Proposal Title: Identification of early (non)functional overreach warning signs following a 3-week lab-controlled cycling protocol

Key Personnel:
Principle Investigator- Thomas Gooding, MEd, ATC, CSCS
Student Advisor- Hans Haverkamp, PhD

Collaborators:
David C Nieman, PhD, FACSM Appalachian State University
Laurie Wideman, PhD, FACSM University of North Carolina-Greensboro

One sentence of what human subjects will be doing:
Subjects will participate in a 3-week lab-controlled cycling training protocol, followed by a 3-week supervised recovery period to identify early biological markers and symptoms of training maladaptation.

Resubmission summary (5,000 characters):
In response to our initial proposal, both reviewers provided critical, helpful and fair feedback. ***General:*** Reviewers expressed concern that the training protocol (stimulus) may not be sufficient to induce overreach. This concern was well placed, given our inability to provide preliminary data due to COVID-19 shutting down human research at our university. In this re-submission we have provided preliminary data with supporting evidence that the protocol can induce overreaching in recreationally active persons (i.e., substantially decreased peak heart rate during exercise, decreased VO_{2max}, and incidence of illness). Additionally, mechanisms causing people of similar training backgrounds to respond differently to heavy training loads are poorly understood. Regardless of (over)training status, the amount of data we collect regarding subjects' physiological responses to high-volume training will be highly valuable. Another concern was that our study groups were potentially not all inclusive. We have revised our group definitions so that all subjects will fall into either "adapted," "functionally overreached," or "non-functionally overreached" status, dependent upon performance changes.

Significance: Reviewers raised concerns regarding our use of a single modality (cycling) protocol to induce overreaching. We do not believe this to be a weakness of our study given that similar training protocols have been described in the literature focusing on overtraining in high-level athletes. Furthermore, overtraining research has almost exclusively focused on high-level male athletes or subjects already diagnosed as overtrained. The nearly ubiquitous focus on these populations has limited our understanding of the progression of training maladaptation. Our proposal studies males and females without extensive training histories and proposes that overtraining is not exclusive to high-level athletes. As shown in the proposal, our exciting preliminary data provide strong evidence that the training protocol will cause training maladaptation in some of the subjects.

Innovation: A reviewer commented on the efficacy of a controlled stimulus to produce overreaching. Previous studies have quantified increases in training volume (e.g., +30% from normal training volume). Our study consists of ~330 minutes of exercise per week, above the recommended weekly moderate physical activity of 150 to 300 minutes by the Physical Activity Guidelines for Americans. To ensure that this training protocol will be a significant increase to subject's typical training load, we collect a six-month exercise history upon recruitment. Additionally, our protocol's overall training weekly load increases ~30% each subsequent week.

Environment: Both reviewers expressed concern regarding a low publication record for the student and no publications from the student advisor in the field of overtraining research. We acknowledge this is a new area of research for the student advisor. However, the advisor has a strong fundamental understanding of the responses and adaptations to aerobic exercise and a demonstrated ability to conduct and publish research findings in high-quality journals. This proposal is directly related to Thomas Gooding's dissertation who has spent the majority of three years immersing himself in this area of research. We have also added David Nieman, PhD, FACSM, and Laurie Wideman, PhD, FACSM as collaborators on this proposal. Both Dr. Nieman and Dr. Wideman are members of Thomas Gooding's dissertation committee and will help guide this research as it progresses Dr. Nieman is a leading researcher in the area of exercise immunometabolism and has extensive experience in proteomics and was a co-author on the most recent ACSM position statement on

overtraining. Dr. Wideman is an exercise physiologist with a strong background in endocrinology and the assessment of biomarkers.

Approach: A reviewer raised the concern that the primarily concentric exercise of cycling may not induce significantly alter the biomarkers we propose to track (testosterone, cortisol, IL-6, TNF-α). The lack of eccentric contraction greatly reduces the risk of exercise-induced rhabdomyolysis reported in untrained individuals undergoing high training volumes. Though eccentric exercise causes more muscle damage than concentric exercise thereby potentially elevating the levels of these biomarkers, previous overtraining studies have employed cycling as a modality and overtraining has been well documented in cycling sports. Furthermore, we believe these markers are worth investigating, given that there are other physiological factors (e.g., illness) that may cause perturbations to these biomarkers.

Additional: We have revised our budget from our initial submission, removing subject compensation as there has been high participant interest in this study. We anticipate having ten subjects completed by May 2022.

Lay summary:
Athletes across many sports follow rigorous, strategically planned training programs designed to improve their performance. High-volume training periods followed by low-load periods (taper) are often used to induce what is known as functional overreach (FOR). FOR is a brief state lasting several days in which an individual exhibits decreased performance and increased fatigue, followed by significantly improved performance. When the imbalance between training and recovery is prolonged, athletes may become non-functionally overreached (NFOR). NFOR is characterized by more severe performance decreases, excessive fatigue, and altered psychological health with recovery lasting weeks to months. If NFOR continues to progress, overtraining syndrome (OTS) may develop, the most severe form of overtraining that may last months to years and potentially end an individual's athletic season, if not their career. Despite a large amount of overtraining research, there are two glaring gaps in the literature: 1) the biological mechanisms leading to overtraining are poorly understood and 2) there is a lack of diagnostic criteria to detect overtraining or predict its occurrence. We believe these gaps persist due previous research overwhelmingly focusing on elite-level male athletes. As such, it is currently unknown if non-elite athletes can experience overtraining. This is concerning as up to 64% of runners have reported experiencing overtraining at least once in their career.

We propose a 3-week, intensive, lab-controlled training protocol designed to identify early signs of training maladaptation (overtraining) in recreationally active adult males and females. This allows for a robust analysis of training load and allows us to measure a larger number of exercise-related variables compared to traditional field-based overtraining research. We hypothesize that progressive decreases in performance will be preceded by progressive increases in inflammatory markers. Subjects who exhibit significant performance decreases will be considered overreached. Subjects whose performance recovers within two weeks will be considered FOR, while subjects who performance deficits take longer than two weeks to recovery will be considered NFOR. Findings from this proposal will generate future, hypothesis-driven studies designed to illuminate the pathophysiological progression of overtraining. Our long-term goals are to develop proactive methods of detecting and preventing impending overtraining.

