use. The plate was then covered with Parafilm and incubated at room temperature for 1

hour while being gently shaken on a Thermo Scientific plate shaker. After incubation, the contents

of the plate were dumped out, and each well was washed three times with 300 μL of dilute wash

buffer each time. Concentrated wash buffer was diluted by a factor of 10 in deionized water prior to

washing the plate. After washing, 150 μL of substrate (provided in the NEOGEN cortisol ELISA

kit) was added to each well, and the plate was incubated at room temperature on the plate shaker for

30 minutes. The plate was read at 650nm on a SpectraMax M5 microplate reader manufactured by

Molecular Devices.

To determine the concentration of cortisol in each saliva sample, a standard curve was

generated from the standard solutions created using the NEOGEN kit run on the ELISA plate. To

generate the curve, the percent maximal binding was determined by dividing each standard

absorbance value by the absorbance of the standard with 0ng/mL cortisol. The maximal binding

was plotted against the known concentration of each standard and the data was fitted with an

exponential function. The equation of this function was used to determine the concentration of

cortisol in each saliva sample from the determined maximal binding value of each sample.

Derivatization Reaction to Quantify Alanine and Glutamine by GC-MS

After initial centrifugation, the supernatant of each saliva sample was further filtered using

Amicon Ultra 0.5 mL Centrifugal Filters (Ultracel 3K Filter). 500 μL of each saliva sample was

added to the filter, and was then spun down using the Eppendorf Centrifuge 5402. The filters were

spun at 12,000 rpm for 15 minutes at a temperature of 4°C. After filtration, approximately 200 to

220 μL had been trapped in the filter.

After centrifugation, the filtrate was transferred to 2mL microcentrifuge tubes with locking

tops. 65 μL of 0.1M HCl was added to each saliva sample to acidify the saliva. The acidified

sample was then dried using a Varian vacuum pump connected to LABCONCO Centrivap Cold Trap and a LABCONCO Centrivap Concentrator.

Once dried, 100 μL of the silylation reagent N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide (MTBSTFA) was added to the dried sample, and then 100 μL of acetonitrile was added. This mixture reacted for 4 hours at 100°C in a Fisher Scientific Isotemp (18). This derivatization reaction had to be run due to the polar nature of amino acids in order to detect alanine and glutamine with the GC-MS.

GC-MS Procedure

The reaction mixture was transferred to a GC-MS vial insert. Reaction mixtures consistently did not fill the insert, and acetonitrile was used to fill the remaining volume. Each sample was run on an Agilent Technologies 7890A Gas Chromatograph with a 5975C Mass Selective Detector. The GC column used was the HP-5MS, with dimensions of 30m by 0.25 mm and 0.25um film thickness. The oven was programmed to hold 100°C for 2 minutes and then ramp to 250°C at 15°C/min and hold for 60 min, with a total run time of 72 min. Helium was the carrier gas used with acetonitrile as the wash solvent.

To assess alanine and glutamine concentration in the saliva samples, a standard curve was created for both metabolites. Standard solutions containing both alanine and glutamine of 100 μg/mL, 50 μg/mL, 25 μg/mL, 10 μg/mL and 5 μg/mL were derivatized and then run on the GC-MS. The instrument was calibrated with these standard solutions to quantify alanine and glutamine in the saliva samples.

Statistical Analysis

To determine if significant changes had occurred between the first data collection time (A) and any of the subsequent collections (B, C, and D) Excel was used to calculate a t-distribution. The t-distribution was used to calculate a two-tailed p-value for each change. P-values less than or equal to 0.05 indicated that the null hypothesis of no change was rejected, and that significant change had occurred in the measured parameter from the first data collection time.

RESULTS

Football Players

Classical measures of fatigue, as well as metabolite concentrations, were quantified for subjects on the Colby College Football Team over the four testing dates (A, B, C, and D). Values with statistically significant differences (as determined by a two-tailed p-value) from the first testing date (A) have an asterisk (*) above them. All p values are reported at the end of the results section (Figure 13).
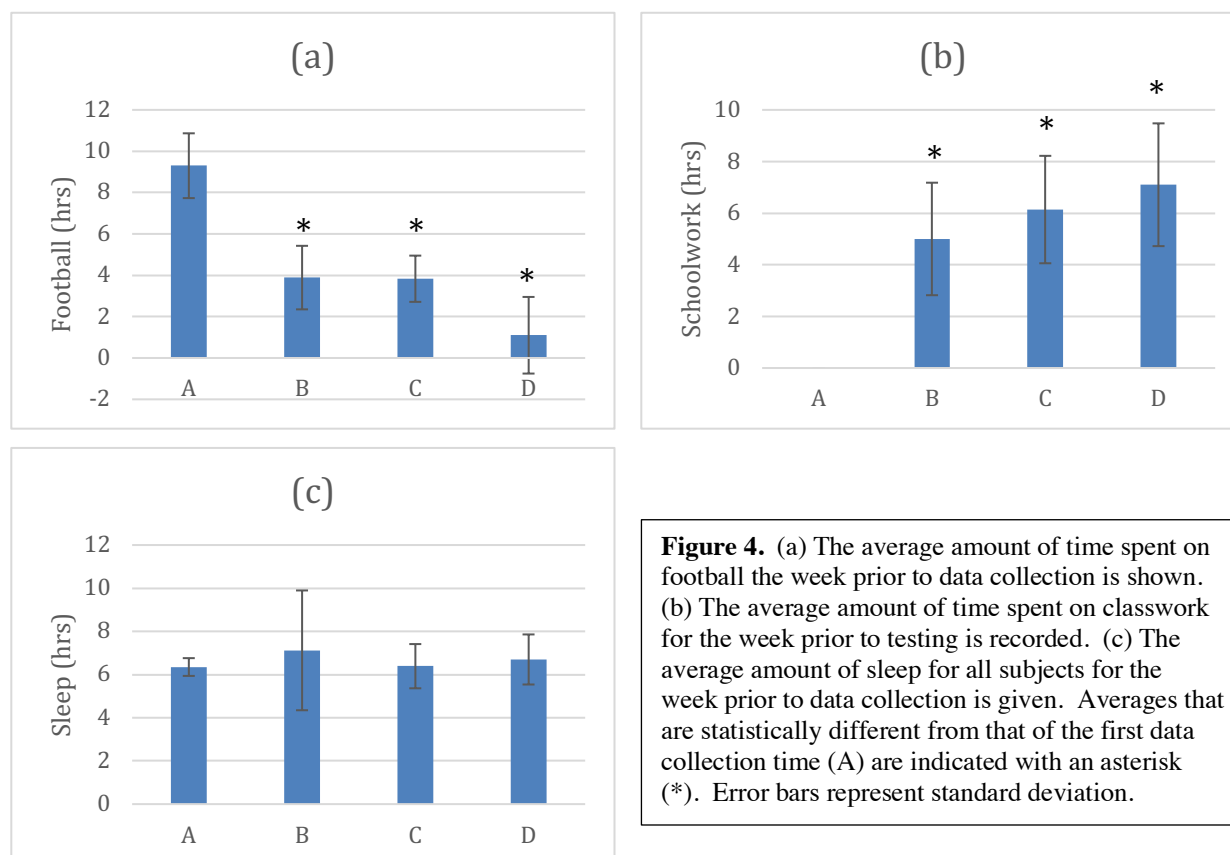
Hours of Sleep, School Work and Football

Each subject was asked to record the average amount of time each day for the week prior to testing they spent on football-related activities, classwork and how many hours of sleep per night they averaged. This was done to understand the source of different stressors affecting the athletes leading up to each testing time point.

