

OVERTRAINING THROUGH A NEW LENS: CHARACTERIZATION OF
OVERREACH IN RECREATIONALLY ACTIVE ADULTS AND
THE HORMETIC IMPLICATIONS

By

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Abstract

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Background. Overtraining (OT) occurs from excessive exercise and inadequate recovery. Different pathophysiological mechanisms of OT have been theorized; however, the biological underpinnings of OT are unclear. Hormesis refers to a biphasic dose-response to stress. Accordingly, any biological system(s) overwhelmed by exercise-related stress may exhibit a maladaptive training response. **Objectives:** This dissertation aimed to 1) determine if recreationally active adults can experience OT and 2) identify potential markers of OT progression. **Methods.** Twenty-one recreationally active adults were randomized into training (TR, n=11) or control (CON, n=10) groups. TR participants underwent a three-week high-intensity training protocol designed to induce short-term OT (overreach [OR]) under laboratory conditions, then monitored over a three-week recovery phase. Both groups performed weekly exercise testing to evaluate performance and metabolic and cardiac responses to training. Sleep was monitored daily using actigraphy and surveys. A pilot proteomic analysis was performed in seven TR participants at baseline (BL), after training (MID), and after recovery (END). **Results.** After training, three TR participants were classified as OR ($-10.3\% \pm 5.4\%$ decrease in

performance from BL), while TR participants without performance decrements were considered adapted (AD, n=8). Throughout training, OR subjects exhibited progressive decreases in maximum heart rate ($-8.5\% \pm 6.4\%$, 180 ± 7.9 to 164 ± 4.6 bpm) and peak lactate ($-33.6\% \pm 15.1\%$, 8.80 ± 1.47 to 5.86 ± 1.69 mmol/L) ($P<0.05$), which returned to baseline values during recovery. At END, peak aerobic capacity improved ~6% in all groups. Compared to CON participants, TR participants obtained less sleep (7.02 ± 1.03 versus 6.68 ± 1.20 hours per night, respectively), exhibited more variable sleep time, and were more likely to accumulate sleep debt ($P<0.05$). OR participants consistently reported higher illness symptoms than CON and AD ($P<0.05$). Proteomics identified 38 proteins upregulated at MID or END including 19 immune-related proteins ($P<0.1$). **Conclusions.** OR participants exhibited markers of OT compatible with both autonomic and immune dysfunction—different theories underpinning the OT response. OT is not a training response exclusive to elite-level athletes. OT as the upper endpoint of exercise-induced hormesis is a viable theory, which suggest that OT can occur from multiple pathophysiological mechanisms.

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Dedication

To Nicole, Julian, Archie, Hugo, and any future members of my pack—

I dedicate the efforts of this PhD to you.

CHAPTER ONE: GENERAL INTRODUCTION

Prelude.

This dissertation does not follow the traditional dissertation format, by which chapter one serves as an introduction to the topic of the dissertation; chapter two, a literature review of said topic; chapter three, methods; chapter four, results; chapter five, discussion; and chapter six, conclusions. Instead, this dissertation follows the “alternative format,” approved by the Graduate Studies Committee of the Washington State university Faculty Senate, whereby individual manuscripts intended for publication replace the standard dissertation chapters. As such, a requisite for the alternative format is the “inclusion of a general introduction, discussion, and/or conclusion sections which serve to integrate the presentation of the chapters (manuscripts) into a single, cohesive body of research.”

The primary focus of this dissertation is overtraining syndrome, a maladaptive process caused by an imbalance between excessive training-related stress and inadequate recovery (i.e., nutrition and exercise). The original research within this dissertation involves a randomized controlled study that used a three-week high-intensity training protocol designed to induce a short-term state of overtraining (overreach [OR]) in recreationally active adults under laboratory conditions. Here, physical activity is defined as any bodily movement that expends energy, whereas exercise training (hereby abridged to ‘exercise’ or ‘training’) is any planned, structured physical activity performed with the intention of maintaining or improving physical fitness (i.e., health- or skill-related improvements).¹ For the purposes of this dissertation, these terms (physical activity, exercise, and training) will be used synonymously to indicate any movement

performed at a sufficient intensity/duration with the intention of improving health outcomes (i.e., well-being or physical fitness).

The general introduction discusses the principles of exercise prescription, factors that influence the exercise dose-response and associated health outcomes, and provides an overview of overtraining syndrome. After introducing the dissertation chapters, this section also introduces the concepts of hormesis theory in relation to exercise. Specifically, this dissertation builds upon existing literature which suggests that overtraining is the upper endpoint of exercise-induced hormesis.

Following the manuscript-style dissertation chapters, the general discussion will expand on exercise-induced hormesis and integrate findings from each chapter and existing literature within the framework of hormesis theory. Finally, the general discussion section will consider how hormesis theory may serve as a ‘unifying theory’ towards the pathophysiology of overtraining, and discuss how this framework can be used in future exercise research.

*Exercise is Medicine*²

*Sola dosis facit venenum*³

(The dose makes the poison)

General Introduction.

Engaging in regular exercise (physical activity) is integral to achieving and maintaining a healthy life as it reduces the risk of life-style related diseases,⁴⁻⁸ increases longevity,⁹ and significantly increases the quality of life.^{10,11} The current Physical Activity Guidelines for Americans recommend that adults should attain 150 to 300 minutes of moderate, 75 to 150 minutes of vigorous physical activity (MVPA) per week, or the equivalent combination thereof, for optimal health.¹² At this dose of physical activity, the steepest reduction of risks occurs for all-cause mortality, cardiovascular disease mortality and incidence, and incidence of type 2 diabetes.⁴ These guidelines further suggest that the health benefits of physical activity continue to increase as physical activity levels increase, albeit with a diminishing rate of return. However, while these

guidelines are primarily intended to reduce the amount of sedentary behavior, these guidelines inadvertently suggest that *more* exercise is better, without limit.^{4,12} This is not always the case.

Sports coaches and researchers alike have long understood that there is variation to how athletes may respond to any given training stimulus.^{13,14} For example, previous research has demonstrated the existence of interindividual variation in training responses to maximal oxygen uptake ($\dot{V}O_{2\text{max}}$),^{15–23} heart rate (HR),^{20,24} systolic blood pressure,²⁴ metabolic thresholds (e.g., anaerobic threshold),^{20,25,26} muscle enzymatic activity and glycogen content,^{15,19,27} and measures of performance (e.g., time-to-exhaustion).¹⁹

There are many factors inherent in any exercise training program which can affect the training dose-response. These factors include: the exercise modalities used (e.g., resistance training, aerobic exercise), exercise intensity, duration, frequency, recovery time, work-to-rest ratios, duration of the entire training program, and overall training volume.²⁸ Additionally, age, genetics, sex, diet, sleep, the time of day in which exercise is performed (circadian rhythm), and the previous training history of the individual have also been shown to influence the training response.^{14,28–31} As such, there is an ongoing debate in the exercise science community regarding the concept of ‘responders’ *versus* ‘non-responders’ to exercise.^{14,32,33} Thus, while the idea of using exercise to improve health outcomes is well established, achieving the optimal dose-response to exercise has yet to be elucidated.

The relationship between physical activity and injury/illness has been studied for decades and follows a ‘J’-shaped curve.^{34,35} As physical activity levels continue to increase beyond moderate levels (i.e., 150 to 300 minutes of MVPA per week), there is an unknown threshold where continued increases in physical activity levels *increase* the risk of injury or illness. In

athletics, the process by which excessive exercise results in a maladaptive response is known as overtraining (**Figure 1.1**).³⁶

The Overtraining Spectrum. Overtraining is the result of an imbalance between excessive training-related stress and inadequate recovery (i.e., nutrition and sleep).^{36,37} Overtraining exists as part of a training continuum.^{36,38} Athletes often undergo periods of intensified training (overload) in order to induce functional overreach (FOR), a short-term state of overtraining, in which performance decrements typically last less than two weeks.³⁶ This performance decrement may also be accompanied by ancillary psychological or physiological symptoms including mood disturbances and upper respiratory illness.³⁴⁻³⁶ Notably, inducing a state of FOR is often thought of as *necessary* for promoting meaningful physiological adaptations and performance supercompensation.^{36,39,40} If the training-recovery imbalance is not addressed, the maladaptive overtraining response may progress beyond FOR into *non-functional* overreach (NFOR), or eventually, overtraining syndrome (OTS). Compared to FOR, NFOR and OTS can be considered pathological states, whereby overtraining symptoms may linger for months or years, respectively.³⁶

Markers and Symptoms of Overtraining. The hallmark symptom of overtraining is a decrease in performance.³⁶ Currently, there is no valid, reliable marker which can identify overtraining, let alone distinguish FOR from NFOR/OTS. The currently accepted method to differentiate FOR from its more pathological counterparts, NFOR and OTS, is to monitor the time required for performance deficits and other symptoms to resolve.³⁹ Common symptoms of overtraining

reported across the overtraining spectrum include impaired mood states,^{36,41} increased respiratory illness,⁴² and sleep disturbances.⁴³ While it is generally believed that the ancillary symptoms of overtraining worsen as an individual progresses beyond FOR toward NFOR and OTS, there is no scientific evidence to confirm this belief.³⁶

Prevalence of Overtraining. Survey research examining overtraining prevalence has suggested that between 20-60% of adult athletes may experience overtraining at least once throughout their careers.^{44–46} Few studies have examined overtraining in youth athletes, but prevalence estimates in the few that have are similar to that of adults.^{47,48} The majority of overtraining research has focused on elite-level, predominately male athletes,^{49–64} though the limited number of overtraining studies which have included female athletes suggest a similar prevalence rate between sexes.^{45,46,65–68}

It is extremely rare to observe athletes while they are actively experiencing OTS^{69–71} and it is unethical to intentionally induce OTS.³⁶ As such, researchers typically observe athletes during training camps,^{58,72–75} extreme athletic events,^{76–80} or induce a state of overreach (OR) using intensified training periods in order to observe the progression of overtraining.^{81–85} The prevalence of OR observed in studies which used intensified training protocols (overload) to observe overtraining progression ranges from 20% to 70%.^{83–87}