Central Hypothesis and Specific Aims:
Overtraining syndrome (OTS) is a disorder existing on a spectrum caused by repetitive imbalance between excessive training load and inadequate recovery. Characterized by performance deficits, increased respiratory illness, and impaired mood states, overtraining may last anywhere from weeks to years, depending on its severity. Despite a large volume of literature, there are two glaring gaps concerning overtraining research: 1) the pathophysiological mechanisms of overtraining are poorly understood and 2) there is an absence of sensitive diagnostic criteria to identify overtraining or predict its impending occurrence. We believe these gaps are largely due to study designs repeatedly examining overtraining in highly-competitive male athletes[1–4] or previously overtrained subjects.[5–7] The primary focus of overtraining on high-level male athletes has limited our understanding of physiological responses associated with overtraining. Well-trained athletes often undergo years of extensive year-round training that causes robust changes to multiple physiological systems (training tolerance). This has also marginalized female athletes who have been largely absent from overtraining research. Furthermore, examining previously diagnosed subjects prevents the ability to establish causal mechanisms for overtraining. These gaps in knowledge and clinical practice are concerning since overtraining is highly prevalent in elite athletes. Previous reports have shown that up to 64% of elite runners have

experienced at least one episode of overtraining during their careers.[8,9] Improving our understanding of the pathophysiological progression of overtraining is critical to identifying effective diagnostic procedures and prevention strategies. Studying a population of males and females without extensive training habituation will yield new insights into the pathological progression of overtraining. **We propose a 3-week, high intensity, lab-controlled training protocol designed to identify early markers of overtraining in recreationally active males and females.**

The Central Hypothesis is that **overtraining is not exclusive to high-level athletes and can occur in recreationally active males and females**. We believe that studying the progression of overreach in recreationally active individuals (i.e., non-elite athletes) will elucidate novel insights regarding the physiological progression of training maladaptation.

Specific Aim 1: Characterize the physiological responses to a 3-week high-intensity exercise protocol in recreationally active adults. *We hypothesize that subjects will exhibit a range of training responses following the training protocol including adaptation, functional overreach, or non-functional overreach*. Following three weeks of high-intensity exercise training, subjects will be placed into one of three categories based on performance changes: 1) Adapted (AD): unchanged or improved performance, 2) functionally overreached (FOR): performance decrements lasting less than two weeks, 3) non-functionally overreached (nFOR): performance decrements lasting longer than two weeks. This aim will generate fundamental and novel data on the progression of training (mal)adaptation by studying a population that has not experienced long-term physiological adaptations due to years of training habituation.

Specific Aim 2: Identify associations between protein production and performance (mal)adaptation during a 3-week high intensity training protocol and subsequent recovery. *We hypothesize that there will be significant changes to the proteome associated with an increased acute phase immune response* ***preceding training maladaptation***. We will perform proteomic analysis at three time points (pre-training, post-training, post-recovery) from blood sampled prior to performance testing.

Specific Aim 3: Identify associations between markers of inflammation (TNFα and IL-6) and performance capacity during a 3-week high intensity training protocol and subsequent recovery. *We hypothesize that proinflammatory cytokines* TNFα and IL-6 *will increase* ***preceding training maladaptation***. Furthermore, we postulate elevations in these markers will associate with decreased testosterone: cortisol. Blood samples will be collected weekly to determine immunological activity during training and subsequent recovery phases.

In this proposal, we will prospectively examine the progression of overtraining in healthy, recreationally active adults. It is unknown whether overtraining can even occur in non-elite athletes and few studies have investigated overtraining in females. Findings from this proposal will be used to generate future hypothesis-driven studies to investigate training maladaptation and could expose the pathophysiological progression of overtraining beyond functional overreach. Our long-term goals are to develop proactive methods of detecting and preventing impending overtraining, allowing a better training and recovery balance in both elite and recreational athletes alike.

Significance:

Overtraining syndrome (OTS) is a disorder existing on a spectrum caused by repetitive imbalance between excessive training load and inadequate recovery. Characterized by performance deficits, increased respiratory illness, and impaired mood states, overtraining may last anywhere from weeks to years, depending on the severity.[10,11] Despite a large volume of literature, there are two glaring gaps concerning overtraining research: 1) the pathophysiological mechanisms of overtraining are poorly understood and 2) there remains an absence of sensitive diagnostic criteria to identify overtraining or predict its impending occurrence. We believe these gaps are largely due to repetitive iterations of similar study design utilizing homogenous populations of highly competitive male athletes[1–4] or previously overtrained subjects.[5,7] The primary focus of overtraining on high-level male athletes has limited our understanding of physiological responses associated with overtraining given that such athletes undergo years of extensive training habituation. This has also marginalized female athletes who have been largely absent from overtraining research. Furthermore, examining previously diagnosed subjects prevents the ability to establish causal mechanisms for overtraining. These knowledge gaps are

concerning as prevalence estimates are high-- 64% of elite runners have reported experiencing at least one episode of overtraining during their careers.[8] Homogenous study populations combined with limited prospective studies and repetitive study designs have prevented identification of sensitive diagnostic criteria that effectively recognize impending training maladaptation.

Physiological Progression of Training Maladaptation. Mechanistic causes of overtraining syndrome have been explored such as glycogen[12] or glutamine depletion hypotheses,[7,13] or the possibility that there exists sympathetic and parasympathetics types of OTS.[14] Although the prevalence of overtraining has grown, there remains an absence of sensitive diagnostic criteria to detect training maladaptation. After decades of research, the underlying pathophysiological mechanisms of overtraining remain poorly understood. This is due to several factors. Early research used inconsistent terminology and criteria used when describing overtraining with terms like "staleness"[15] or "burnout."[16] Additionally, there was a lack of well-controlled investigations that included performance measures, allowing athletes to be considered overtrained if they reported unexplained performance deficits and fatigue.[17,18] Furthermore, the majority of overtraining research has focused almost exclusively on high-level male athletes who have built years of tolerance to high-volume training. The continued use of a homogenous population with many years of training experience has created a myopic understanding of the physiological response to periods of high-volume training. Our proposal seeks to study the physiological response to high training volume in *recreationally active males and females* without extensive training habituation using a 3-week intensive lab-controlled protocol.