A statistically significant change was found between how many hours the subjects spent on football-related activities each day during the first collection date (9.3 hours per day) compared to 3.8 hours for collection dates B and C (Figure 4a). Subjects spent an average of 1.1 hours per day on football during the week of the last collection.

During the week of the first collection time, subjects spent no time on classwork (because classes had not started), which increased significantly for the next three collection times (Figure 4b). Although average time spent on school work for testing dates B, C, and D, was significantly greater than time spent during the first testing week, there was no statistical difference between B and C or B and D.

No statistically significant change was detected for the average nightly hours of sleep during
data collection weeks B, C, and D compared to the first week (Figure 4c).

Figure 4. (a) The average amount of time spent on football the week prior to data collection is shown. (b) The average amount of time spent on classwork for the week prior to testing is recorded. (c) The average amount of sleep for all subjects for the week prior to data collection is given. Averages that are statistically different from that of the first data collection time (A) are indicated with an asterisk (*). Error bars represent standard deviation.

Heart Rate

Resting heart during each testing date was the first parameter analyzed. Averages for all
subjects were looked at as well as individually by group. A statistically significant rise in average
resting heart rate for all participants was observed during collection B (Figure 5a). Average heart
rate of all subjects rose from 70.9 bpm to 81.4 bpm, a statistically significant increase (p=0.0371).

The average resting heart rate of the "less likely to be fatigued" group did not change
significantly from the first collection time (Figure 5b). In contrast, the average resting heart rate of
the "more likely to be fatigued" group rose 13 bpm from A to C with p=0.0327. Although the
"more likely to be fatigued" group has a noticeably higher average resting heart for B and C than

the "less likely to be fatigued" group (Figure 5b), this difference in resting heart rate between the two groups for B and C is not considered statistically significant.
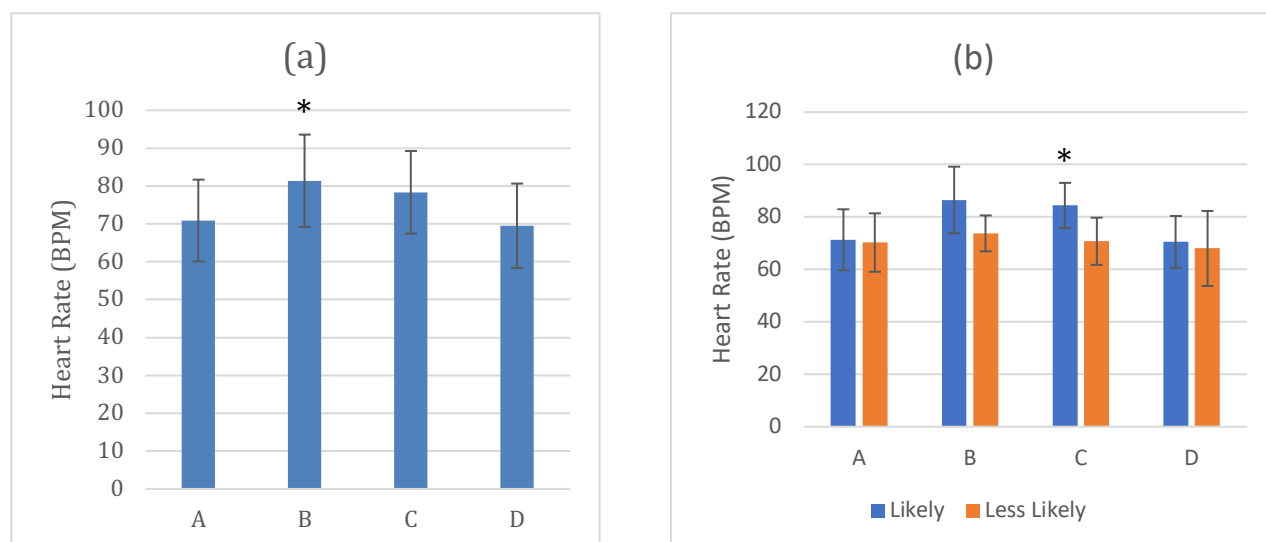

Figure 5. (a) The average resting heart rate is shown for all subjects. (b) The average resting heart rate of the subjects in the "more likely to be fatigued" group (blue) are compared to the average resting heart rate of the subjects in the "less likely to be fatigued group" (orange). Averages statistically different from those during the first data collection (A) are indicated with an asterisk (*). Error bars represent standard deviation.

Body Mass

Body mass at each testing date was recorded. Differences in average body mass between the "more likely" and "less likely to be fatigued" groups were not examined due to the large gap in average body mass. "More likely to be fatigued" subjects had a much higher average body mass.

The average body mass of all subjects dropped from 212 lbs during collection date A to 208.3 lbs on collection date B with $p=0.0162$ (Figure 6a). Average body mass dropped on collection date C to 202.8 but this change was not considered to be significant ($p=0.901$), most likely because data was not collected for one subject.

Average body mass of the "more likely to be fatigued" subjects dropped significantly 3.8lbs from A to C with p=0.0143 (Figure 6b). No significant change in mass was found for subjects in the "less likely to be fatigued" group (Figure 6c).
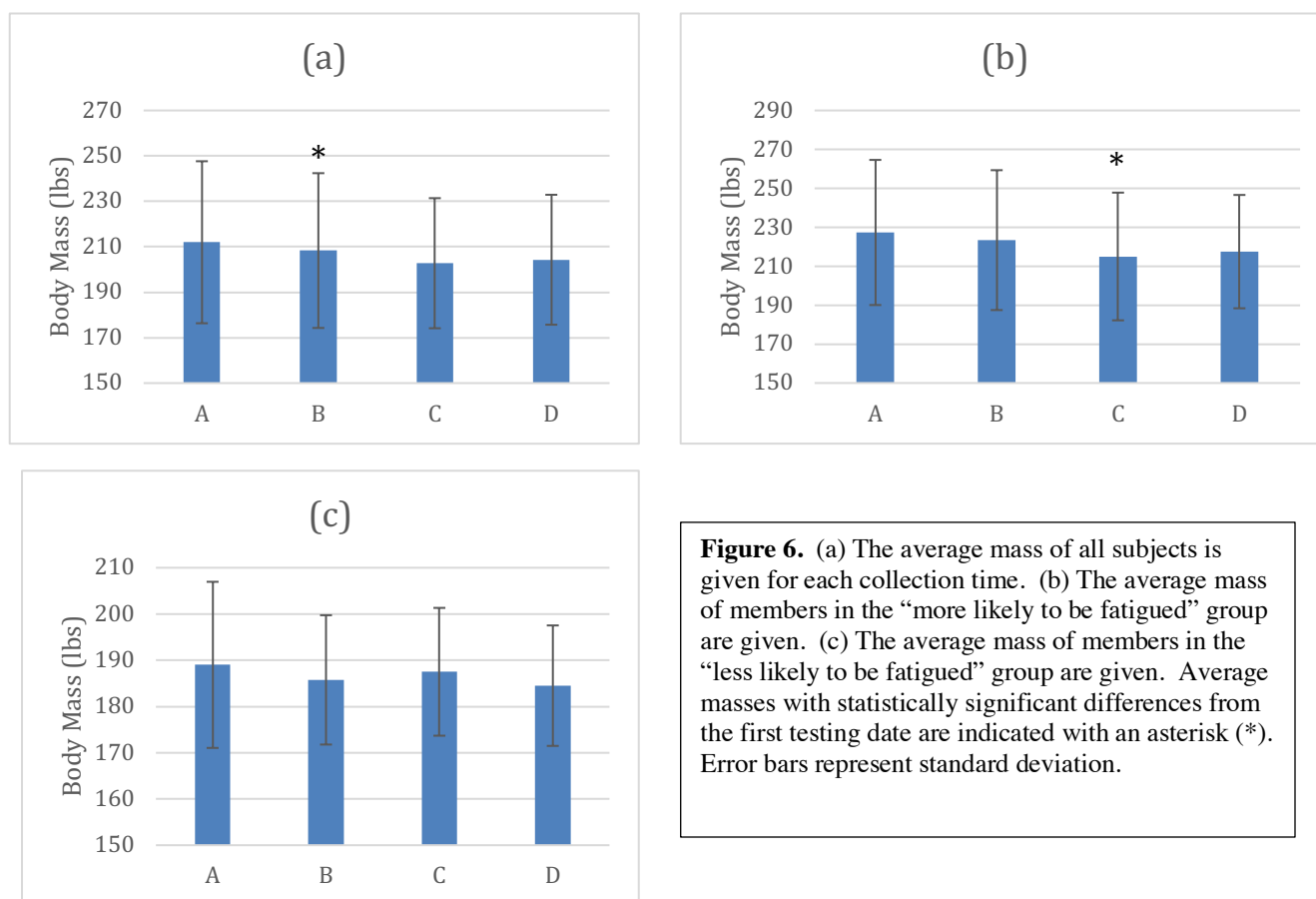