Pathophysiology of Overtraining– Previous Hypotheses. The pathophysiological mechanisms of overtraining are unclear. Numerous subjective and objective markers have been suggested in

previous research. Alongside these potential markers, multiple theories have been proposed and investigated during attempts to elucidate the etiological mechanisms of overtraining.^{36,88} Previous hypotheses include central fatigue, excessive oxidative stress, glycogen depletion, autonomic nervous system dysfunction, immune system dysfunction, and hormonal dysfunction pertaining to the hypothalamic-pituitary-adrenal (HPA-axis) and hypothalamic-pituitary-gonadal axes (for a detailed review of these hypotheses, see Kreher and Schwartz⁸⁸). Each of these theories has merit and provides biological plausibility to the decreased performance (fatigue) observed with overtraining. However, none of these theories have accounted for the large inter-individual variation in symptoms associated with the overtraining response. Previous attempts (i.e., systematic reviews, meta-analyses) to consolidate the evidence surrounding potential markers of overtraining have repeatedly found mixed, often conflicting, results regarding hormonal responses,^{37,89,90} autonomic HR measures,^{91–93} and immune system-related responses.⁸⁹ For instance, Cadegiani & Kater³⁷ conducted a systematic review regarding the hormonal responses linked to overtraining. Of the studies included in this review, multiple hormones were found to exhibit a blunted, decreased, or normal response to intensified training (i.e., inducing a state of overreach).³⁷ Moreover, these authors, along with other reviews of overtraining literature, have repeatedly highlighted inconsistent methodologies among overtraining studies which have contributed to inconsistent findings. These issues include performance testing heterogeneity, failure to measure or report performance changes, small sample sizes, and a lack of consistent cut-off values in the biomarkers used to distinguish FOR from NFOR or OTS^{36,37,39,94,95}

Summary of the Gaps in Overtraining research. In more than 40 years of focused research, little progress has been made towards uncovering the pathophysiological mechanisms of overtraining. The majority of overtraining studies have focused on high-level, predominately male athletes.^{49–64} Additionally, investigations into the theoretical mechanisms of overtraining pathophysiology have resulted in mixed and often conflicting results, which is partially attributed to methodological differences among overtraining studies. Thus, besides a prolonged and unexplainable decrease in performance, researchers, coaches, and athletes alike are left with few reliable tools to detect the presence of overtraining, let alone distinguish FOR from NFOR/OTS. As such, overtraining is a diagnosis of exclusion, made after disease pathologies which could explain the decreased performance and ancillary symptoms have been ruled out.^{36,37,94}

Thus, there is a need for well-controlled, prospective investigations to elucidate the pathophysiological mechanisms of overtraining. Furthermore, there is a need for a unifying framework to better understand the highly variable symptoms reported among overtrained individuals, as well as the highly variable prevalence of overtraining found in studies which have induced short-term OR. At the elite level, training volumes in endurance athletes often exceed 30h/week.^{96–98} As such, prospective cohort studies aimed at inducing OR in elite athletes under laboratory conditions are impractical. However, training adaptations are influenced by age, genetics, sex, diet, and the training history of the individual,^{29–31} and have been found to differ in moderately trained compared with elite athletes.⁹⁹ Incidentally, few studies have explored the progression of overtraining in recreationally active^{82,100,101} or sedentary populations.¹⁰² Thus, it may be feasible to observe the progression of overtraining using a standardized training protocol

under laboratory conditions in moderately trained or untrained individuals unaccustomed to high-intensity training.

Accordingly, the central hypothesis of this dissertation is that overtraining is not a condition exclusive to high-level athletes. Therefore, it may be feasible to observe the overtraining response under laboratory conditions in moderately fit populations. To test this hypothesis, this study employed a three-week high-intensity training protocol designed to induce symptoms of OR in recreationally active adults under laboratory conditions, compared to a control condition. We hypothesized that a subset of training subjects (TR) would exhibit signs and symptoms of OR, as evidenced by decreased performance and increased mood disturbance. This would then allow us to identify potential subjective and objective markers of overtraining, by comparing changes in physiological markers among healthy controls (CON), OR subjects, and non-overreached subjects (adapted, [AD]) who did not exhibit signs of overtraining following the prescribed training protocol.

Introduction to Hormesis Theory. Previous theories of overtraining mechanisms have failed to fully explain both the variable prevalence of overtraining following intensified training and heterogeneous, sometimes paradoxical, physiological responses observed in overtrained athletes. Hormesis theory offers the potential to explain why exercise can both improve health outcomes (as promoted by the current physical activity guidelines^{4,12} and the *Exercise is Medicine*² initiative) as well as lead to a maladaptive training response (overtraining).

Hormesis refers to a process in which a low ‘dose’ of a chemical agent or environmental factor, that is damaging at high doses, induces an adaptive and beneficial response on a cell or

organism.¹⁰³ Simply put, the term hormesis encompasses the concept of a ‘J’-shaped dose-response to a stressor (i.e., exercise). Originally, the concept of hormesis was first proposed in the 16th century by Paracelsus who stated, *sola dosis facit venenum*, or “the dose makes the poison.” The term hormesis itself was largely confined to the field of toxicology until the early 2000s following the seminal paper *Defining Hormesis*¹⁰⁴ by Calabrese and Baldwin. Since then, usage of the term has rapidly expanded and examples of hormesis theory have been found throughout multiple fields of science and medicine.¹⁰⁵ Notably, terms beyond toxicology that describe a hormetic response include: a U-shaped dose-response, Arndt-Schulz Law, biphasic dose response, preconditioning/adaptive response, overcompensation responses, rebound effect, repeat bout effect, and steeling effect, among others.¹⁰⁵

Exercise and Hormesis Theory. The term hormesis was originally introduced into the exercise physiology lexicon in 2005.¹⁰⁶ As such, hormesis is relatively new to exercise science when considering that for nearly two centuries, lactate was erroneously viewed as a metabolic waste product (until the efforts of George Brooks in the 1980s, among others).^{26,107,108} The concepts of hormesis theory closely align with the core principles of exercise training and the adaptive response (**Table 1.1**). Additionally, the concepts of conditioning and adaptive responses of hormesis theory can be reflected in the early works of Hans Selye,¹⁰⁹ who later proposed General Adaptation Syndrome—a tenet of the exercise training response.^{110–112} In *A Syndrome Produced by Diverse Nocuous Agents* (1936), Selye wrote:

Experiments on rats show that if the organism is severely damaged by acute non-specific nocuous agents such as...excessive muscular exercise...a typical syndrome appears, the

symptoms of which are independent of the nature of the damaging agent or the pharmacological type of the drug employed, and represent rather a response to damage as such. This syndrome develops in three stages...If the treatment be continued with relatively small doses of the drug or relatively slight injuries, the animals will build up such resistance that in the later part of the second stage...but with further continued treatment, after a period of one to three months (depending on the severity of the damaging agent) the animals lose their resistance and succumb with symptoms similar to those seen in the first stage, this phase of exhaustion being regarded as the third stage of the syndrome...We consider the first stage to be the expression of a general alarm of the organism...and therefore term it the 'general alarm reaction'. Since the syndrome as a whole seems to represent a generalized effort of the organism to adapt itself to new conditions, it might be termed the 'general adaptation syndrome'. It might be compared to other general defence [*sic*] reactions such as inflammation or the formation of immune bodies...It seems to us that more or less pronounced forms of this three-stage reaction represent the usual response of the organism to stimuli such as temperature changes, drugs, muscular exercise, etc., to which habituation or inurement can occur.¹⁰⁹

The original argument to suggest that exercise-induced oxidative stress produced a hormetic response was as follows:¹⁰⁶ 1) Cardiorespiratory fitness is a strong independent predictor of all-cause mortality and chronic disease,¹¹³ and it is well-established that regular exercise has a protective effect against chronic illnesses.⁴ 2) Reactive oxygen species (ROS) are linked to a number of chronic diseases including atherosclerosis, cancer, ischemia, inflammation, and neurodegenerative diseases.¹¹⁴ 3) Exercise at a certain intensity or duration increases the production of ROS.^{115,116} 4) The oxidative cellular milieu generated by exercise results in the

activation of endogenous antioxidant systems,¹¹⁷ stimulates the oxidative damage-repair system,^{118–121} increases the proteasome complex,¹²² and upregulates enzymatic activities to promote DNA repair.^{119–121,123} As such, an oxidative stress which produces ROS would stimulate physiological adaptations including the upregulation of antioxidant systems, promotion of damaged cell turnover, and promotion of DNA repair. These physiological adaptations, in turn, would provide a protective effect against future oxidative stress. Thus, to explain the exercise training response through the lens of hormesis theory, an individual engaging in regular exercise (stressor) at an appropriate dose (duration, intensity, training volume etc.) will experience an adaptive physiological (pre-conditioning) response specific to the training program undertaken (dose-response). However, if an individual engages in acute or chronic physical activity beyond the tolerance of their biological systems (toxic dose), they may experience an adverse response to the exercise stimulus (overtraining or injury).

Besides oxidative stress, exercise can challenge the body's homeostatic mechanisms through multiple inflammatory mediators such as thermal, metabolic, hypoxic, and mechanical stressors. As a result of these stressors, multiple biochemical messengers are released (e.g., growth factors, cytokines), which then activate various signaling pathways, protein kinases, phosphatases, and deacetylases, which in turn regulate the molecular machinery controlling gene expression to elicit an adaptive response.¹²⁴ Thus, if the exercise dose is appropriate, meaningful adaptations to training such as mitochondrial biogenesis or hypertrophy can occur.^{125–127} However, excessive ROS and other inflammatory mediators from prolonged or excessive exercise could instead overwhelm the signaling pathways and endogenous defense mechanisms,

resulting in an attenuation of the training response^{83,85,128} or an adverse training response.^{125,129–}

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In summary, the ‘J’-shaped relationship observed between physical activity and health outcomes is a hormetic response, whereby both low and excessive levels of physical activity (sedentarism and overtraining, respectively) are associated with an adverse physiological response, whereas moderate levels of physical activity can generate a beneficial adaptive response and promote longevity. Finally, it is worth noting that exercise-based hormesis literature has already claimed that overtraining is the upper endpoint of exercise-induced hormesis,^{106,133–137} however, the term hormesis is absent in overtraining-specific literature. As such, exercise-induced hormesis as a theory to describe the pathophysiology of overtraining has yet to be embraced, or even acknowledged, in contemporary overtraining research.