Markers of Training Maladaptation. The past four decades of research has investigated numerous subjective and objective criteria to monitor overtraining. Several markers have shown promise towards monitoring overtraining. Autonomic heart rate regulation appears to be a promising non-invasive marker to observe overtraining. Decreased peak heart rate coupled with decreased peak lactate concentrations following exercise have been used to distinguish overtrained and non-overtrained athletes.[19] These directional changes have been inconsistent across the literature, however, since similar findings have also been found as a result of positive training adaptations.[17,20] A recent meta-analysis[21] has revealed that there has been an extensive examination of hormonal and immunological markers associated with training maladaptation including testosterone, cortisol, and proinflammatory cytokines. Though some evidence has been found to associate perturbations of these markers following high-volume training, these results have not been consistent across the literature due to limited samples sizes and a lack of reported performance outcomes.[21,22] Further well-controlled investigations that regularly report performance outcomes in tandem with the observation of biological markers is warranted. In our proposal, all exercise sessions will take place in a laboratory, allowing a more robust analysis of exercise-related variables, compared to traditional overtraining studies. Our protocol intends to observe the immune system's response to high-volume training through an in-depth combination of proteomic analysis biological markers not previously utilized in overtraining literature. Specific aim 2 seeks to observe the anabolic/catabolic balance (testosterone: cortisol) during training and recovery in conjunction with proinflammatory cytokine levels (IL-6, TNF-α), while specific aim 3 will integrate the novel use of proteomic analysis at baseline, post-training, and post-training to assess the immune response at its earliest stages of activity.

We believe that examining recreational male and female adults, who do not have extensive histories of training habituation will elucidate new insights into the pathophysiological mechanisms of training maladaptation. Findings from this proposal will generate future hypothesis-driven studies and could expose the pathophysiological progression of overtraining beyond functional overreach. This would have far-reaching impacts for training-recovery paradigms in several physically active populations including 'weekend warriors' (e.g., first time marathoners), basic military training populations, and the traditional competitive athletes who often undergo year-round training. Our long-term goals are to develop future proactive methods for detecting and preventing impending training maladaptation, allowing a better training and recovery balance for athletes of all caliber. If successful, the findings in this study would support a framework for detecting training maladaptation through a combination of performance testing and markers generally accessible to most sports medicine settings.

Innovation:

This proposal is highly innovative as it *will investigate the progression of training maladaptation in both male and female subjects*. Previous overtraining research has overwhelmingly focused overtraining in highly-competitive male athletes[1–3] or previously overtrained subjects.[5–7] Female subjects are largely absent from

biomedical research as a whole, which has resulted in inequality regarding the understanding, diagnosis, and treatment of disease between sexes.[23] This same trend has continued in overtraining research.

Our proposal focuses on recreationally active males and females without an extensive training history. We believe that the inability to determine sensitive diagnostic criteria have been in part due to previous overtraining research overwhelmingly primarily revolving around high-level male athletes. This population would have gone through years of training adaptations, which may allow their body systems to be more tolerant to high volumes of training, compared to those of untrained persons. By examining the physiological response to high training volume in a population without extensive training habituation, we intend to elucidate novel insights towards the progression of training maladaptation.

The biological antecedents to overtraining are currently unknown. *We will employ a novel proteomics approach to explore the biological mechanisms of overtraining*. Recent field-based studies have identified multiple upregulated and downregulated proteins associated with impaired immune function following exhaustive exercise.[24,25] Examining the immune system during its initial response to high volume training could uncover new insights into the pathophysiological mechanisms of overtraining. Additionally, we will measure proinflammatory cytokine levels via TNF-α, IL-6 during the same timepoints. Both are cytokines involved in systemic inflammation and have been well documented in overtraining literature.[21,22,26] We will also measure testosterone: cortisol (T/C) to assess the body's anabolic/catabolic response to high-volume training and subsequent recovery.[27] T/C has previously been shown to decrease following demanding training periods.[28–30]

We will collect blood weekly during training and recovery phases, allowing for longitudinal during both training and recovery phases. A shortcoming of the previous overtraining research is the limited frequency of performance testing and data collection (e.g., only pre- and post-training measurements).[2,31,32] These simple designs have not monitored the progression of training maladaptation *during* periods of high training volume. Additionally, our entire training protocol is performed in a laboratory setting, allowing us to monitor a more robust set of exercise-related variables, compared to traditional field-based overtraining research. The combined observation of the proteome, proinflammatory cytokine levels (TNF-α, IL-6), testosterone:cortisol, and respiratory illness symptoms (WURSS-11 survey) during periods of high training volume would provide large amounts of information regarding the immune response during periods of high-training volume not previously shown in overtraining research. We intend to monitor protein expression at the onset of training (visit 2), immediately post-training (visit 20), and post-recovery (visit 23). This will be combined with weekly assessment of testosterone, cortisol, TNF-α, and IL-6 and daily assessment of respiratory illness symptoms.