Figure 6. (a) The average mass of all subjects is given for each collection time. (b) The average mass of members in the "more likely to be fatigued" group are given. (c) The average mass of members in the "less likely to be fatigued" group are given. Average masses with statistically significant differences from the first testing date are indicated with an asterisk (*). Error bars represent standard deviation.

Blood Lactate

The concentration of lactate in the blood of each subject was determined from a finger prick during each testing date. Average blood lactate for all subjects and for more and less likely to be fatigued subjects was determined for each date.

Average blood lactate for all subjects was highest during the first collection time with a value of 4.63mmol/L. This average dropped significantly to 1.93mmol/L during collection time B with p=0.0315 (Figure 7a). When looking at the more likely and less likely to be fatigued subjects

by group, no statistically significant difference was found between average lactate levels during collection A and those during B, C, or D (Figure 7b). In addition, no significant difference was found between the lactate levels of the "more likely to be fatigued" and "less likely to be fatigued" subjects.
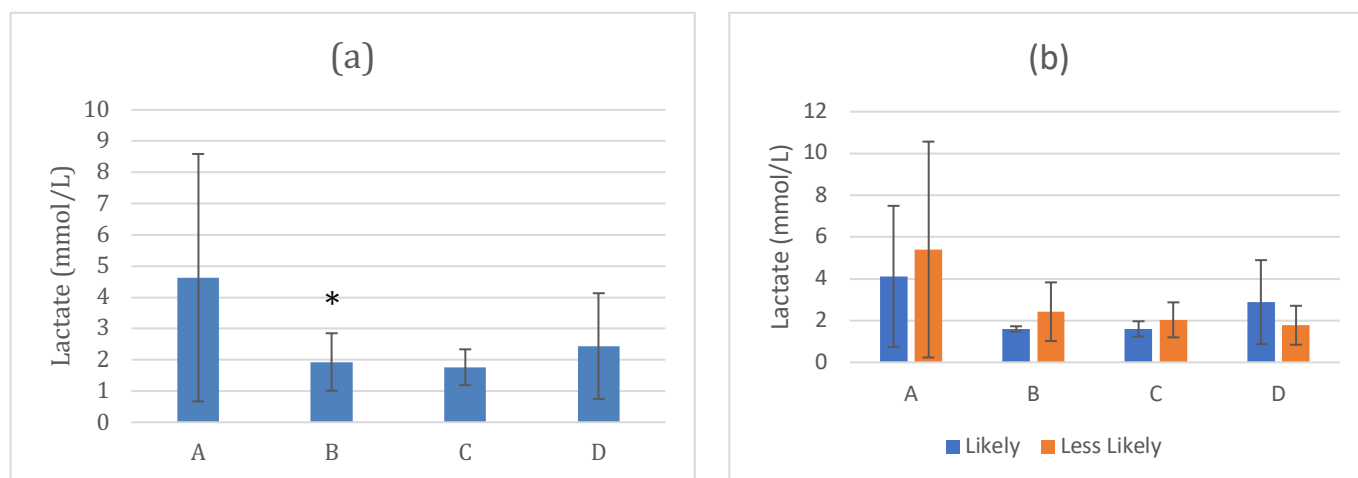

Figure 7. (a) The average blood lactate of all subjects at each testing time are given. (b) The average blood lactate levels of the "more likely to be fatigued" group are shown in blue and the average lactate levels of the "less likely to be fatigued" group are shown in orange. Values with statistically significant differences from the first testing point are indicated with an asterisk (*). Error bars represent standard deviation.

Blood Pressure

The next classical measure of fatigue recorded was systolic and diastolic blood pressure for each subject.

Statistical analysis showed that there was no significant change in systolic blood pressure from the first testing date to all later testing dates (Figure 8a). In addition, changes in average systolic blood pressure of each group was not significant, and no significant differences between the groups was detected (Figure 8b).

Although average diastolic blood pressure for all subjects showed a downward trend over A, B and C, these changes were shown not to be statistically significant (Figure 8c). When looking at

the averages of subjects in the "more likely to be fatigued" and "less likely to be fatigued" groups separately, only the drop in diastolic blood pressure from A to C for the "less likely to be fatigued" group was statistically significant with p=0.0124 (Figure 8d).
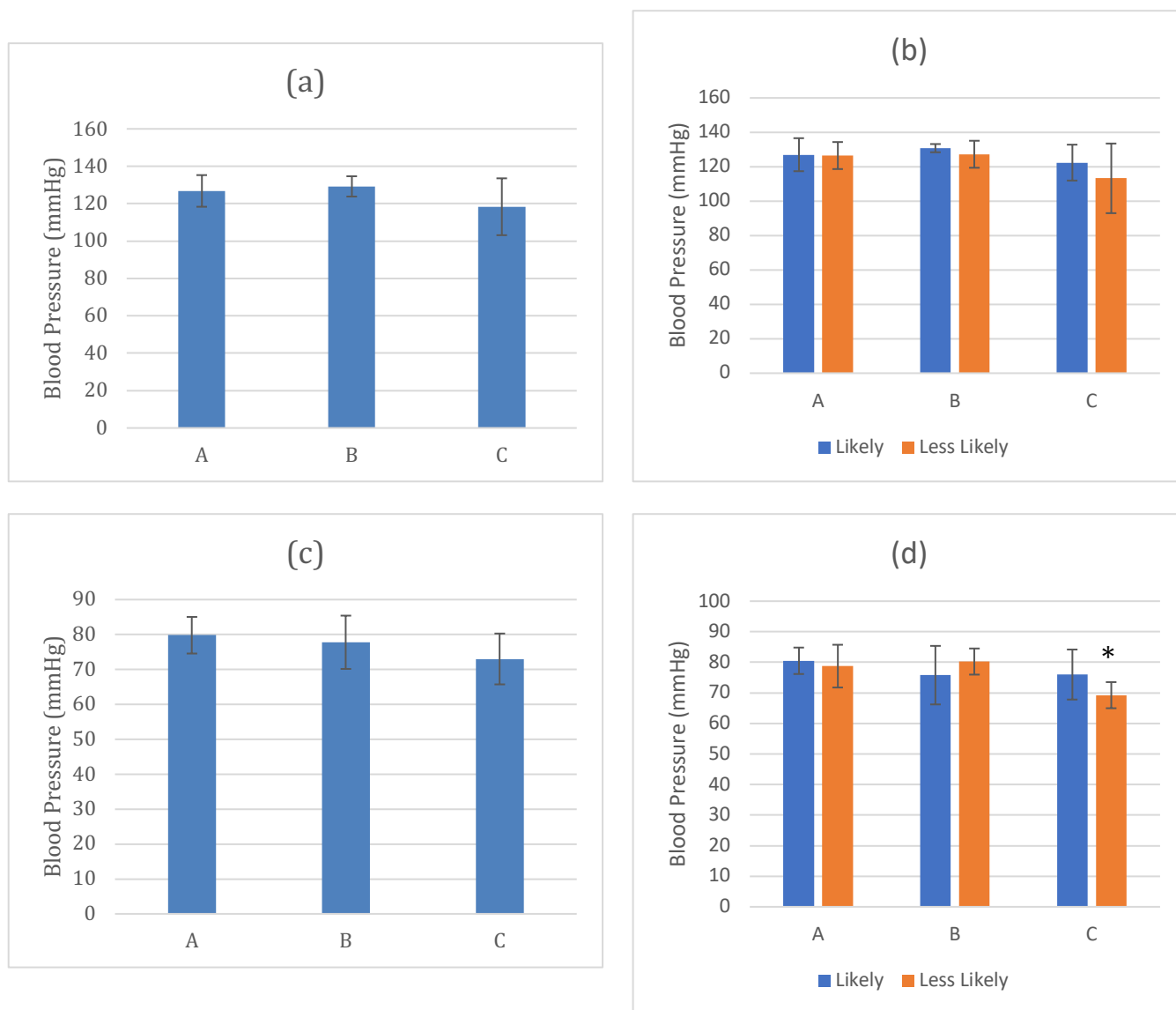