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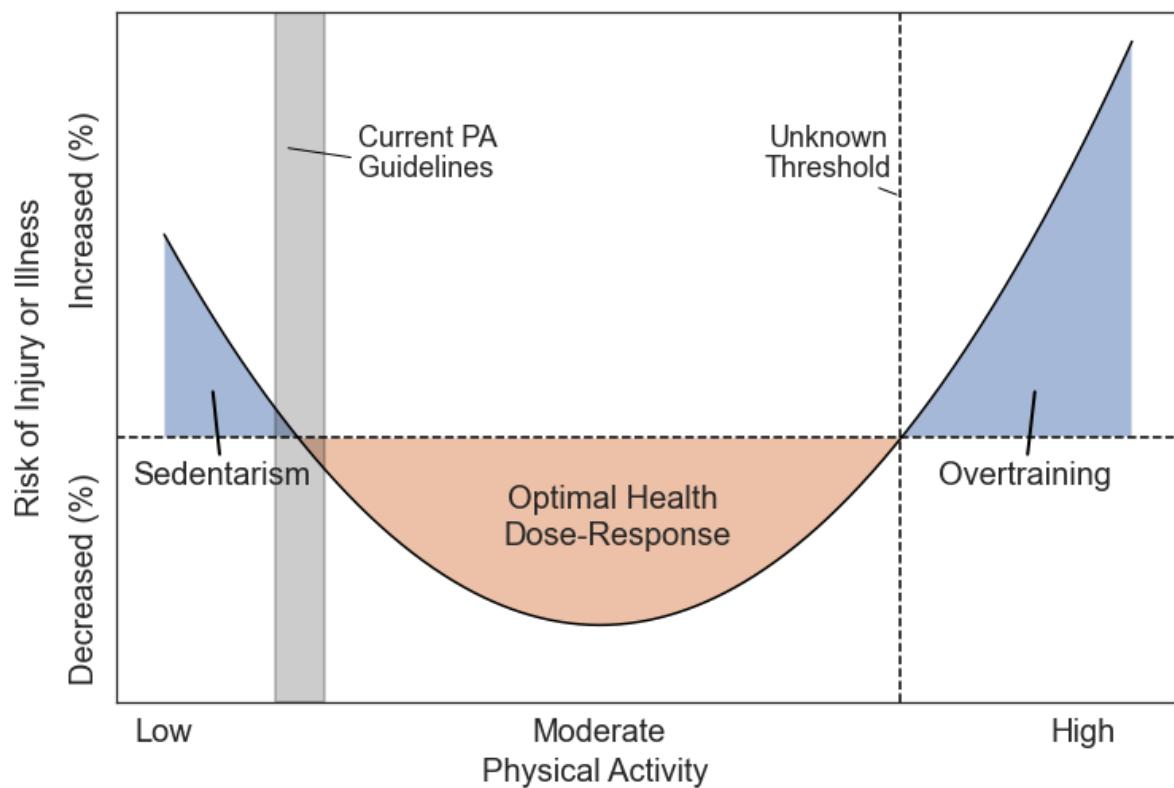
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Table 1.1. Related concepts in hormesis and exercise literature.

Hormetic Term/Concept	Basic Definition	Related Exercise Science Concept	Example in Exercise
Hormesis 'J'-shaped dose-response	Low doses of a stressful stimuli generate an adaptive, protective response; high (toxic) doses of a stimuli can inhibit or harm an organism's homeostatic mechanisms.	Relationship between Physical Activity and associated health outcomes appear to follow a J-shaped dose-response. ^{34,138}	Low levels of physical activity are linked to chronic illness; ⁴ moderate levels of physical activity have shown to promote health and longevity; excessive exercise can lead to overtraining. ³⁶
Pre-conditioning (adaptive response)	A titrated exposure to a stressor at an appropriate dose will generate an adaptive response to protect against similar doses of the stressor in the future.	FITT Principle (Frequency, Intensity, Type, Time); SAID principle (Specific Adaptations to Imposed Demands); Progressive Overload; ²⁸ General Adaptation Syndrome. ¹¹⁰	Engaging in a running training program to train for a marathon. Increasing mileage and pace overtime will result in better tolerance to running distances and speeds; Aerobic training over time can improve aerobic capacity. ¹³⁹
Post-conditioning	Exposure to stressor following damaging event will lead to future protective adaptive response.	Exercise is used as a therapeutic modality following injury or surgery.	Physical therapy rehabilitation after tearing an ACL to regain quadriceps function.
Remote conditioning	Hormetic signals emanating from a tissue under stress can communicate to distant tissues.	Physiological response to exercise is systemic.	Interleukin-6 and Lactate generated from muscles during exercise will perfuse through the body, acting as signaling molecules to various tissues beyond muscle. ^{140,141}
Early- and late-phase hormetic responses	Some adaptive responses to hormesis are observed acutely following exposure to stressor, whereas other adaptations appear delayed.	There are both acute and chronic physiological responses to exercise, which may provide a protective mechanism.	An acute exercise bout can immediately improve cognitive function. ¹⁴² "Repeat-bout effect" in which a single bout of eccentric exercise confers a protective effect against future bouts of exercise-

			induced muscle damage. ₁₄₃
Hormesis terms in this table are summarized from Calabrese and Mattson. ¹⁰⁵			

Figure 1.1. Relationship between physical activity and Injury/Illness risk.



CHAPTER TWO: RECREATIONALLY ACTIVE ADULTS EXHIBIT SYMPTOMS OF OVERTRAINING FOLLOWING A THREE-WEEK LAB-CONTROLLED OVERREACHING TRAINING PROTOCOL

Introduction.

Optimizing training adaptations requires a meticulous balance between training stimuli and recovery. Athletes commonly undergo periods of intensified training with the intention of improving athletic performance by inducing a physiological response known as functional overreach (FOR). Inducing FOR is generally thought to be necessary for promoting meaningful physiological adaptations and performance supercompensation.¹⁻³ However, when training demands are persistently met with insufficient recovery, athletes may experience a more prolonged maladaptive response known as *non-functional* overreach (NFOR),¹ resulting in performance decrements lasting several weeks to months. Eventually, if the training-recovery imbalance is not addressed, this maladaptive training response may escalate beyond NFOR to manifest as overtraining syndrome (OTS).^{3,4} Collectively, FOR, NFOR, and OTS comprise the training-overtraining continuum.^{1,5}

The requisite symptom of overtraining is an unexplained decrease in performance, though overtrained athletes often report secondary symptoms including general fatigue, sleep disturbances, reduced training motivation, and mood disturbances.^{1,3} It is generally believed that the ancillary symptoms associated with overtraining, such as mood disturbances, worsen as an individual progresses beyond FOR and approaches OTS; however, there is no scientific evidence to confirm this belief.¹ The currently accepted method to differentiate FOR from its more pathological counterparts, NFOR and OTS, is to monitor the time required for performance deficits and other symptoms to resolve.³ However, a period of complete rest is often rejected by both coaches and athletes for concerns that prolonged rest may

lead to detraining.³ Moreover, this approach for monitoring overtraining fails to *prevent* training maladaptation. As currently defined in overtraining literature,¹ the time-course of recovery distinguishing the different overtraining substates is vague, imprecise, and lacks connection to causal mechanisms or physiological markers which may be objectively monitored.

Survey research examining overtraining prevalence has suggested that between 20-60% of athletes may experience overtraining at least once throughout their careers.⁶⁻⁸ Unfortunately, the pathophysiological mechanism(s) of overtraining are unclear, and sensitive objective diagnostic criteria that can reliably detect overtraining substates have yet to be validated.^{1,9} Researchers typically observe athletes during training camps,¹⁰⁻¹⁴ extreme athletic events,¹⁵⁻¹⁹ or intensified training periods in order to observe the progression of overtraining.^{2,20-23} While FOR, NFOR, and OTS are considered to exist on a continuum, it is extremely rare to observe athletes while they are actively experiencing OTS,²⁴⁻²⁶ and it is unethical to intentionally induce OTS.¹ Therefore, studies with training protocols designed to induce overtraining solely focus on inducing FOR/NFOR, collectively termed overreach (OR). When investigating potential physiological or biochemical mechanisms of overtraining, researchers have often focused on autonomic function,²⁷⁻³² immune system function,³³⁻³⁵ or hormonal status that is primarily related to the hypothalamic-pituitary-adrenal (HPA) axis.³⁶⁻³⁹ However, the observed responses among subjects classified as overtrained have not been consistent throughout the overtraining literature.^{40,41} Reviews have underscored several design and methodological issues among overtraining studies that contribute to the inconsistent findings in the literature. These issues include performance testing heterogeneity, failure to measure or report performance changes, small sample sizes, and a lack of cut-off values for biomarkers which would distinguish FOR from NFOR or OTS.^{1,3,40,42,43} Furthermore, the majority of overtraining research has concentrated on elite, predominately male athletes.^{13,28,29,37,44-55} Confining the focus of overtraining research to such an exclusive demographic limits the applicability of findings to broader populations.

There is a need for well-controlled, prospective investigations to elucidate the pathophysiological mechanisms of overtraining. At the elite level, training volumes in endurance athletes often exceed 30h/week.⁵⁶⁻⁵⁸ As such, prospective cohort studies aimed at inducing OR in elite athletes under laboratory conditions are impractical. However, training adaptations are influenced by age, genetics, sex, diet, and the training history of the individual,⁵⁹⁻⁶¹ and have been found to differ in moderately trained and elite athletes.⁶² Incidentally, few studies have explored the progression of overtraining in recreationally active^{20,63,64} or sedentary populations.⁶⁵ Thus, it may be feasible to observe the progression of overtraining using a standardized training protocol under laboratory conditions in moderately trained or untrained individuals unaccustomed to high-intensity training.