Approach:

Experimental Approach for SA1: Subjects will be randomized upon recruitment into an experimental or control group. Control subjects will attend the lab once weekly for six weeks to complete exercise testing and provide resting blood samples. For the duration of this study, control subjects will be asked to maintain a consistent lifestyle routine including diet and exercise habits. Experimental subjects will undergo an in-lab cycling training protocol for three weeks. At the beginning of each week (3 weeks training phase, 3 weeks recovery phase) experimental subjects will undergo the same exercise testing as control subjects. In addition to weekly performance testing during recovery, experimental subjects will undergo performance testing 48-hours post-training to assess for any immediate signs of training maladaptation

Determination of Training (Mal)adaptation*:* Performance throughout the training and recovery phases will be compared to visit 2 (onset of training protocol), which will serve as a reference point when determining significant performance changes. Our primary performance variable will be time-to-exaction (TTE) determined by a maximal incremental ramp test (*see performance testing protocol*). The variance shown in TTE performance by control subjects will be used create a threshold for determining significant changes in performance. This will be calculated by determining the smallest worthwhile change (SWC) in performance, which has been used in previous overtraining research.[33] Experimental subjects whose performance increases or remains within the SWC threshold will be considered "adapted" (AD), while those who show performance decreases beyond the SWC will be considered overreached. Overreached subjects whose performance recovers within two weeks will be considered "functionally overreached" (FOR), while those whose performance takes longer than two weeks to recover will be considered "non-functionally overreached" (NFOR).

Performance Testing Protocol (all subjects): A two-bout exercise test will be performed on a magnetically controlled cycle ergometer. Bout 1: Subjects will at either 100W (males) or 75W (females) with an increase 45W or 30W, respectively, every two minutes until volitional exhaustion. Bout 2: following a five-minute recovery period, subjects will cycle at a constant workload (110% of the peak workload achieved during their bout 1) to volitional exhaustion. This second bout of exercise allows us to verify maximal effort and variables such as maximal oxygen uptake (VO_{2peak} vs. VO_{2max}) were achieved during the first round of exercise.[34] During all cycling, subjects will breathe through a one-way facemask (Hand-Rudolph) allowing breath-by-breath expired gases to be sampled continuously via a metabolic cart (ParvoMedics). Heart rate will be monitored continuously during exercise via a chest strap heart rate monitor (Polar).
3-week Cycle Training Protocol (Table 1): All visits include a 5-min warm-up and cool-down at 25% of the subject's peak workload (PWL) achieved during their maximal incremental exercise test. Experimental subjects will return to the lab for the same performance testing procedures 2-,7-,14-, and 21-days post-training to monitor recovery. All exercise visits are personalized to each subject's PWL achieved during their initial exercise testing. If a subject outperforms their initial performance testing during weeks two or three of training, all subsequent exercise workloads will correspond to the new peak workload. If a training visit becomes too difficult for a subject to complete, workloads will be decreased by 20W increments and increased by 10W every three minutes until the original workload is regained or upon completion of the training session.

3-week Cycle Training Protocol		
Week 1	**Week 2**	**Week 3**
Performance Testing	Performance Testing	Performance Testing
50-min ride @ 60% PWL	50-min ride @ 65% PWL	50-min ride @ 70% PWL
5x5-min @75% PWL	5x5:15-min @75% PWL	5x5:31-min @75% PWL
2x20min @65% PWL	2x25min @65% PWL	2x30min @65% PWL
12x45sec @130% PWL	12x50sec @130% PWL	12x55sec @130% PWL
50-min at 3mmol lactate	55-min at 3mmol lactate	60-min at 3mmol lactate
Rest day	Rest Day	Rest Day

Table 1: 3-week Cycle Training Protocol

Experimental Approach for SA2&SA3: Once weekly, four vials (2 serum, 2 plasma) containing ~4mL venous blood will be collected by a researcher holding a valid phlebotomy certification (Haverkamp). Plasma and serum will be centrifuged at 2500RPM x15minutes (serum samples will sit for 60 minutes prior to centrifuge) then aliquoted and stored in triplicate Eppendorf tubes at -80°C for future analysis. In the event that veinous blood sampling is not available dried blood spot (DBS) cards will be used to collect blood via lancet fingerprick.

- *Blood Lactate Collection.* Blood lactate levels will be assessed during at rest and during every stage of exercise performance testing. This process involves sterilizing a fingertip with an alcohol pad and collecting 1 droplet of blood into a lactate analyzer strip.
- *Dried Blood Spotting Collection.* Dried Blood Spots (DBS) are performed via a lancet to fingerprick, like lactate collection or hematocrit blood collection. Guided by the researcher, subjects will place one drop of blood within the designated collection circles on the blood spotting cards (manufacturer), which will dry and be stored under dark, ambient (room temperature) conditions. For storage longer than 6 months, DBS cards will be stored in a -80°C freezer.

Analysis of Biological markers: the initial performance testing day (visit two) will be used as a reference point for changes to all proteomic activity and biomarker levels (testosterone, cortisol, IL-6, and TNF-α) throughout training and subsequent recovery. These changes will also be compared to daily upper respiratory illness survey symptoms (WURSS-11)

- *Plasma Protein Isolation/Proteomics Analysis.* Plasma will be prepared for analysis by performing a protein crash using 100uL of specimen and 500uL acetonitrile (5:1 v/v ratio) in a microcentrifuge tube. The samples will be incubated for 60 min at 4°C to precipitate proteins. The samples will be centrifuged at 16,000 x g for fifteen minutes. The supernatant will be transferred to a new tube, evaporated, and reconstituted in solvent. Following preparation, plasma proteins will be sent to the Washington State University Tissue Imaging, Metabolomics and Proteomics Laboratory for analysis. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) will be used to identify protein production linked to immune system activity in maladapted subjects. STRING is a database of known and predicted

physical/functional protein associations based on genomic context, high-through put experiments, co-expression, and previous knowledge.[35]

- *Hormonal/Biological Marker Analysis.* Standard enzyme-linked immunosorbent assay (ELISA) kits will be used to analyze testosterone, cortisol, IL-6, and TNF-α. At all sampling times, samples will be run in duplicate for each marker. In instances where venous blood cannot be collected for hormonal marker analysis (e.g., limited phlebotomist availability), dried blood spotting (DBS) will be collected.