Figure 8. (a) The average systolic blood pressure of all subjects is given for each data collection date. (b) The average systolic blood pressure for each subject group is given. "More likely to be fatigued" averages are given in blue and "less likely to be fatigued" averages are shown in orange. (c) The average diastolic blood pressure of all subjects at each date is given. (d) The average diastolic blood pressure of each group is shown. "More likely to be fatigued" averages are shown in blue and "less likely to be fatigued" averages are shown in orange. Averages that are statistically different from the first testing date are indicated with an asterisk (*). No blood pressure measurements were taken during the last collection date. Error bars represent standard deviation.

POMS Assessment

All subjects completed a POMS assessment at each testing date. The two most important parameters for the present study were the fatigue and vigor scores of each subject. The POMS assessment allows subjects to report how fatigued and how vigorous (among other things) they feel. High fatigue scores and low vigor scores indicate the subject is feeling fatigued.

No significant change was found in the average "fatigue" score of all subjects over the four testing dates (Figure 9a). In addition, no significant change was found over the four testing dates in the fatigue scores of the more likely or less likely to be fatigued subject groups (Figure 9b). While the "more likely to be fatigued" group had a larger average fatigue score at every testing date than the "less likely to be fatigued" group, these differences were not shown to be statistically significant (Figure 9b).

The average vigor score of all subjects did not change significantly over all four testing dates (Figure 9c). When looking at the more likely and less likely to be fatigued groups separately, there was no change in the average vigor score from the first testing date (Figure 9d). Additionally, while the average vigor score of the "less likely to be fatigued" group was higher for every testing date than that of the "more likely to be fatigued" group, this difference was not shown to be statistically significant.
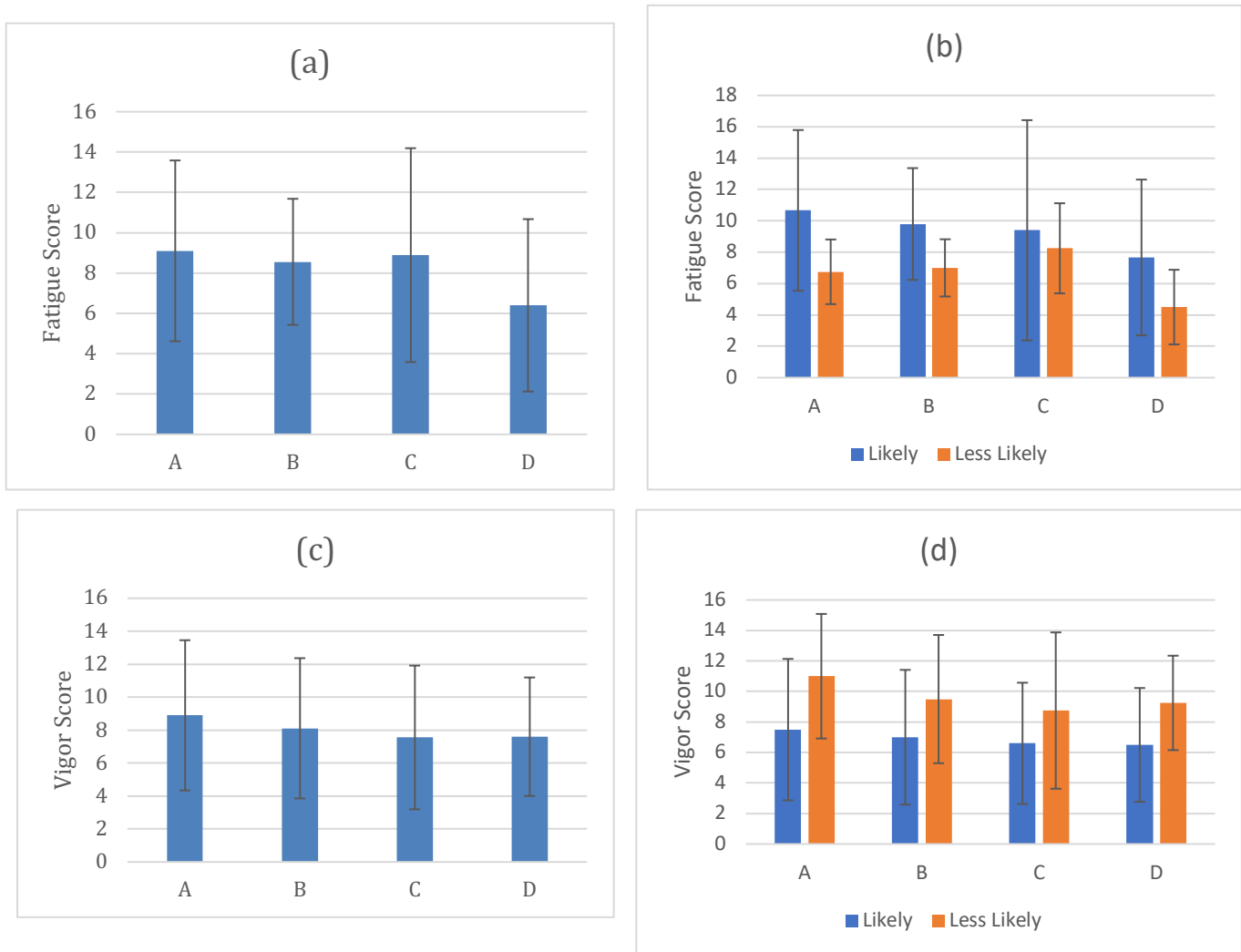
Figure 9. (a) The average fatigue score given calculated from the POMs assessment for all subjects is shown for each data collection time. (b) The average fatigue scores of the "more likely to be fatigued" group (blue) is compared to the average fatigue scores of the "less likely to be fatigued" group (orange). (c) The average vigor score calculated by the POMs assessment of all subjects at each testing time is shown. (d) The average vigor scores of the "more likely to be fatigued" subjects (blue) are compared to those of the "less likely to be fatigued" subjects (orange). Values with statistically significant differences from the first collection date are indicated with an asterisk (*). Error bars represent standard deviation.