Accordingly, this study employed a three-week high-intensity training protocol designed to induce symptoms of OR in recreationally active adults under laboratory conditions. We hypothesized that a subset of subjects randomized to a training group (TR) would exhibit signs and symptoms of OR as evidenced by decreased performance and increased mood disturbance, compared to subjects in a control group (CON). This would then allow us to identify potential biomarkers of overtraining by comparing changes in physiological markers among CON, OR, and non-overreached (adapted, [AD]) subjects who did not exhibit signs of overtraining following the prescribed training protocol.

Materials and Methods.

Participants. Twenty-four recreationally active adults (19 females, 5 males) volunteered to participate in this study. Upon recruitment, subjects were randomly assigned to TR (n=12) or CON (n=12). It was confirmed during recruitment that participants were unaccustomed to cycling training to ensure the training protocol would be a novel training stimulus. Three subjects (2 CON, 1 TR; 3 females) withdrew from the study due to scheduling conflicts. These subjects' data were excluded, and the final sample size

was n=21 (10 CON, 11 TR). Female subjects started the study at random time points in their menstrual cycle so that hormonal status would not affect the outcomes. The study was approved by the Washington State University (WSU) Institutional Review Board (#18860) and was conducted in accordance with the Declaration of Helsinki. Before study participation, subjects completed health screening questionnaires, lung function testing, and exercise performance testing during an initial intake visit to assess overall health and establish baseline measures.

Experimental Design. A diagram of the overall study design is shown in **Figure 2.1**. TR subjects underwent a three-week high-intensity training protocol (**Table 2.1**) exercising six days per week under laboratory conditions. This protocol was designed to induce a state of OR. All training sessions were performed in the exercise physiology research lab at the WSU-Health Sciences Spokane campus and consisted of a mix of long-duration, interval, and sprint-like training sessions using a cycle ergometer. Workloads for all training sessions were calculated as a percentage of each individual's peak workload (PWL) achieved during weekly performance testing. TR subjects completed a total of seven performance tests: one per week for six consecutive weeks and one test at 48-hours after the three-week training protocol. CON subjects attended the lab once each week for a total of six consecutive weeks, where they completed the same weekly performance testing procedures as TR subjects. CON participants were instructed to maintain their normal sleep and dietary habits for the duration of observation and were allowed to maintain their habitual exercise routines during the study.

Measurements.

Performance Testing Procedures and Peak Oxygen Uptake ($\dot{V}O_{2\text{peak}}$). All participants underwent weekly performance testing using a magnetically braked cycle ergometer (Ergoselect 200, Ergoline GmbH, Bitz, Germany) to determine $\dot{V}O_{2\text{peak}}$ and cycling performance PWL. To mitigate the risk of a

learning effect in the maximal incremental exercise test, an initial performance test was performed during an intake visit. The second performance test at the onset of the training phase established the baseline for measuring changes in outcome measures during all subsequent weekly performance tests. For all lab visits, subjects wore a face mask (Hans Rudolph, Kansas City, MO, USA) for exhaled breath collection. Expired oxygen and carbon dioxide concentrations were continuously measured breath-by-breath (ParvoMedics TrueOne 2400, Salt Lake City, UT, USA) and recorded in 15 second averages.

The performance test was a graded exercise test (GXT) until volitional exhaustion. Warm-up included cycling for five minutes at 25% PWL from the baseline performance test performed during intake screening. The GXT began immediately following warm-up with starting workload set at 75W (females) or 100W (males). Workload increased every two minutes by 30W (females) or 45W (males) until volitional exhaustion, or until participants were unable to maintain a cadence greater than 60rpm. Subjects then proceeded through a five-minute cooldown using the same power output as the warm-up. After a five-minute intermission off the bike, participants completed a second performance test at a constant, supramaximal workload to confirm $\dot{V}O_{2\text{peak}}$, as suggested by Pool and Jones.⁶⁶ The second round included a two-minute warm-up and a constant workload time-to-exhaustion cycling test. Resistance was set at 110% of each participant's PWL achieved during the GXT.

Maximal effort was determined by observing a plateau in the $\dot{V}O_{2\text{peak}}$ data. If a plateau in $\dot{V}O_2$ was not observed during the GXT, maximal effort was determined by identifying at least two secondary criteria commonly used to determine maximal effort.⁶⁷ Secondary criteria included reaching a respiratory exchange ratio of at least 1.10, a peak heart rate over 90% of the age-predicted maximal heart rate (MHR), or a peak lactate (LA^-_{peak}) of at least 8 mmol/L. If a participant did not meet two of these three criteria, maximal effort was considered achieved if a participant's $\dot{V}O_{2\text{peak}}$ from the two exercise rounds differed by less than 0.15 L.

Body Composition. Body composition was measured using the BodPod system (Life Measurement, Concord, CA, USA) before exercise during every performance testing visit. Each participant underwent body composition measures and performance testing at the same time of day throughout the study; however, these times were not uniform among all participants. Some participants were tested in the morning while others in the afternoon. As such, all participants were instructed to attend body composition and performance testing visits under the same post-prandial or fasted conditions as their initial performance test for all subsequent lab visits.

Heart Rate Measures. During all exercise sessions, HR was continuously monitored using a Bluetooth chest strap (Polar, Polar Electro Oy, Kempele, Finland) and recorded in 15 second averages. MHR was determined as the highest 15s average recorded at the end of performance testing. Heart rate recovery (HRR) was defined as the decline in HR from MHR in the first minute after the cessation of exercise.

Profile of Mood States. Weekly, on the day of performance testing, participants completed a short-form version of the Profile of Mood States (POMS).⁶⁸ The original POMS⁶⁹ is a 65-item Likert scale questionnaire that assesses vigor, depression, fatigue, anger, anxiety, and confusion. The short-form POMS consists of 37 items from the original POMS. Survey data were collected and managed using REDCap electronic data capture tools hosted at Washington State University.^{70,71}

Metabolic Equivalents of Task (MET-min) per week. To assess the volume of physical activity in the training protocol, metabolic equivalents of task (MET-min) were calculated as follows:

$$\text{MET-min} = \left(\sum \frac{\text{V' O}_2 \text{ during exercise}}{\text{resting V' O}_2} \right) \times \text{exercise duration (minutes)}$$

For all TR subjects, MET-min per week was calculated by summing the calculated MET-min for all exercise sessions throughout each week of the three weeks during the training protocol. During all exercise training sessions, breath-by-breath gas exchange was collected using a facemask (Hans Rudolph, Kansas City, MO, USA) and metabolic cart (TrueOne 2400, ParvoMedics) and recorded in 15 second averages.

Lactate. During performance testing, blood lactate concentration (LA^-) was measured via fingerprick during the last 30 seconds of each cycling stage using a lactate analyzer (Lactate Plus Meter, Nova Biomedical, Waltham, MA). $\text{LA}_{\text{Peak}}^-$ was collected two minutes after the cessation of exercise.

Assessment of Overreaching. Following the training protocol, TR participants were subdivided into AD and OR subgroups according to their performance response during the recovery phase. In line with previous research,^{21,23,72,73} the smallest worthwhile change (SWC) was used to determine a performance threshold to indicate OR (OR threshold). SWC was calculated as 0.3 times the coefficient of variation (CV) for PWL from all performance tests completed by CON subjects during the study. TR subjects who exhibited a decrease in PWL beyond the OR threshold during at least one performance test in the recovery phase, accompanied by an overall increase in POMS, were classified as OR. The remaining subjects in the TR group who maintained or increased performance after training were considered adapted (AD).

Statistical Analysis. Data analysis was conducted using Scikit-learn (1.3.2),⁷⁴ Statsmodels (0.13.2),⁷⁵ SciPy (11.1.4),⁷⁶ and Pingouin (0.5.4)⁷⁷ libraries in Python. Levene's test was used to assess homogeneity of variance and QQ plots and residual plots were inspected to determine if data was normally distributed

among groups. Baseline characteristics between CON and TR groups were compared using independent samples T-tests. Depending on the distribution of the data, Pearson or Spearman correlation coefficients were used to evaluate selected relationships among variables in CON and TR subjects. Mixed-effects regression models were employed to investigate the impact of group status (CON, AD, OR) and time (training and recovery phase) on outcome measures. Mixed-effects regression models, compared to traditional repeat-measures analysis of variance (ANOVA), are more appropriate for handling longitudinal measures when there is a specific temporal arrangement (i.e., order and intervals).⁷⁸ Moreover, these models are well-suited to handle unequal sample sizes and support empirical Bayes estimation of individual subject parameters (random effects).⁷⁹

The following linear mixed-effect model was fitted to the data for each outcome measure Y :

$$Y_t \sim \mathbf{b} \times t + \mathbf{a}$$

Where t denotes time (phase). The b coefficient represents the fixed effect of the predictor variables (i.e., group and phase) and describes the directional change in the outcome measure (Y) from baseline across time. The a parameter represents the random intercept, accounting for variability in the outcome measures specific to the reference group (i.e., CON group) or individual variability (random effects) in the outcome measure. A single model was fitted for all outcome measures. F-tests were performed to assess the significance of regression model effects and interactions ($\alpha \leq 0.05$). For outcomes with significant F values, Tukey's Honest Significant Difference (HSD) post hoc tests were conducted to compare group differences between time points. Regression model fixed effects (β) and p-values are reported in text. All other data presented in text are reported as mean \pm standard deviation (SD), unless otherwise specified.

Missing Data. Instances of missing data were imputed using multiple imputation chained equations (MICE).⁸⁰ Multiple imputation is considered a modern and principled technique for dealing with missing

data, which considers the circumstances surrounding missing data. MICE can provide more reliable model estimates than listwise or pairwise deletion.⁸¹ As outlined in Azur et al.,⁸² MICE assumes that missing data are missing at random and the chained equation process can be broken down into four steps: 1) single imputations (e.g., mean) are performed for any missing value, which can be thought of as “place holder” values. 2) Next, “place holder” imputations are set back to missing, one variable “var” at a time. 3) The non-missing values in “var” are treated as the dependent variable in a regression model and other variables in the dataset act as independent predictor variables. 4) Lastly, missing values in “var” are replaced with the imputed data from the regression model. The process continues through iterative cycles, sequentially imputing missing data in other variables using a combination of previously imputed and observed values. This sequence repeats steps two through four in multiple cycles, aiming for convergence. Convergence indicates stability in the imputed model, ensuring its accuracy in predicting both observed and imputed values.⁸²

Results.