Preliminary Data: Besides, decreased performance, alterations to multiple physiological variables during exercise have been associated with training maladaptation including attenuated oxidative capacity,[33] along with increased parasympathetic tone indicated increased heart rate recovery and decreased peak heart rate.[19,36] Lastly, overtraining literature has reported decreased peak blood lactate concentrations associated with overtraining.[31] Additionally, peak blood lactate concentrations lower than 8mmol*L^{-1} has shown high sensitivity in distinguishing overtrained from non-overtrained subjects.[37] Our subject's post-training peak lactate with maximal exercise was found to be 7.3mmol*L^{-1}. Given these previous findings we believe that at least one experimental subject has experienced overreaching following our training protocol (Table 2).

Experimental Subject Data	Baseline Value	Post-training (% change)	Post-recovery (% change)
Time-to-exhaustion (min)	4.72	4.58(-5.5%)	4.98 (+2.75%)
Peak Heart Rate (bpm)	189	159 (-15.87%)	185 (-4.71%)
Heart Rate Recovery (bpm)	21	31 (+44.19%)	26 (+20.93%)
Aerobic Capacity (L/min)	1.57	1.29 (-17.83%)	1.68 (+7.01%)
Peak Lactate (mmol)	10.5	7.3 (-30.48%)	9.1 (-13.33%)

Table 2: Preliminary data supporting evidence of overreaching in a subject

Anticipated Problems and Pitfalls: A primary concern regarding our training protocol is the initial uncertainty that this protocol provides sufficient stimulus to induce overreaching (FOR, NFOR) in subjects. We have provided preliminary data that at least one subject exhibited physiological responses compatible with overreaching. This suggests that our training protocol is sufficient training stimulus to induce overreaching in at least a portion of subjects who complete this study. Even if subjects do not achieve NFOR and are only separated into adapted or FOR groups, data from this proposal would still provide detailed knowledge as to the body's responses to high-volume in subjects unaccustomed to such work.

We acknowledge that this research protocol will require a large time commitment on the part of the research participants. We have allotted three absences (85% adherence) from training days to retain subjects should protocol adherence become an issue. Should a subject miss a performance testing day with biomarker collection, these procedures will be performed on the next testing day, prior to exercise.

COVID-19**,** shut down essentially all human research Washington State University until August 2021. As the pandemic continues on throughout 2022, is some concern that COVID variants such as Omicron may once again temporarily halt overtraining research. We six subjects (3 control, 3 experimental) have completed this study protocol, with the anticipation of having at least ten subjects through the protocol by May 2022. If research were to halt, we would have the ability to run the proteomic and biological analyses that this proposal seeks to perform. We continue to practice the use of personal protective equipment and regularly sanitize lab equipment during and between subject visits. Should one of our research team become ill, others have been trained on the lab equipment and may be able to step in for data collection.

Personal Narrative: (5,000 characters)

I will serve as the PI for the proposed project titled, "Identification of early (non)functional overreach warning signs following a 3-week lab-controlled cycling protocol." I am confident I am well-suited to serve in this role for a number of reasons pertaining to my education/training, experience with the literature & proposed methods, and experience & support from my key personnel and advisor/co-investigator

I am a third-year graduate student in the Nutrition & Exercise Physiology department at Washington State University (WSU) in Spokane, WA. Prior to WSU, I have received baccalaureate and master's in Athletic Training, with over 5 years active clinical experience. I have provided care for numerous athletes dealing with illness, chronic fatigue, and musculoskeletal injuries during all points in their seasons. I have dealt with several athletes in my clinical career whom I've suspected of being overtrained due to their symptoms of excessive fatigue, illness, and performance decrements. I was successfully able to help these athletes recover, despite not having the resources to properly diagnose these disorders. From a sports performance standpoint, I have

served in roles as a personal trainer and performance coach for hundreds of athletes over the past 10 years. I have designed, implemented, and tracked training programs, honing the ability to prescribe the proper training loads at key times to achieve the desired training stimulus and effect

The courses and curriculum I have studied in my previous undergraduate, master's, and current doctoral pursuits have suited my background knowledge regarding states of overtraining. As a senior athletic training student, I led a senior study project. This initially piqued my interest in pursuing research long-term. My master's degree was an intensive 13-month program including a full-time course load, clinical athletic training position, and thesis requirement. I was able to develop and carry out a novel research study with subsequent publication in that time frame. Finally, in the past three years of my doctorate program at WSU, I have focused my studies towards an emphasis on training load, overreach, and training adaptation literature, in order to familiarize myself & key personnel with the topic. This has allowed us to understand the literature on overtraining, understand how it has evolved over the past four decades, identify current gaps and issues in the literature, and formulate a novel plan aimed at solving those gaps in overreach & overtraining literature.

Regarding collaborators and the scientific environment, I have worked regularly for three semesters in Dr. Haverkamp's lab, familiarizing myself with the lab equipment and methods that will be implemented, and developing our proposal's training protocol. This includes use of lab-related equipment to perform pulmonary function testing, exercise testing on a cycle ergometer, fluid sample collection procedures, and use of whole-body plethysmography. Since the summer of 2021, I have pilot tested this study protocol, was approved by the IRB, and have begun collecting data on several subjects. At the time of this proposal, I have successfully taken three control and three experimental subjects through the study protocol.

Personnel: Dr. Hans Haverkamp, associate professor in the Nutrition & Exercise Physiology (NEP) department at Washington State University, is the student advisor/co-investigator of the proposal. Dr. Haverkamp is a whole-body exercise physiologist with an extensive background in research, emphasizing a focus on airway diseases such as asthma and certified phlebotomist. Dr. Haverkamp's lab regularly has at least one additional undergraduate and graduate student each assisting him in lab-related work. Dr. Wideman is a distinguished professor at the University of North Carolina-Greensboro with extensive experience investigating hormonal and biological biomarkers associated with exercise and disease. She has been collaborating with our lab group since the fabrication of this research study; currently one of her doctoral mentees is also investigating overtraining as a component of their dissertation. Dr. Wideman has recently examined hypothalamic dysfunction in overtrained subjects due to high training volumes. Her expertise in the endocrine system and in biomarker analysis will be highly beneficial as we look to analyze hormones (testosterone, cortisol) and cytokine (IL-6, TNF-α) levels from collected fluid samples. Dr. Nieman is a pioneer in the research areas of exercise and nutrition immunology. Over the past several years, Dr. Nieman has been spearheading the use of proteomics to determine immunological activity in relation to extreme bouts of exercise. His knowledge and expertise in exercise immunometabolism will be of great benefit to investigating the immune system (proteomics and cytokines) during this study.