Cortisol

The salivary cortisol concentration of each subject was determined using an ELISA plate. This was the first metabolite of interest analyzed. The average salivary cortisol concentration of all

subjects did not increase significantly from A to B, but did increase significantly on testing date C and again on testing date D (Figure 10a). Concentration increased from 3.48ng/mL during the first collection time to 4.36ng/mL on collection C with p=0.0324 and again to 6.69ng/mL on collection D with p=0.014. Although date B appears higher than date C, it was determined not to be statistically significant with p=0.1827.

When looking at the groups individually (Figure 10b), there was no statistically significant change in the salivary cortisol concentration of the "more likely to be fatigued" group over the four testing dates. In contrast, the average salivary cortisol of the "less likely to be fatigued" group rose from 2.34ng/mL during the first testing date to 6.88ng/mL on the second with p=.0008 and then dropped to 4.01ng/mL on testing date C with p=.0144.
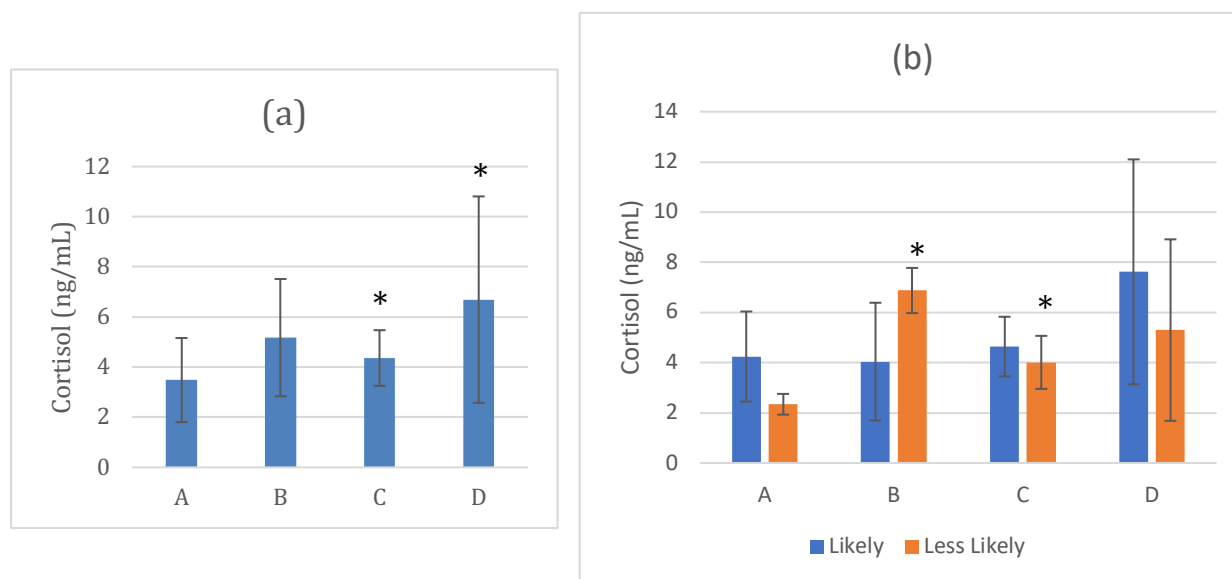
Figure 10. (a) The average concentration of salivary cortisol of all subjects is shown. (b) The average cortisol concentration of subjects in the "more likely to be fatigued" group (blue) is compared to that of the subjects in the "less likely to be fatigued" group. Average cortisol concentrations with statistically significant differences from those on the first testing date are indicated with an asterisk (*). Error bars represent standard deviation.

Alanine

The next metabolite assessed was salivary alanine concentration. Alanine was derivatized with a silylating reaction and quantified using the GC-MS.

When looking at all subjects, the average salivary alanine concentration dropped significantly from testing date A only during testing date C (Figure 11a). Alanine concentration dropped from 10.655ug/mL on A to 9.17ug/mL on C with p=0.0138.

No statistically significant change in salivary alanine concentration from the testing date A was observed for the "less likely to be fatigued" group (Figure 11b). In contrast, the "more likely to be fatigued" group's average alanine concentration dropped significantly from 10.71ug/mL on A to 9.05ug/mL on C with p=0.0479.
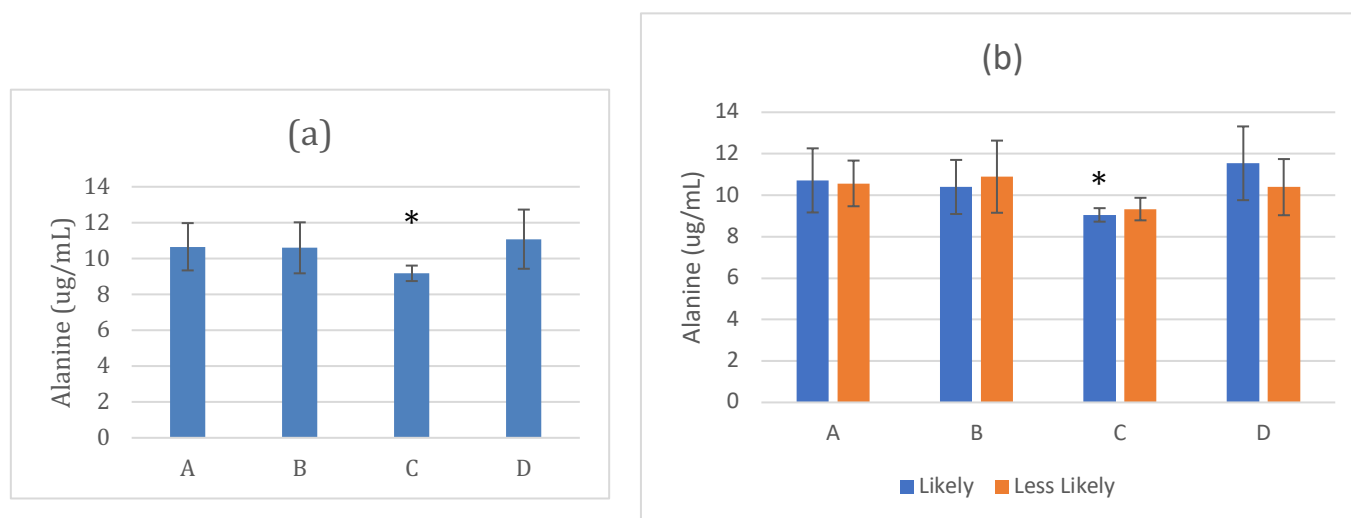
Figure 11. (a) The average concentration of salivary alanine of all subjects is shown for the four data collection times. (b) The average salivary alanine concentrations of the "more likely to be fatigued" group (blue) are compared to the averages for the "less likely to be fatigued" group (orange). All values with statistically significant differences from the first testing date are indicated with an asterisk (*). Error bars represent standard deviation.

Glutamine

The last salivary metabolite of interest to be analyzed was glutamine. As with alanine, glutamine was first silylated and then quantified using the GC-MS.