Group characteristics at baseline are shown in **Table 2.2**; there were no group differences. Both body mass and body composition (i.e., percent body fat) remained consistent among all three groups throughout the study ($P>0.05$). Weekly averages for all outcome measures discussed are shown in **Table 2.3**.

Training Volume and Prevalence of Overreaching. On average, the TR group performed 1631 ± 351 , 1835 ± 368 , and 2020 ± 432 MET-min of exercise during each of the three weeks of training, respectively (**Figure 2.2**). The average PWL for all performance tests among CON subjects was 222.13 ± 7.44 W (CV=3%), which set the OR threshold to a 1.00% (0.3*CV) decline in performance from baseline. Following the training protocol, three female TR subjects (27%) exhibited a decline in performance

beyond the OR threshold and were subsequently classified as OR. Subsequently, TR subjects not exhibiting performance decrements were classified as AD (73%). The average maximal decrease in PWL in OR subjects was $-10.3 \pm 5.4\%$ from baseline values, though the largest individual decreases in performance occurred at different timepoints. Compared to CON subjects, PWL in OR subjects decreased from baseline during both the training ($b=-0.098$, $P<0.001$) and recovery phases ($b=-0.089$, $P=0.002$; **Figure 2.3A**). At the end of the recovery phase, performance in OR subjects had returned to baseline levels ($-0.19\% \pm 4.10\%$), while PWL in CON and AD subjects improved $7.7\% \pm 6.6\%$ and $7.3\% \pm 6.1\%$ from baseline, respectively. The pooled average of POMS scores across all visits were higher in OR compared to CON subjects (Welch's ANOVA $F(1,50.28)=10.31$, $P<0.001$, $h^2=0.188$; Games-Howell, adjusted $P<0.001$). However, there were no significant group differences in POMS scores during any specific week or phase ($P>0.05$). OR subjects exhibited a non-significant increase in POMS scores across the training phase with peak POMS scores in each OR subject occurring during the recovery phase, albeit at different timepoints ($\text{POMS}_{\text{Baseline}}=18.3 \pm 22.4$, $\text{POMS}_{\text{Peak}}=42.3 \pm 16.5$; **Figure 2.3B**). In contrast, POMS scores in CON and AD groups remained near baseline values throughout the study (CON $\text{POMS}_{\text{baseline}}=3.3 \pm 15.7$, $\text{POMS}_{\text{peak}}=13.2 \pm 12.3$; AD $\text{POMS}_{\text{baseline}}=8.9 \pm 12.3$, $\text{POMS}_{\text{peak}}=17.1 \pm 19.2$, $P>0.05$).

Physiological Measures.

Peak Aerobic Capacity. A main effect of time was observed for aerobic capacity indicating that $\dot{\text{V}}\text{O}_{2\text{Peak}}$ increased from baseline values during the recovery phase in all groups ($b= 0.05$, $P=0.011$; **Figure 2.4A-B**). At the end of the recovery phase, $\dot{\text{V}}\text{O}_{2\text{Peak}}$ in AD and OR subjects had improved $5.9\% \pm 10.5\%$ and $6.4\% \pm 10.2\%$ from baseline, respectively (AD $\dot{\text{V}}\text{O}_{2\text{Peak}} 37.67 \pm 6.11$ to 39.78 ± 6.83 ml/kg/min; OR $\dot{\text{V}}\text{O}_{2\text{Peak}} 32.23 \pm 5.52$ to 34.06 ± 4.87 ml/kg/min). Similarly, by the end of the recovery phase, $\dot{\text{V}}\text{O}_{2\text{Peak}}$ had improved $5.6\% \pm 6.4\%$ in CON subjects (39.77 ± 7.35 to 41.66 ± 9.29 ml/kg/min).

Blood Lactate Measures. LA_{Peak} in CON subjects remained near baseline levels (9.38 ± 1.72 mmol/L) throughout the study. From baseline, LA_{Peak} decreased in OR subjects throughout both the training ($b=-0.253$, $P=0.012$) and recovery phases ($b=-0.322$, $P=0.001$; **Figure 2.4C-D**), with the greatest reduction occurring 48-hours post-training ($-33.6\% \pm 15.1\%$, 8.80 ± 1.47 to 5.86 ± 1.69 mmol/L). Similarly, LA_{Peak} in AD subjects decreased from baseline during both the training ($b=-0.145$, $P=0.049$) and recovery phases ($b=-1.168$, $P=0.043$), with the greatest reduction occurring 48-hours post-training ($-12.7\% \pm 16.1\%$, 11.19 ± 1.26 to 9.79 ± 2.22 mmol/L).

Heart Rate Measures. Throughout the study, MHR remained near baseline levels in both CON and AD groups (184 ± 6.9 bpm and 181 ± 8.8 bpm, respectively). Average MHR declined in OR subjects during both the training ($b=-0.064$, $P<0.001$) and recovery phases ($b=-0.037$, $P=0.028$; **Figure 2.5A-B**), with the greatest reduction occurring 48-hours post-training ($-8.5\% \pm 6.4\%$, 180 ± 7.9 bpm to 164 ± 4.6 bpm). A main effect of time was observed for HRR indicating that it decreased in all groups during the training phase from baseline ($b=0.0133$, $P=0.039$). During the recovery phase, a group-time interaction was observed indicating that HRR increased in OR subjects from baseline ($b=0.251$, $P=0.040$; **Figure 2.5C-D**); however, pairwise comparisons did not detect specific group differences.

Missing Data. There were 137 possible observations of each outcome measure for all performance testing visits among all subjects (10 Con, 11 TR) and 198 possible observations of MET-min outcome measures (11 TR subjects, 18 training sessions each). Missing data were assumed to be missing at random and included two POMS surveys, and two body composition, two LA_{Peak}, one MHR, and eight HRR measures. One missing LA_{Peak} measure was due to a lactate analyzer malfunction, whereas the other was

due to human error. Missing heart rate metrics were partly the result of a malfunctioning chest strap HR sensor. Additionally, HRR data were not collected in one TR and one CON subject. Consequently, these two subjects were removed from HRR analysis. All other instances of missing data were imputed using the described methods (MICE).

Discussion.

The purpose of this study was to investigate whether overtraining could be induced in recreationally active adults, evidenced by decreased exercise performance and increased mood disturbances following training. Using specific criteria, we were able to identify OR subjects, and they exhibited several significant maladaptive responses during high-intensity training including: 1) a progressive decrease in LA_{peak} during training, which persisted throughout the recovery phase, and 2) a reduction in MHR during training, which then rebounded during recovery with a concomitant increase to HRR. These findings provide evidence that a standardized training protocol under laboratory conditions is an effective method to induce overreach and supports our hypothesis that moderately fit adults (i.e., non-elite athletes) can exhibit symptoms of overtraining.

After three weeks of high-intensity exercise, three TR subjects were classified as OR. Following the recovery phase, both AD and CON groups demonstrated a minor improvement in performance, whereas performance in OR subjects returned to baseline levels (**Figure 2.3**). At the end of the study, both AD and OR groups, as well as the CON group, exhibited a modest increase in aerobic capacity (~6%) from baseline. Participants were unaccustomed to cycling as a primary exercise modality, and the three-week training protocol consisted of a mixture of interval and constant-duration workouts. This resulted in TR subjects performing an average of 1,600-2,000 MET-min of exercise per week. The current Physical Activity Guidelines for Americans recommends at least 500 MET-min of physical activity (150

minutes of moderate-to-vigorous aerobic activity) per week for optimal health,⁸³ and it has been estimated that maximal cardiovascular health benefits are obtained at exercise volumes approximately three to four times these recommendations.^{84,85} As such, the modest improvements in $\dot{V}O_{2\text{Peak}}$ were less than expected considering the increases in aerobic capacity demonstrated in previous exercise intervention studies. For example, Hickson and Holloszy⁸⁶ found $\dot{V}O_{2\text{max}}$ increased 5% after one week of moderate-intensity exercise (50-60% of $\dot{V}O_{2\text{max}}$); a trend which continued for 10 weeks wherein average $\dot{V}O_{2\text{max}}$ had increased 44% (16.8 ml/kg/min) from baseline. Similarly, high-intensity interval training (HIIT) has been found to increase $\dot{V}O_{2\text{max}}$ by ~7% in as little as six HIIT sessions over five days. These studies highlight the lack of an aerobic training response found in the present study—particularly as these two studies included participants of similar fitness levels as the current investigation. Current training paradigms assert that inducing a state of FOR is *necessary* in order to achieve meaningful adaptations and performance supercompensation;¹⁻³ however, there is some evidence that refutes this notion.^{21,23} For instance, well-trained athletes classified as FOR have exhibited attenuated improvements to aerobic capacity after intensified training compared to non-overreached (acute fatigued) triathletes undergoing the same training loads.²³ Additionally, others have found impairments to both muscle oxidative capacity in high-level runners⁸⁷ and mitochondrial function in moderately-trained individuals⁸⁸ after high-volume training periods. Thus, the small increase in aerobic capacity in TR participants after three-weeks of high-intensity training provides evidence that at least a portion of TR subjects experienced an overtraining response, and supports the notion that excessive exercise can attenuate aerobic training adaptations.