Budget & Budget Justification: budget form (separate attachment) justification
Subject remuneration: $0. We have been fortunate that recruitment interest has been high despite a current lack of funds to support subject remuneration. Our current plan is to continue on with the study without funding for compensation. This would allow crucial funding to go towards analysis of biomarkers and proteomics. We currently have six subjects (3 control, 3 experimental) completed and several more slated for participation in the upcoming months. We aim to have approximately 12 subjects completed with this study by May 2022.

Assays: Assay kits are estimated to cost $500 per kit. 16 wells/assay will be used for calibration leaving 80 wells/assay available for data collection & analysis. There will be 7 days (baseline, visits 2,8,14,20,21,22,23) where biomarkers: TNF-a, IL-6, testosterone, and cortisol will be collected and analyzed. Each biomarker will be analyzed twice to ensure validity. This leaves a total of 224 wells needed for accurate sampling, barring any errors. Approximately three kits will be required costing ~$1400.

Assay supplies including reagents for the assays are estimated to cost ~$500.

Proteomic Analysis: Proteomics through the Washington State University Tissue Imaging, Metabolomics, and Proteomics Laboratory costs $210 per analysis. Three analysis time points (pre- and post-training and post-recovery) for four subjects will cost $2,520.

Expendable data collection supplies including personal protective equipment (gloves, masks, face shields), alcohol swabs, gauze, cleaning solution, pipet tips, face mask mouth pieces are estimated to cost ~$80

Phlebotomy certification: This study is time-consuming and there are times where a certified phlebotomist (Dr. Haverkamp) is not available to draw blood. As such, we would request $500 towards the student investigator (Gooding) pursuing a phlebotomy certification. These certifications with training cost ~$700.

Description of Facilities: Institutional Resources & Environment
Research Laboratories: WSU investigators for the proposed project are housed in the Elson S. Floyd College of Medicine on the Spokane Campus The lab is located in the same building and on the same floor as the PIs office. The state-of-the-art lab space (920 sq ft) contains the necessary equipment and items for collecting all data in this proposal. A wet lab with four freezers (-20 and -80 degrees Celsius) is located directly next door to the research lab. The PIs department has a full-time laboratory manager who aids in maintaining the labs, placing orders, equipment maintenance, and troubleshooting. Major lab equipment related to this planned study includes:
-Cycle Ergometer (Ergoline, Ergoselect 200)
-Body Plethysmography (COSMED, Q-Box)
-BodPod (COSMED)
-Parvo Medics metabolic cart (TrueOne 2400)
-Lactate Analyzer (Nova Biomedical, Lactate Plus)
-Chest strap heart rate monitor (Polar)
-Tremoflo (Thorasys)
-Fractional Exhaled Nitric Oxide analyzer (Niox)
-Nebulizer (ULTRA-NEB'99, DeVILBISS)
WSU provides e-mail software, telephone support, videoconferencing, secure computing and network resources, technical assistance, and library services for faculty and staff. The WSU – Health Sciences Spokane Library provides access to over 2 million books and more than 30,000 journals. Information technology staff facilitate high quality computing resources, network access, and videoconferencing. Collectively, these resources provide valuable infrastructure for study support, data security, and assuring successful communication among members of the research team

Personnel: Primary personnel besides the student investigator include Dr. Haverkamp (student advisor) as well as Dr. Laurie Wideman and Dr. David Nieman (collaborators). This proposal is a component of Thomas Gooding's dissertation at Washington State University and as such, he will devote 100% of his efforts towards this research. He will be guided by Dr. Haverkamp who will also serve as the phlebotomist when blood collection is needed. Dr. Nieman will serve as a guide and mentor when examining immunological activity, particularly with regard to proteomic analysis. Dr. Wideman will serve as a guide and mentor when examining biological markers including hormonal (testosterone, cortisol) and cytokines (IL-6, TNF-α).

Description of Risks for subjects:
Confidentiality: If data is lost or stolen, subjects could experience invasion of privacy. To minimize the potential invasion of privacy, we are not collecting social security numbers so that the potential economic impact is greatly minimized. All of our files will be kept in a locked filing cabinet to prevent theft and data will be de-identified. Data acquired on computers will be password protected. As such, the ***probability*** of the adverse outcomes discussed above is low, and the ***severity*** is minimal.

Musculoskeletal injury: With exercise, there always exists the possibility of musculoskeletal injury during activity. Subjects will always perform a warm-up prior to all exercise. Additionally, all exercise will be performed on a cycle ergometer. Cycling consists of predominantly concentric muscle contractions, which inherently cause less stress to muscle fibers, reducing the risk of injury. Additionally, a state licensed & certified athletic trainer will be present during all exercise sessions to triage any concerns of musculoskeletal injuries and to refer for appropriate care, if necessary. However, the risk of injury associated with stationary cycling is minimal.

Venipuncture: Subjects will have venous blood drawn for determination of serum biomarker levels. Risks associated with a venipuncture include pain and/or bleeding during needle insertion and discomfort, bruising, and/or infection at the insertion site. To minimize these risks only individuals trained to perform venipuncture will obtain blood samples using sterile equipment.

Rhabdomyolysis: Though extremely rare, intensity exercise bouts have been documented to cause exercise-induced rhabdomyolysis (ExRML). Cycling is largely a concentric muscle activity, which is much less stressful to muscle tissue compared to eccentric contractions, thereby minimizing the risk of rhabdomyolysis. During the training phase of this study daily urine will be collected and analyzed for color, hydration status (specific gravity), and protein content to detect any onset ExRML. Should there be a high protein content and a dark red/black urine color, subject will be immediately referred to medical services to assess concern of ExRML.