Average salivary concentrations of glutamine for all subjects did not change significantly from the first testing date (Figure 12a). When looking at the more likely and less likely to be fatigued groups separately, glutamine concentration did not change significantly from testing date A for the less likely to be fatigued group either (Figure 12b). For the "more likely to be fatigued" group, glutamine concentration decreased significantly from 23.36ug/mL on A to 11.42ug/mL on C with p=0.0364.
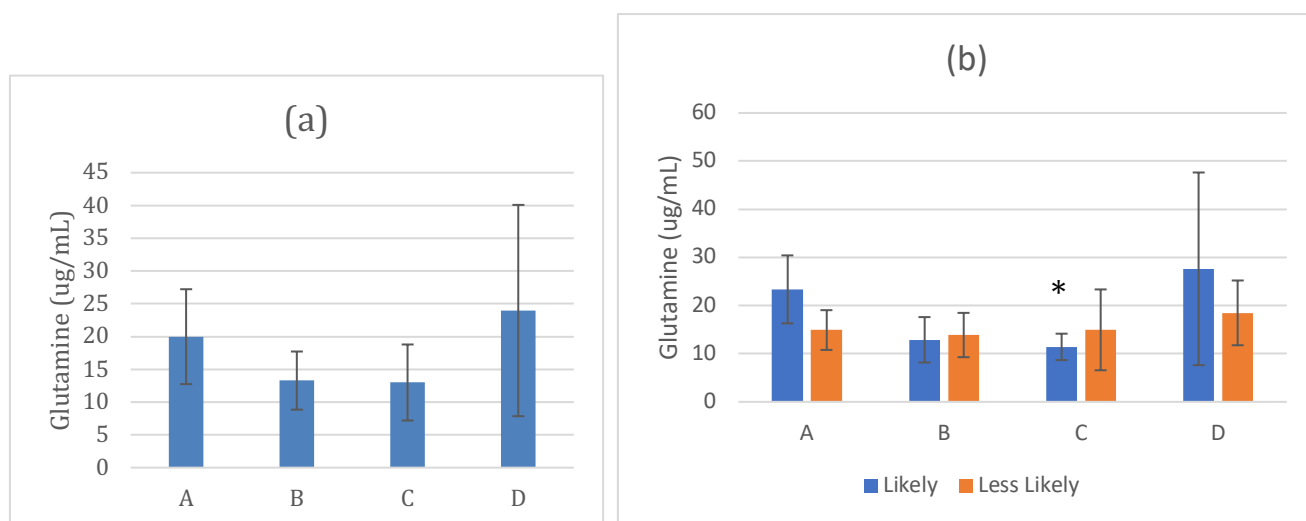
Figure 12. (a) The average salivary concentration of glutamine in all subjects is shown. (b) The average salivary concentrations of glutamine for subjects in the "more likely to be fatigued group" (blue) are compared to those of the subjects in the "less likely to be fatigued" group (orange). Averages with statistically significant differences from the first data collection date are indicated with an asterisk (*). Error bars represent standard deviation.

Marathon Runners

Fatigue parameters and metabolites for the marathon runners were not able to be quantified and analyzed due to the Covid-19 pandemic. On campus learning at Colby College was cancelled before samples and data were able to be quantified.

P Values

Hours	ΔB	ΔC	ΔD
Football	0.0001	0.0001	0.0001
Classwork	0.0001	0.0001	0.0001
Sleep	0.3974	1	0.3901

Systolic Blood Pressure	ΔB	ΔC	ΔD
Full Group	0.5565	0.1528	
More Likely	0.6526	0.6452	
Less Likely	0.7645	0.1679	

Heart Rate	ΔB	ΔC	ΔD
Full Group	0.0371	0.0984	0.8142
More Likely	0.0541	0.0327	0.9085
Less Likely	0.4868	0.9511	0.8571

Diastolic Blood Pressure	ΔB	ΔC	ΔD
Full Group	0.453	0.0655	
More Likely	0.333	0.5347	
Less Likely	0.3751	0.0124	

Body Mass	ΔB	ΔC	ΔD
Full Group	0.0162	0.0901	0.0514
More Likely	0.0503	0.0143	0.109
Less Likely	0.262	0.6689	0.3751

Fatigue Score	ΔB	ΔC	ΔD
Full Group	0.4028	0.4751	0.0796
More Likely	0.3602	0.8928	0.1876
Less Likely	0.6376	0.4444	0.3539

Blood Lactate	ΔB	ΔC	ΔD
Full Group	0.0315	0.0765	0.1103
More Likely	0.1196	0.143	0.4174
Less Likely	0.2293	0.6353	0.2183

Vigor Score	ΔB	ΔC	ΔD
Full Group	0.3968	0.3331	0.4859
More Likely	0.6597	0.6653	0.706
Less Likely	0.5269	0.4166	0.5849

Cortisol	ΔB	ΔC	ΔD
Full Group	0.1827	0.0324	0.014
More Likely	0.8989	0.3361	0.0667
Less Likely	0.0008	0.0144	0.1919

Alanine	ΔB	ΔC	ΔD
Full Group	0.9308	0.0138	0.5876
More Likely	0.7194	0.0479	0.4929
Less Likely	0.8123	0.1959	0.8638

Glutamine	ΔB	ΔC	ΔD
Full Group	0.0593	0.1127	0.5197
More Likely	0.0552	0.0364	0.6829
Less Likely	0.7673	0.9939	0.5113

Figure 13 P-values calculated for the paired differences in means between each parameter from the first testing date (A) to each of the next dates (B, C, and D) using t values and degrees of freedom for each group.

DISCUSSION

Fatigue can cause a significant drop in athletic performance as well as academic performance for student athletes. In addition, fatigue can increase the risk of illness of affected athletes. For this reason, the present study tracked classical measures of fatigue, as well as changes in specific metabolites, of ten football players on the Colby College Football Team. The classical fatigue measurements taken were heart rate, body mass, blood lactate, POMS assessment scores, and blood pressure. The metabolites of interest that were quantified were cortisol, as an indicator of stress, and alanine and glutamine, as indicators of protein catabolism. The primary goal of the study was to find a reliable marker of fatigue.

Statistical Significance of the Data

While statistical analysis showed that much of the data did not show significant changes from the first testing date, this is primarily due to the small subject pool. However, what is more important is that many classical measures of fatigue and salivary metabolites showed at least one or two significant changes. In a subject pool as small as the one for this study (ten subjects), the presence of any significant data at all indicates that these measures can be used to effectively track fatigue. Future experiments with much larger subject groups should show more statistically significant changes in the metabolome and classical measures of fatigue.

In addition to the small subject pool, large standard deviations in the data may also have contributed to a lack of statistical significance. Also, data for some parameters was not collected on all subjects. No data was collected on subject 3 for collection date C due to an injury sustained during competition. In addition, no blood lactate measurement was taken on collection C for

subject 7. Furthermore, no POMS assessment or average time spent on football, school work and sleep for the week of collection C were collected for subject 6.

Hours

One of the primary goals of the study was to determine the effects of a lack of sleep on the metabolome, specifically on the metabolites cortisol, alanine and glutamine. However, there was no significant change in the average hours of sleep over the four testing dates. We hypothesized that the hours of sleep would be lowest on collection date C, right after midterm week. Unexpectedly, not all subjects had midterms that week. Exams may have occurred the week before or after, or subjects may not have had any exams at all, but rather were required to write papers for their classes.

As expected, the average time spent on football dropped significantly from A (preseason) to B and remained the same from B to C, which were the testing dates during the regular season of play when the subject's practice schedule did not change. Time spent playing football on date D was very low, as the season had ended at this point, and subjects only counted daily weight training as time spent on football.

Another goal of the study was to determine how academic stress effected levels of cortisol, alanine and glutamine. During the first collection date, football camp had started, but classes had not, so no subject spent time on classwork. This increased to an average of 5 hours per day for the week of collection B, which was done before midterm week started. The study had anticipated a significant difference in the amount of time spent on school work between testing dates B and C, but this was not observed. On dates B and C, time spent on football was held constant, and it was hoped a change in the metabolome could be correlated to an increase in academic course load and not physical fatigue. Instead, we observed that 9 hours of football activities per day during

preseason were replaced with 3.8 hours of football per day and 5 hours of schoolwork per day throughout the regular season.