In the present study, LA_{Peak} levels progressively decreased throughout the training phase in both OR and AD groups, which returned to near baseline levels during the recovery phase. A reduction in LA_{Peak} during exercise is one of the most consistent findings in athletes having OTS.^{1,42} LA^- is an important signaling molecule that can mediate exercise-induced adaptations related to mitochondrial biogenesis.⁸⁹ Previous research has demonstrated that decreased LA^- accumulation in response to repeated

administration of dichloroacetate reduced the mitochondrial adaptations to high-intensity interval training in mice.⁹⁰ In high-level runners, Bellinger et al.²¹ observed attenuated improvements to oxidative capacity in runners experiencing FOR after high-volume training, compared to acutely fatigued runners who underwent the same training volume. As in the present investigation, authors also observed reductions in LA_{Peak} during high-volume training, which led the authors to speculate that a diminished LA⁻ response with training can inhibit adaptations to mitochondrial function associated with aerobic training.²¹ However, neither study monitored mitochondrial function (i.e., mitochondrial structure, content, quality). As such, further research is needed to confirm or refute this theory. Nevertheless LA⁻ alone is not sensitive enough to solely determine overtraining status. A rightward shift of the lactate curve (lower LA⁻ at a given workload) is associated with an increase in aerobic capacity;⁹¹⁻⁹³ however, depleted glycogen stores may also produce a similar shift in LA⁻ curves,⁹⁴⁻⁹⁶ which could then incorrectly be interpreted as an adaptation from endurance training.^{97,98} It has been theorized that the reduction in LA_{Peak} observed in overtraining literature could result from reduced glycogen stores;^{99,100} however, previous research has not found evidence to support this theory.^{2,53,101} Additionally, simultaneous arterial and venous blood sampling of LA⁻ has demonstrated that endurance training further improves LA⁻ clearance rates,^{102,103} but this sampling method was beyond the purview of this study.

Progressive decreases in MHR, concomitant to the progressive decreases in LA_{Peak}, were observed in OR subjects throughout the training phase. Reductions in maximal physiological measures during incremental exercise to exhaustion may simply be the result of a reduction in exercise time and not related to abnormal physiological function *per se*. Nevertheless, reductions to both MHR and LA_{Peak} have consistently been found in athletes classified as overtrained.^{13,22,28,54,104,105} Additionally, while reduced LA⁻ levels alone are insufficient to distinguish the overtraining states from one another,¹⁰¹ concomitant decreases in MHR and LA_{Peak} have successfully been used to determine overreaching status (OR *versus* non-OR) in well-trained triathletes.²² As such, autonomic dysfunction has been theorized as a potential

mechanism of overtraining^{1,106} since the autonomic nervous system directly influences HR through sympathetic and parasympathetic modulation.¹⁰⁷ A reduced chronotropic response during exercise would inhibit cardiac output and thus, impair an individual's ability to meet the metabolic demands of working skeletal muscle. Testing this hypothesis, Le Meur et al.²⁸ assessed the cardiac response to exhaustive exercise in triathletes undergoing a three-week overload training period compared to controls undergoing normal training. The authors found that, after training, FOR athletes exhibited transient decreases to $\dot{V}O_{2\max}$ and cardiac output with concomitant decreases in epinephrine. Importantly, these physiological changes did not occur in non-OR (acutely fatigued) or control athletes and reversed after a taper period, which led researchers to conclude that the reduced cardiac output and subsequent $\dot{V}O_{2\max}$ and performance decreases were due to an adrenal insufficiency.²⁸

In addition to progressive the decreases in MHR and \bar{LA}_{Peak} observed during the training phase, OR subjects exhibited an increased post-exercise HRR response during the recovery phase, which has also been shown in athletes classified as FOR.^{29,45,108} Post-exercise HRR reflects the coordinated interaction between parasympathetic reactivation and sympathetic withdrawal,^{109,110} caused by physiological adjustments related to hemodynamics in relation to body position, blood pressure regulation, and the metaboreflex after the cessation of exercise.^{111,112} Previous studies have found HRR to be directly associated with $\dot{V}O_{2\max}$, irrespective of age,¹¹³ and have found HRR to increase after endurance training.^{114,115} Accordingly, increased vagal tone indicates an enhanced ability to return to homeostasis and is generally considered a positive training adaptation.^{50,116} Conversely, a diminished post-exercise HRR response is a strong predictor of both cardiovascular^{117,118} and all-cause mortality,¹¹⁹ and acute increases to training load have shown to have a dampening effect on HRR.¹²⁰ As such, overtraining can cause a paradoxical response of parasympathetic HR indices. It is plausible that the increase in HRR observed in OR participants in the present study is indicative of a positive training adaptation; however, this physiological response was not reciprocated in AD subjects who had similar

reductions in LA_{peak} yet no overt reductions to MHR during the training protocol. Furthermore, at the end of the study, both groups showed similar improvements in aerobic capacity despite these differences in physiological responses and performance during the training protocol. Specific measures of the sympathetic and parasympathetic indices during exercise (e.g., catecholamines) could elucidate whether the training responses observed were due to sympathetic withdrawal or parasympathetic hyperactivity;^{13,105,116,121} however, the resources to measure these indices were not available during this study. Nevertheless, the progressive decreases in MHR and LA_{peak} suggest that OR participants exhibited physiological responses consistent with impaired autonomic cardiac responses during training and exhibited improvements to HRR during recovery whereas AD subjects did not experience a similar increase in HRR. Thus, the findings of this study provide some evidence that autonomic dysfunction may be an underlying mechanism of overtraining and warrants further investigation. Accordingly, future studies investigating the role of the autonomic nervous system regarding the overtraining response should seek to measure both sympathetic and parasympathetic branches.

Limitations.

This study has several limitations. Three TR participants were classified as OR after the training protocol. With a larger sample size, more participants would likely be classified as OR after training, thus improving the applicability of this study's findings to the broader population. Nonetheless, post-hoc power analysis of our mixed-effect models did indicate that a portion of outcome measures discussed were adequately powered ($B \geq 0.8$) (**Table S2.3**). Participants were randomized upon recruitment; however, the sample was dominated by female volunteers and only one male was randomized into the TR group, which prevented us from comparing differences in study outcomes between sexes. Previous overtraining studies have employed overload training protocols which typically last up to four weeks.²⁰⁻²³ It is possible more TR participants would have demonstrated symptoms of overtraining In the present

study had the training protocol gone longer; however, we refrained from a longer training protocol to avoid concerns of causing ‘undue harm’ to study participants, considering the differences in training tolerance between recreationally active adults and elite athletes. Similarly, this study exclusively used cycling as a training modality which has minimal eccentric movement and mitigates the risk of exercise-induced muscle damage,¹²² compared to other activities such as running or resistance training. Athletes normally perform a combination of aerobic, resistance, and cross-training during habitual training. As such, the training stimulus and associated responses in this study may not reflect real-world training responses shown in athletes. CON subjects were instructed to maintain their habitual physical activity levels, yet the average $\dot{V}O_{2\text{Peak}}$ improved in this group as much as in both AD and OR groups. As we did not monitor physical activity in CON subjects, it is possible that CON subjects performed exercise beyond their habitual levels, despite restrictions of this study.

Conclusions.

The pathophysiological mechanisms of overtraining are unclear and no single biomarker can effectively distinguish an overtraining response following high-intensity training. The majority of overtraining research to date has focused on elite, predominately male athletes. To understand the progression of overtraining, we were successfully able to induce symptoms of OR in a group of moderately fit adults through the application of a high-intensity training protocol under laboratory conditions. OR subjects, characterized by decreased exercise performance and heightened mood disturbances, demonstrated concomitant and progressive decreases in LAP_{Peak} and MHR, which is compatible with autonomic dysfunction. Additionally, both AD and OR subjects exhibited only modest improvements in aerobic capacity, which reflects previous attenuations to aerobic capacity found in elite-level athletes experiencing FOR. Together, these findings demonstrate that inducing OR in recreationally active adults is feasible, and specific physiologic measures can be used to monitor the progression and resolution of

overtraining. Moreover, this study provides evidence that overtraining is not a training response exclusive to highly-trained (i.e., elite) athletes. Future well-controlled investigations with larger sample sizes should investigate the apparent link between high-intensity training and autonomic dysfunction as a possible mechanism of overtraining.

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Table 2.1. Three-week Cycle Training Protocol

Week 1 (visits 1 to 7)	Week 2 (visits 8 to 13)	Week 3 (days 14 to 19)
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 3-min active recovery	5x5:15 @ 75% PWL 3-min active recovery	5x5:31 @ 75% PWL 3-min active recovery
2x20-min @ 65% PWL 5-min active recovery	2x25-min @ 65% PWL 5-min active recovery	2x30-min @ 65% PWL 5-min active recovery
12x45s @ 130% PWL 2-min active recovery	12x50s @ 130% PWL 2-min active recovery	12x55s @ 130% PWL 2-min active recovery
50-min Lactate ride ³ 3mmol*L ⁻¹	55-min Lactate ³ 3mmol*L ⁻¹	60-min Lactate ride ³ 3mmol*L ⁻¹
Rest Day	Rest Day	Rest Day

Table 2.2. Baseline characteristics of CON and TR subjects

Group	n (sex)	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_{2\text{Peak}}$ (ml/kg/min)	$\dot{V}O_{2\text{Peak}}^{123}$ (% Predicted)	W_{\max} (W)
CON	10 (6f)	28.8 ± 8.7	168 ± 11.1	68.9 ± 12.9	39.7 ± 9.8	97.0 ± 22.9%	211.7 ± 48.1
TR	11 (10f)	28.4 ± 8.1	168.8 ± 8.4	72.8 ± 17.3	36.2 ± 6.2	105.3 ± 29.3%	199.9 ± 40.5
All values are reported from the baseline visit. Values are presented as mean ± SD.							