Altered sleep and mood states: Potential psychological risks from exercise include loss of sleep due to fatigue and soreness, as well as impaired mood states. Daily sleep journals will be kept, and mood states will be tracked using the Profile of Mood States= short form (POMS-SF) questionnaire.[38] Subjects are able to discontinue the protocol at any time should they become concerned regarding their sleep schedule or mood state. Should two consecutive POMS-SF scores be greater than 100, the subject will be disenrolled from the study and referred to mental health services for follow up. The risk of altered mood changes is moderate, but such changes would be transient and integral to the main findings in this proposal. Furthermore, any changes observed should return to normal with cessation of high training volumes and appropriate rest.

Upper respiratory illness: There is emerging evidence that high-volume exercise may temporarily suppress the immune system, allowing an increased susceptibility to upper respiratory illness.10 Symptoms of upper respiratory illness will be tracked daily using the Wisconsin Upper Respiratory Illness survey.10 to mitigate risk of COVID-19, researchers will wear appropriate PPE, lab coats or scrubs, safety glasses, and gloves. Additionally, proper social distancing can be maintained during most instances within the protocols described above, i.e., maintaining 6 feet between participants and researchers. While the severity of COVID-19 infection has the potential to be severe, the measures described to mitigate this risk make the likelihood of an event low.

Safety and Emergency Procedures: All research personnel affiliated with this proposal are CPR/First aid/AED certified. During all exercise and recovery periods, subjects will be continuously monitored for vital signs. In the event of a non-emergent event, testing will be discontinued, and the subject will be moved to an exam table to lay down with legs and feet elevated. If symptoms do not subside after a reasonable period of time (~10 min) the AED will be connected to determine cardiac rhythm and then assess whether or not emergency personnel should be contacted. In the unlikely event of an emergent event (i.e., subject loses consciousness) emergency personnel will be contacted immediately, and the subject connected to the AED. If indicated by the AED, defibrillation will be administered according to device guidelines until EMS arrives.

References

1. Bellenger CR, Thomson RL, Robertson EY, et al. The effect of functional overreaching on parameters of autonomic heart rate regulation. *European Journal of Applied Physiology*. 2017;117(3):541-550. doi:10.1007/s00421-017-3549-5
2. Hausswirth C, Louis J, Aubry A, Bonnet G, Duffield R, le Meur Y. Evidence of disturbed sleep and increased illness in overreached endurance athletes. *Medicine and Science in Sports and Exercise*. 2014;46(5):1036-1045. doi:10.1249/MSS.0000000000000177
3. Bourdillon N, Nilchian M, Millet GP. Photoplethysmography Detection of Overreaching. *Medicine and Science in Sports and Exercise*. 2019;51(4):701-707. doi:10.1249/MSS.0000000000001836
4. Tanskanen MM, Kyröläinen H, Uusitalo AL, et al. Serum sex hormon-binding globulin and cortisol concentrations are associated with overreaching during strenuous military training. *Journal of strength and conditioning research*. 2011;25(3):787-797.
5. Cadegiani FA, Kater CE. Basal hormones and biochemical markers as predictors of overtraining syndrome in Male athletes: The EROS-Basal study. *Journal of Athletic Training*. 2019;54(8):906-914. doi:10.4085/1062-6050-148-18
6. Ackerman KE, Paula Pinto A, Ribeirão Preto P, Anthony Hackney BC, Cadegiani FA, Kater CE. Eating, Sleep, and Social Patterns as Independent Predictors of Clinical, Metabolic, and Biochemical Behaviors Among Elite Male Athletes: The EROS-PREDICTORS Study. *Frontiers in Endocrinology | www.frontiersin.org*. 2020;1:414. doi:10.3389/fendo.2020.00414
7. Parry-Billings M, Budget R, Koutedakis Y, Al. E. Plasma Amino Concentrations in the overtraining Syndrome: Possible Effects on the Immune System. Published online 1992.
8. Morgan WP, O'Connor PJ, Ellickson KA, Bradley PW. Personality structure, mood states, and performance in elite male distance runners. *International Journal of Sport Psychology*. 1988;19(4):247-263. https://psycnet.apa.org/record/1989-41579-001
9. Morgan WP, O'Connor PJ, Sparling PB, Pate RR. Psychological characterization of the elite female distance runner. *International journal of sports medicine*. 1987;8 Suppl 2:124-131. doi:10.1055/s-2008-1025717
10. Meeusen R, Duclos M, Foster C, et al. Prevention, diagnosis, and treatment of the overtraining syndrome: Joint consensus statement of the european college of sport science and the American College of Sports Medicine. *Medicine and Science in Sports and Exercise*. 2013;45(1):186-205. doi:10.1249/MSS.0b013e318279a10a
11. Bellinger P. Functional Overreaching in Endurance Athletes: A Necessity or Cause for Concern? *Sports Medicine*. 2020;(0123456789). doi:10.1007/s40279-020-01269-w
12. Snyder AC. Overtraining and glycogen depletion hypothesis. *Medicine and science in sports and exercise*. 1998;30(7):1146-1150. doi:10.1097/00005768-199807000-00020
13. Keast D, Arstein D, Harper W, Fry RW, Morton AR. Depression of plasma glutamine concentration after exercise stress and its possible influence on the immune system. *The Medical journal of Australia*. 1995;162(1):15-18.
14. Kuipers H, Keizer HA. Overtraining in elite athletes. Review and directions for the future. *Sports medicine (Auckland, NZ)*. 1988;6(2):79-92. doi:10.2165/00007256-198806020-00003
15. Morgan WP, Brown DR, Raglin JS, O'Connor PJ, Ellickson KA. Psychological monitoring of overtraining and staleness. *British journal of sports medicine*. 1987;21(3):107-114. doi:10.1136/bjsm.21.3.107
16. Mackinnon LT. Overtraining effects on immunity and performance in athletes. *Immunology and Cell Biology*. 2000;78(5):502-509. doi:10.1111/j.1440-1711.2000.t01-7-.x
17. Urhausen A, Kindermann W. Diagnosis of overtraining: what tools do we have? *Sports Medicine*. 2002;32(2):95-102. http://articles.sirc.ca/search.cfm?id=S-811818%5Cnhttp://search.ebscohost.com/login.aspx?direct=true&db=s3h&AN=SPHS-811818&site=ehost-live%5Cnhttp://www.adis.com
18. Halson SL, Jeukendrup AE. Does overtraining exist? An analysis of overreaching and overtraining research. *Sports Medicine*. 2004;34(14):967-981. doi:10.2165/00007256-200434140-00003