Heart Rate

Heart rate was expected to rise from A during collection dates B and C, and then drop again during collection date D when the football season had ended and the subjects did not have daily practice. It was also expected that the "more likely to be fatigued" group would have a higher average heart rate than the "less likely to be fatigued" group indicating a higher level of fatigue. Average heart rate was shown to be highest on B for the whole group, but was not significantly different than the first testing time on date C (midterms week). It was expected that subjects would have the highest average heart rate on C, and not B. There may have been other stressors and external factors that the study could not account for that affected the resting heart rate of each subject. These could include social pressures in the subject's lives or the quality of practice the day the measures were taken.

In contrast, the "more likely to be fatigued" group had a significant increase from A to C, which was expected, because the academic course load was predicted to be the highest on date C.

Future experiments should include a larger subject pool to obtain statistically significant increases from the first testing date, as well as statistically significant differences between likely and less likely to be fatigued groups. A larger subject pool may show more significant increases in heart rate, or it may not, indicating that resting heart rate is not a good measure of fatigue. In addition, this study measured resting heart rates of the subjects after practice had occurred. It was not feasible for researches to take resting heart rates of subjects the moment they woke up in the morning. Future studies should attempt to take resting heart rate measurements directly after subjects wake up to obtain more reliable measures.

Body Mass

Many athletics coaches and athletic trainers monitor their athletes' weights to track fatigue level, looking for a loss in body weight to indicate a rising level of fatigue. It was expected that all subjects would lose body weight over the course of the season, manifested in decreasing averages from A to B to C, with a possible weight gain from C to D once the season ended. What was observed was a significant drop in average weight from A to B, but not for C. These results are unexpected, and may be attributed to the small group size and large standard deviations.

In addition, the "more likely to be fatigued" group showed a significant drop in body mass from date A only for date C, instead of a steady decline. This would be more significant if a larger difference was seen in the academic load from B to C, but this difference was not seen. This could mean that body mass is not an effective measure of fatigue as previously thought, or that a larger subject pool is needed to show statistically significant changes in body mass over the course of a season. Another explanation is that the hydration state of each subject was not accounted for. Water weight is lost during athletic exercise, and if subjects did not replenish their bodies by drinking more, their body weights would have shown a larger decrease. It is possible that subjects did not focus on rehydration during exam week (date C), which would account for the large and significant decrease from A.

For the "less likely to be fatigued" group, a decline in mass was expected over the four testing dates, but a smaller drop than that of the "more likely to be fatigued" group. Instead, data showed that there was no significant change in the body mass of the subjects in the "less likely to be fatigued" group over the course of the season. These players are under less physical stress than the "more likely to be fatigued" group, and indicates that less physical stress leads to less weight loss over a competition season.

Blood Lactate

Blood lactate increases as anaerobic metabolism increases. It was expected that over the course of the season, the average blood lactate of the subjects would increase as they became more fatigued, with the subjects in the "more likely to be fatigued" group having larger increases than the "less likely to be fatigued" group. Data showed that this was not the case. Blood lactate significantly dropped from A to B for all subjects. Average lactate on C was also low, but oddly enough this was not statistically different from blood lactate concentrations on the first testing date. Nevertheless, this decrease in blood lactate may be rationalized in a different way. Instead of more stress being put on anaerobic metabolism, as the season went on, the subjects may have gotten in better shape. It is possible that this led their bodies to clear lactate from the blood faster than it did in preseason. Another explanation is that blood lactate is not a good mark of long-term fatigue, but rather of short term. Preseason practice is longer and more physically taxing than practices during the regular season. Testing date A was in the middle of preseason, and it showed the highest blood lactate levels for all subjects. The drop in blood lactate for date B may be because the subjects were not under as much physical stress as they were in the preseason.

Statistically significant changes in blood lactate were not found over the course of the season when subjects were grouped by expected fatigue level, and differences between the two groups also were not significant. It is expected that larger subject pools might show more statistically significant differences.

Blood Pressure

Similar to other metrics of fatigue, very little significant change from the first testing date was seen for either systolic or diastolic blood pressure. The only significant change from A was a decrease on date C for the average diastolic blood pressure of the "less likely to be fatigued" group.

Even if the data were all taken to be significant, both systolic and diastolic seem to rise and fall without a pattern. This could indicate that blood pressure is not a good indication of long-term fatigue, but may instead be an indication of acute fatigue and physical stress. Unfortunately, no blood pressure measurements were taken for the last testing date, during final exam week. This would have been interesting to see how blood pressure changes when there is almost no physical fatigue placed on the subjects but only academic stress instead. Future experiments should explore this scenario.

POMS

The most important measurements from the POMS mood assessment are the fatigue and vigor scores. These scores are important to the present study because they allow the subjects to report how fatigued or vigorous they feel. These scores offer qualitative information on how fatigued each subject is, as opposed to impersonal quantitative measurements. Over the course of the football season (testing dates A, B, and C) we expected to see an increase in the fatigue score and a decrease in the vigor score to indicate an increased level of fatigue in the subjects. We did not know how the scores would change for testing date D which was during final exams, when there was almost no time spent on football.

Interestingly, no significant change in either score was observed for all subjects from the first testing date. This makes more sense when we look at subjects in the likely and less likely to be fatigued groups separately. On all four testing dates, the "more likely to be fatigued" group had higher fatigue scores and lower vigor scores than the "less likely to be fatigued" group. It was surprising to find that these differences in average fatigue and vigor scores for each group was not statistically significant for any testing date, likely because of the small subject pool. Although statistical analysis showed the disparities in the scores of the likely and less likely to be fatigued

groups was not significant, they do not seem to be a coincidence. A future experiment including more subjects should be conducted to determine if the difference in fatigue and vigor scores of athletes who are likely and less likely to be fatigued can be replicated and observed again.

Cortisol

The results of the salivary cortisol concentrations are ambiguous. Because cortisol is released as a stress hormone and can also act as an anti-inflammatory in the body, cortisol concentrations were expected to increase as the season went on and subjects became increasingly fatigued. It was thought that cortisol concentrations would be highest during midterm week (testing date C) because subjects would be experiencing the stress of an increased academic work load, as well as fatigue from a long football season. This was hypothesized to cause in increase in stress response as well as an increase in need for an anti-inflammatory response. Because there was no significant change in school work from B to C, the effects of academic stress on cortisol were difficult to gauge.

For the whole group, the significant increase in cortisol from A to C indicates that the initial hypothesis was correct, meaning physical fatigue and academic stress caused in increase in cortisol concentrations. Interestingly, cortisol concentrations for all subjects were highest during testing date D, when subjects spent only 1.1 hours a day on football but 7 hours on class work. This suggests that an increase in cortisol is more closely related to mental stress than physical fatigue. This is a significant finding. This may indicate that midterm weeks occurring in-season for student athletes places them at higher risk of becoming sick due to high levels of cortisol and its immunosuppressive properties. It is possible that high levels of cortisol are more indicative of academic stress because it is a different type of stress than physical. Physical stress from a practice or game may elicit an acute stress response and release of cortisol, one which the body can deal

with and recover from. In contrast, increased academic workload may act as a chronic stressor, placing student athletes in a extended state of stress. Chronic stress may lead to prolonged cortisol release and overall higher levels of cortisol (19).

When looking at cortisol change by group, on testing dates A, C, and D the "more likely to be fatigued" group had a higher cortisol concentration than the "less likely to be fatigued" group. Statistical analysis showed that no change in cortisol for the "more likely to be fatigued" group was significant. In contrast, average salivary cortisol concentration was significantly increased for the "less likely to be fatigued" group on testing date B, when midterms were not occurring. There was also a significant increase from A to C for this group, although it was a smaller increase than from A to B. These results appear to be scattered and inconclusive. Further experiments with larger subject pools should be conducted to determine if cortisol is more strongly correlated with an increase in academic or mental stress as opposed to physical fatigue as this study indicates.