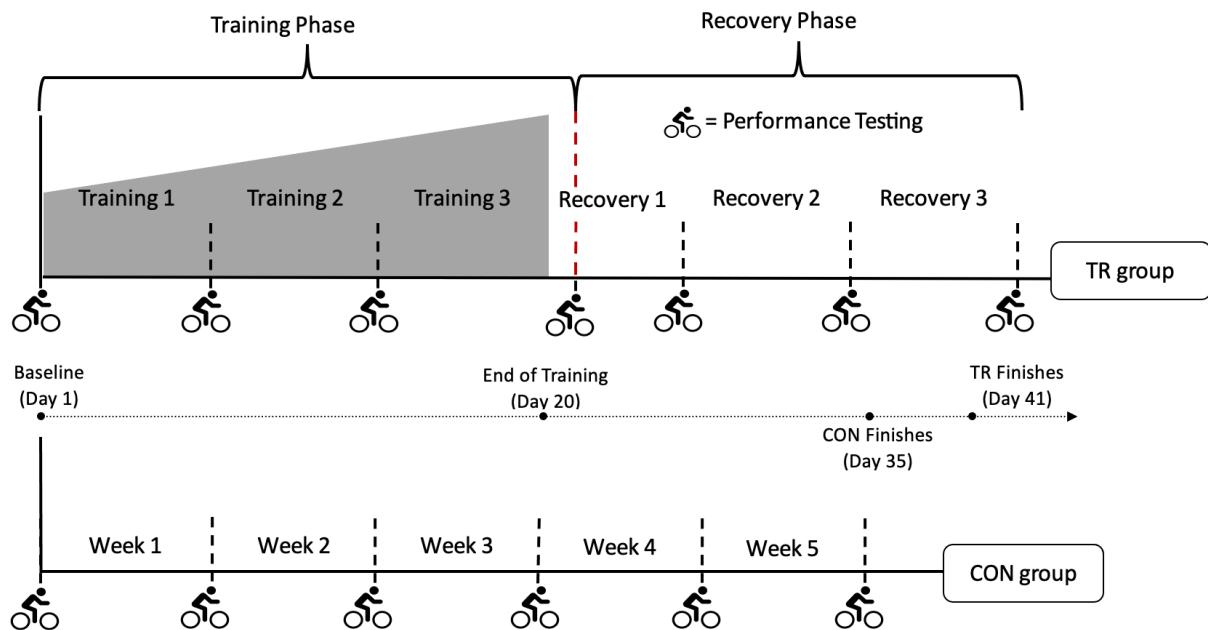
Table 2.3. Weekly Lab-based measures in CON, AD, and OR subjects.

	Baseline	Week 2	Week 3	Post-48h	Post-7	Post-14	Post-21
Peak Workload (W)							
CON	211.69 ± 48.1	220.24 ± 47.3	225.27 ± 52.21	---	223.5 ± 48.83	224.88 ± 51.19	227.19 ± 48.54
AD	213.3 ± 31.9	223.8 ± 31.8	227.4 ± 28.6	226.6 ± 24.6	232.8 ± 24.9	229.3 ± 25.5	227.5 ± 25.4
OR	162.17 ± 42.5	156.17 ± 41.63	156.83 ± 38.62	150.0 ± 31.4	158.17 ± 46.26	155.17 ± 42.4	161.75 ± 43.3
Δ% Peak Workload (W)							
CON	100.0 ± 0.0	104.4 ± 3.5	106.5 ± 4.7	---	105.8 ± 5.2	106.3 ± 5.8	107.7 ± 6.6
AD	100.0 ± 0.0	105.0 ± 4.0	107.1 ± 5.4	107.0 ± 7.3	109.9 ± 8.4	108.1 ± 6.1	107.3 ± 6.1
OR	100.0 ± 0.0	96.3 ± 2.9	97.0 ± 1.7	93.6 ± 8.7	97.4 ± 8.1	95.7 ± 4.8	99.8 ± 4.1
̇VO₂Peak (ml/kg/min)							
CON	39.77 ± 9.84	41.20 ± 9.44	40.90 ± 9.30	---	41.09 ± 10.44	41.97 ± 9.69	41.66 ± 9.29
AD	37.68 ± 6.11	37.70 ± 5.63	39.02 ± 6.92	38.82 ± 7.56	39.51 ± 7.13	39.79 ± 7.60	39.78 ± 6.82
OR	32.23 ± 5.52	30.62 ± 5.48	32.05 ± 6.05	31.18 ± 8.54	31.04 ± 2.65	30.99 ± 3.31	34.06 ± 4.87
Δ% ̇VO₂Peak (ml/kg/min)							
CON	100.0 ± 0.0	104.2 ± 4.9	103.5 ± 5.8	---	103.4 ± 5.3	106.0 ± 4.6	105.6 ± 6.4
AD	100.0 ± 0.0	100.3 ± 5.7	103.7 ± 9.0	103.1 ± 11.3	105.0 ± 9.7	105.7 ± 11.0	105.9 ± 10.5
OR	100.0 ± 0.0	95.0 ± 4.8	99.2 ± 1.9	95.8 ± 11.8	97.6 ± 11.7	97.3 ± 12.4	106.4 ± 10.2
̇VO₂ (L/min)							
CON	2.66 ± 0.61	2.76 ± 0.60	2.77 ± 0.62	---	2.78 ± 0.66	2.84 ± 0.62	2.82 ± 0.56
AD	2.78 ± 0.42	2.82 ± 0.39	2.90 ± 0.33	2.86 ± 0.28	2.91 ± 0.30	2.94 ± 0.34	2.94 ± 0.38
OR	2.03 ± 0.43	1.85 ± 0.35	2.00 ± 0.44	1.93 ± 0.48	1.95 ± 0.35	1.98 ± 0.40	2.14 ± 0.51
Δ% ̇VO₂Peak (L/min)							
CON	100.0 ± 0.0	103.9 ± 5.5	104.5 ± 4.9	---	104.3 ± 4.4	107.0 ± 3.9	106.5 ± 5.5
AD	100.0 ± 0.0	101.8 ± 4.7	105.3 ± 8.2	104.0 ± 9.9	105.9 ± 8.8	106.8 ± 10.6	107.0 ± 10.7
OR	100.0 ± 0.0	91.2 ± 2.2	98.5 ± 2.4	94.9 ± 13.1	97.2 ± 11.3	98.1 ± 7.7	105.5 ± 7.9
Maximum Heart Rate (bpm)							
CON	184.2 ± 7.0	184.4 ± 7.4	183.6 ± 8.2	---	183.8 ± 8.1	184.6 ± 6.5	183.55 ± 7.88

AD	181.44 ± 8.78	180.62 ± 8.77	177.75 ± 7.15	175.0 ± 10.97	178.12 ± 8.92	179.75 ± 7.32	178.38 ± 8.65
OR	180.0 ± 7.9	168.0 ± 7.9	171.91 ± 2.0	164.33 ± 4.62	170.0 ± 9.0	170.0 ± 5.3	179.0 ± 11.3
Δ% Maximum Heart Rate							
CON	100.0 ± 0.0	100.1 ± 2.5	99.7 ± 3.5	---	99.8 ± 3.0	100.3 ± 3.2	99.7 ± 2.6
AD	100.0 ± 0.0	99.6 ± 2.1	98.0 ± 2.5	96.4 ± 3.2	98.2 ± 2.6	99.1 ± 1.6	98.3 ± 2.6
OR	100.0 ± 0.0	93.5 ± 7.2	95.6 ± 4 .9	91.5 ± 6.4	94.5 ± 5.9	94.6 ± 5.3	99.5 ± 5.0
Peak Lactate (mmol/L)							
CON	9.38 ± 1.72	10.30 ± 1.71	9.76 ± 1.42	---	10.50 ± 1.93	10.38 ± 1.64	10.09 ± 1.14
AD	11.19 ± 1.25	11.01 ± 2.24	10.75 ± 2.43	9.79 ± 2.22	11.46 ± 2.21	10.02 ± 1.97	10.72 ± 1.77
OR	8.80 ± 1.47	7.93 ± 1.55	8.07 ± 2.04	5.87 ± 1.69	7.27 ± 2.10	6.83 ± 2.50	7.20 ± 2.63
Δ% Peak Lactate							
CON	100.0 ± 0.0	111.0 ± 15.7	105.3 ± 13.5	---	114.4 ± 25.9	112.3 ± 17.9	110.5 ± 22.8
AD	100.0 ± 0.0	98.2 ± 16.0	95.6 ± 16.5	87.3 ± 16.1	102.0 ± 12.7	89.2 ± 10.4	95.6 ± 9.5
OR	100.0 ± 0.0	91.2 ± 20.7	90.9 ± 9.8	66.4 ± 15.1	82.3 ± 19.1	77.0 ± 24.0	81.2 ± 25.7
Heart Rate Recovery (bpm)							
CON	31.3 ± 8.5	27.9 ± 10.9	26.7 ± 9.4	---	30.1 ± 8.8	28.1 ± 9.8	29.7 ± 8.1
AD	28.5 ± 6.8	25.9 ± 5.5	27.7 ± 8.1	30.1 ± 6.5	28.7 ± 6.6	30.1 ± 6.7	26.9 ± 5.2
OR	35.7 ± 20.0	31.3 ± 14.5	31.7 ± 20.1	35.9 ± 12.2	36.2 ± 9.7	45.3 ± 17.8	37.5 ± 13.3
Δ% Heart Rate Recovery							
CON	100.0 ± 0.0	87.7 ± 17.1	85.7 ± 17.6	---	97.9 ± 21.1	89.7 ± 16.9	97.8 ± 26.1
AD	100.0 ± 0.0	91.8 ± 10.5	98.6 ± 20.8	106.3 ± 5.9	101.3 ± 8.6	106.5 ± 14.7	96.6 ± 17.8
OR	100.0 ± 0.0	91.1 ± 10.3	86.3 ± 14.5	110.1 ± 34.1	112.4 ± 33.7	135.6 ± 40.6	112.5 ± 20.8
Body Weight (kg)							
CON	68.9 ± 12.8	69.0 ± 13.1	69.1 ± 13.0	---	68.8 ± 13.0	68.9 ± 12.7	68.8 ± 12.8
AD	76.6 ± 18.7	76.7 ± 18.4	76.9 ± 18.6	76.5 ± 18.5	76.3 ± 18.2	76.5 ± 18.3	76.6 ± 18.2
OR	62.8 ± 7.6	62.7 ± 6.7	62.6 ± 6.7	62.4 ± 6.7	62.4 ± 6.7	62.3 ± 6.9	62.5 ± 6.8