19. le Meur Y, Hausswirth C, Natta F, Couturier A, Bignet F, Vidal PP. A multidisciplinary approach to overreaching detection in endurance trained athletes. *J applied physiology*. 2013;114(3):411-420. doi:10.1152/japplphysiol.01254.2012
20. Bellenger CR, Fuller JT, Thomson RL, Davison K, Robertson EY, Buckley JD. Monitoring Athletic Training Status Through Autonomic Heart Rate Regulation: A Systematic Review and Meta-Analysis. *Sports Medicine*. 2016;46(10):1461-1486. doi:10.1007/s40279-016-0484-2
21. Greenham G, Buckley JD, Garrett J, Eston R, Norton K. Biomarkers of Physiological Responses to Periods of Intensified, Non-Resistance-Based Exercise Training in Well-Trained Male Athletes: A Systematic Review and Meta-Analysis. *Sports Medicine*. 2018;48(11):2517-2548. doi:10.1007/s40279-018-0969-2
22. Cadegiani FA, Kater CE. Hormonal aspects of overtraining syndrome: A systematic review. *BMC Sports Science, Medicine and Rehabilitation*. 2017;9(1):1-15. doi:10.1186/s13102-017-0079-8
23. Uhl K, Parekh A, Kweder S. Females in clinical studies: Where are we going? *Clinical Pharmacology and Therapeutics*. 2007;81(4):600-602. doi:10.1038/sj.clpt.6100112
24. Nieman DC, Groen AJ, Pugachev A, Vacca G. Detection of functional overreaching in endurance athletes using proteomics. *Proteomes*. 2018;6(3). doi:10.3390/proteomes6030033
25. Antona GD', Louis J, Roberts MD, et al. Proteomic Markers of Non-functional Overreaching During the Race Across America (RAAM): A Case Study. Published online 2019. doi:10.3389/fphys.2019.01410
26. Sullivan GW, Carper HT, Novick WJJ, Mandell GL. Inhibition of the inflammatory action of interleukin-1 and tumor necrosis factor (alpha) on neutrophil function by pentoxifylline. *Infection and immunity*. 1988;56(7):1722-1729. doi:10.1128/iai.56.7.1722-1729.1988
27. Adlercreutz H, Härkönen M, Kuoppasalmi K, et al. Effect of training on plasma anabolic and catabolic steroid hormones and their response during physical exercise. *International journal of sports medicine*. 1986;7 Suppl 1:27-28. doi:10.1055/s-2008-1025798
28. Coutts AJ, Reaburn P, Piva TJ, Rowsell GJ. Monitoring for overreaching in rugby league players. *European journal of applied physiology*. 2007;99(3):313-324. doi:10.1007/s00421-006-0345-z
29. Booth CK, Probert B, Forbes-Ewan C, Coad RA. Australian army recruits in training display symptoms of overtraining. *Military Medicine*. 2006;171(11):1059-1064. doi:10.7205/MILMED.171.11.1059
30. Urhausen A, Kullmer T, Kindermann W. A 7-week follow-up study of the behaviour of testosterone and cortisol during the competition period in rowers. *European journal of applied physiology and occupational physiology*. 1987;56(5):528-533. doi:10.1007/BF00635365
31. Hedelin R, Kentta G, Wiklund U, Bjerle P, Henriksson-Larsen K. Short-term overtraining: Effects on performance, circulatory responses, and heart rate variability. *Medicine and Science in Sports and Exercise*. 2000;32(8):1480-1484. doi:10.1097/00005768-200008000-00017
32. Schaal K, le Meur Y, Louis J, et al. Whole-body cryostimulation limits overreaching in elite synchronized swimmers. *Medicine and Science in Sports and Exercise*. 2015;47(7):1416-1425. doi:10.1249/MSS.0000000000000546
33. Bellinger PM, Sabapathy S, Craven J, Arnold B, Minahan C. Overreaching Attenuates Training-induced Improvements in Muscle Oxidative Capacity. *Medicine and science in sports and exercise*. 2020;52(1):77-85. doi:10.1249/MSS.0000000000002095
34. Poole DC, Jones AM. Measurement of the maximum oxygen uptake Vo2max: Vo2peak is no longer acceptable. *Journal of Applied Physiology*. 2017;122(4):997-1002. doi:10.1152/japplphysiol.01063.2016
35. Szklarczyk D, Morris JH, Cook H, et al. The STRING database in 2017: Quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Research*. 2017;45(D1):D362-D368. doi:10.1093/nar/gkw937
36. Aubry A, Hausswirth C, Louis J, Coutts AJ, Buchheit M, le Meur Y. The development of functional overreaching is associated with a faster heart rate recovery in endurance athletes. *PLoS ONE*. 2015;10(10):1-16. doi:10.1371/journal.pone.0139754
37. Meeusen R, Nederhof E, Buyse L, Roelands B, de Schutter G, Piacentini MF. Diagnosing overtraining in athletes using the twobout exercise protocol. *British Journal of Sports Medicine*. 2010;44(9):642-648. doi:10.1136/bjsm.2008.049981

38. Shacham S. A shortened version of the Profile of Mood States. *Journal of personality assessment*. 1983;47(3):305-306. doi:10.1207/s15327752jpa4703_14
39. Wisconsin Upper Respiratory Symptom Survey. https://www.fammed.wisc.edu/wurss/