Alanine

Salivary alanine was expected to increase as subjects were under increasing physical stress as a product of muscle breakdown and stress on the glucose-alanine cycle. Results show that average salivary alanine concentrations for all subjects remained nearly the same for A, B, and D, but dropped significantly from A to C. When looking at the likely and less likely to be fatigued groups, alanine levels were almost the same for all testing dates, but statistical analysis showed that only the more likely to be fatigued subjects had decreased alanine on date C.

Although alanine was expected to increase as a result of physical stress, our data showed that a decrease in salivary alanine may indicate fatigue. Date C was the latest in-season saliva sample collected, and the lowest salivary alanine concentration was detected on date C. This could

mean that decreases in alanine instead of increases indicate fatigue, although the mechanism that may cause this is not known.

There is another plausible explanation for this drop in alanine concentration during C. After the silylation reaction was complete, derivatized saliva samples were loaded onto the GC-MS. Unfortunately, due to technical problems with the instrument, the samples were not immediately analyzed, and were stored in the laboratory freezer until the issue was resolved, which took two weeks. This brings into question the stability of the derivatized alanine, and that may account for the detected decrease in subjects' salivary alanine concentrations. A study into the stability of derivatized alanine would help to validate these findings.

Glutamine

As described in previous literature, glutamine concentrations were expected to drop as a subject became increasingly fatigued. Although our study did not yield many statistically significant results due to the small subject pool, a significant decrease in glutamine was observed for the "more likely to be fatigued" group on date C.

This may indicate that glutamine is a reliable biomarker of fatigue in student athletes, and a decrease in its salivary concentration indicates an increase in fatigue. Alternatively, because salivary levels of alanine and glutamine were assessed by the GCMS at the same time, the drop in glutamine concentration may be inaccurate because the samples remained untested for two weeks. Similar to alanine, the observed drop in glutamine may have actually been caused by a break-down of the derivatized compounds, and all the glutamine in the saliva samples were not detected by the GC-MS. Similar to alanine, a study of the stability of derivatized glutamine over multiple weeks would help to confirm the findings of the present study.

Key Findings

- Average salivary cortisol concentration of all subjects increased significantly from A to C (midterm week) and from A to D (final exam week). No consistent differences in salivary cortisol between more and less likely to be fatigued subjects were detected. This suggests that an increase in cortisol is more indicative of academic stress than physical fatigue.

- Salivary alanine concentration significantly decreased from A to C when all subjects were grouped together and for the "more likely to be fatigued" group. This indicates alanine concentration may decrease in response to physical fatigue.

- Salivary glutamine concentration decreased significantly from A to C for the "more likely to be fatigued" group. This drop in glutamine likely corresponds to an increase in an athlete's physical fatigue.

REFERENCES

1. Jones, C. M., Griffiths, P. C., & Mellalieu, S. D. (2017). Training load and fatigue marker associations with injury and illness: A systematic review of longitudinal studies. *Sports Medicine, 47*(5), 943-974. doi:10.1007/s40279-016-0619-5

2. Budgett, R. (1998). Fatigue and underperformance in athletes: The overtraining syndrome. *British Journal of Sports Medicine, 32*(2), 107-110. doi:10.1136/bjsm.32.2.107

3. Koutedakis, Y., Budgett, R., & Faulmann, L. (1990). Rest in underperforming elite competitors. *British Journal of Sports Medicine, 24*(4), 248-252. doi:10.1136/bjsm.24.4.248

4. Dressendorfer, R. H., Wade, C. E., & Scaff Jr., J. H. (1985). Increased morning heart rate in runners: A valid sign of overtraining? *Physician and Sportsmedicine, 13*(8), 77-86. doi:10.1080/00913847.1985.11708858

5. Ra, S. -., Maeda, S., Higashino, R., Imai, T., & Miyakawa, S. (2014). Metabolomics of salivary fatigue markers in soccer players after consecutive games. *Applied Physiology, Nutrition and Metabolism, 39*(10), 1120-1126. doi:10.1139/apnm-2013-0546

6. Sakaguchi, C. A., Nieman, D. C., Signini, E. F., Abreu, R. M., & Catai, A. M. (2019). Metabolomics-based studies assessing exercise-induced alterations of the human metabolome: A systematic review. *Metabolites, 9*(8) doi:10.3390/metabo9080164

7. Santone, C., Dinallod, V., Paci, M., D'Ottavio, S., Barbato, G., & Bernardini, S. (2014). Saliva metabolomics by NMR for the evaluation of sport performance. *Journal of Pharmaceutical and Biomedical Analysis, 88*, 441-446. doi:10.1016/j.jpba.2013.09.021

8. Thau L, Sharma S. Physiology, Cortisol. [Updated 2019 Feb 15]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2019 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK538239/

9. Dickerson, S. S., & Kemeny, M. E. (2004). Acute stressors and cortisol responses: A theoretical integration and synthesis of laboratory research. *Psychological Bulletin, 130*(3), 355-391. doi:10.1037/0033-2909.130.3.355

10. Lac, G., & Maso, F. (2004). Biological markers for the follow-up of athletes throughout the training season. [Les marqueurs biologiques pour la surveillance des athlètes à l'entraînement] *Pathologie Biologie, 52*(1), 43-49. doi:10.1016/S0369-8114(03)00049-X

11. Nelson, David L., et al. *Lehninger Principles of Biochemistry*. 17th ed., W.H. Freeman and Company, 2017

12. Keast, D., Arstein, D., Harper, W., Fry, R. W., & Morton, A. R. (1995). Depression of plasma glutamine concentration after exercise stress and its possible influence on the immune system. *Medical Journal of Australia, 162*(1), 15-18. Retrieved from www.scopus.com

13. Reilly, T., & Piercy, M. (1994). The effect of partial sleep deprivation on weight-lifting performance. *Ergonomics, 37*(1), 107-115. doi:10.1080/00140139408963628

14. Benedict, C., Hallschmid, M., Lassen, A., Mahnke, C., Schultes, B., Schiöth, H. B., . . . Lange, T. (2011). Acute sleep deprivation reduces energy expenditure in healthy men. *American Journal of Clinical Nutrition, 93*(6), 1229-1236. doi:10.3945/ajcn.110.006460

15. Mah, C. D., Mah, K. E., Kezirian, E. J., & Dement, W. C. (2011). The effects of sleep extension on the athletic performance of collegiate basketball players. *Sleep, 34*(7), 942-950. doi:10.5665/SLEEP.1132

16. Akazawa, N., Kobayashi, N., Nakamura, Y., Kumagai, H., Choi, Y., & Maeda, S. (2019). Effect of sleep efficiency on salivary metabolite profile and cognitive function during

exercise in volleyball athletes. *European Journal of Applied Physiology, 119*(10), 2215-2223. doi:10.1007/s00421-019-04205-7

17. Grove, J.R., & Prapavessis, H. (1992). Preliminary evidence for the reliability and validity of an abbreviated Profile of Mood States. *International Journal of Sport Psychology, 23*, 93-109.

18. Stenerson, K.K. (2020). The Derivatization and Analysis of Amino Acids by GC-MS. *Millipore Sigma, Reporter US Volume 25.3*.

19. Hannibal, K. E., & Bishop, M. D. (2014). Chronic stress, cortisol dysfunction, and pain: a psychoneuroendocrine rationale for stress management in pain rehabilitation. *Physical therapy*, *94*(12), 1816–1825. https://doi.org/10.2522/ptj.20130597