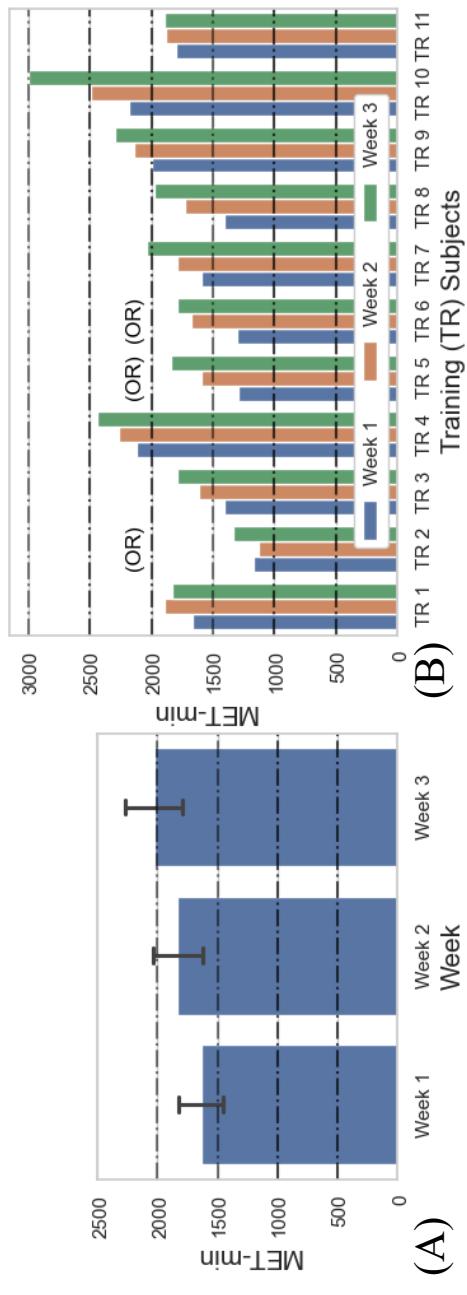
% Body Fat							
	26.4 ± 5.0	26.3 ± 4.9	26.1 ± 4.8	---	26.2 ± 5.4	25.6 ± 5.1	25.8 ± 5.2
AD	29.3 ± 10.3	19.2 ± 10.9	29.0 ± 10.8	28.8 ± 10.5	28.9 ± 10.8	28.6 ± 10.2	28.7 ± 10.7
OR	28.9 ± 5.3	28.5 ± 5.4	29.5 ± 3.8	28.3 ± 5.5	28.4 ± 4.4	27.9 ± 5.9	28.3 ± 4.5

Figure 2.1. Diagram of study design.



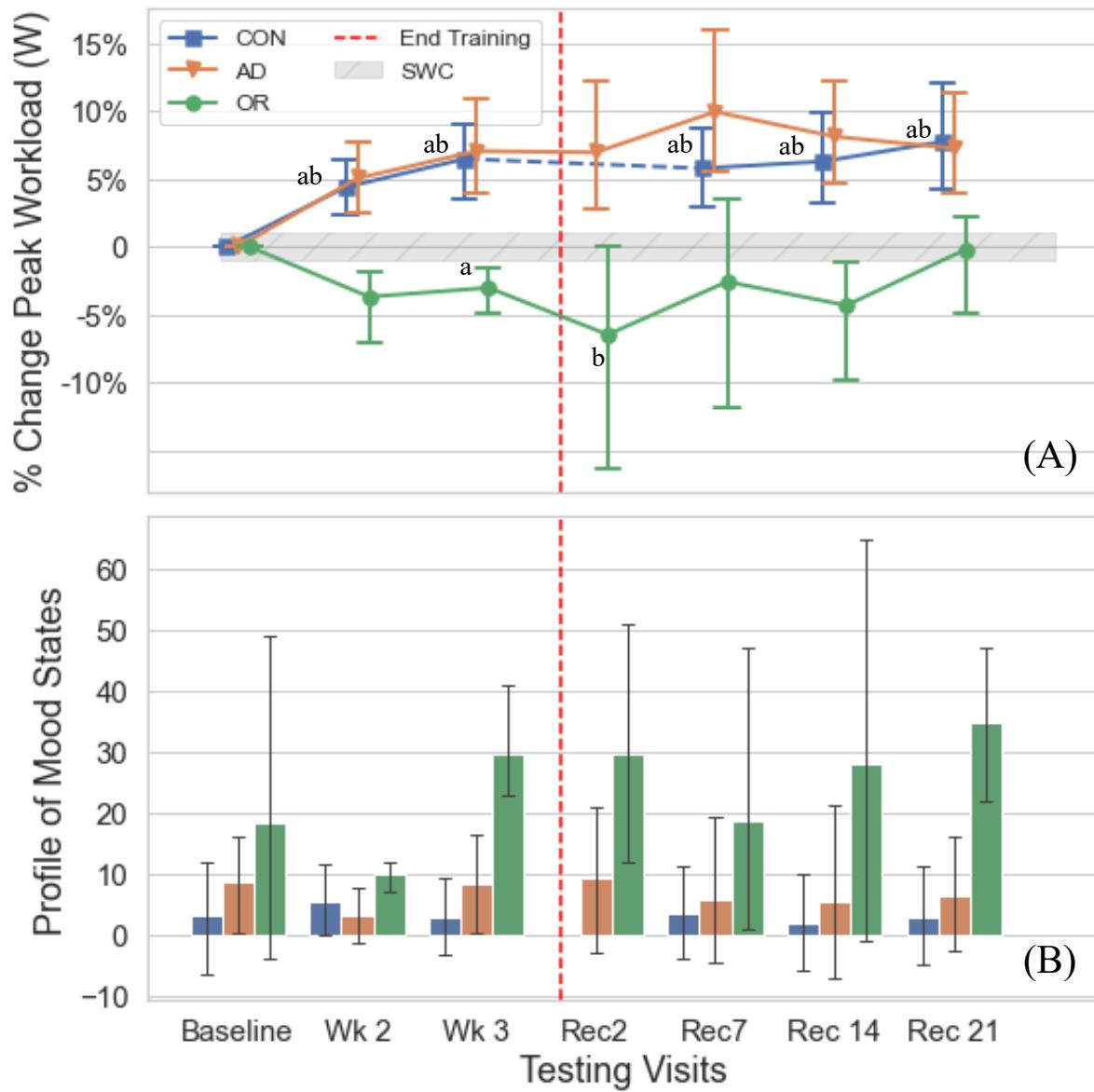
Training (TR) subjects underwent a three-week training protocol on a cycle ergometer. Workloads for all training sessions were calculated as a percentage of each individual's peak workload (PWL) achieved during weekly performance testing. Control (CON) subjects completed the same weekly performance testing procedures as TR subjects for six consecutive weekly visits. Shaded grey region depicts progressive overload of training protocol.

Figure 2.2. MET-min per week of exercise from training protocol.



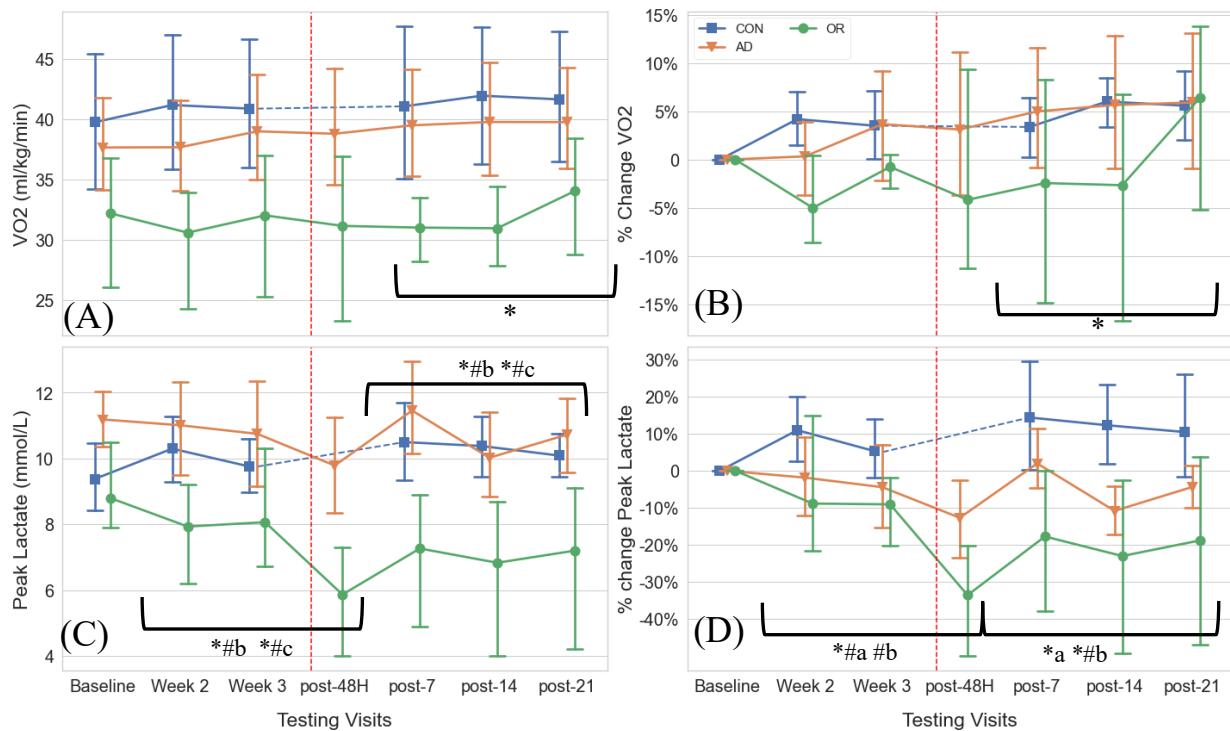
(A) Average (\pm 95% CI) exercise in MET-min performed by the training (TR) group per week of the three week training protocol. (B) Individual MET-min per week for each subject. Values were obtained by measuring gas exchange during rest and throughout all exercise sessions. 500 MET-min per week is equivalent to 150 minutes of moderate-to-vigorous physical activity per week as recommended by the Physical Activity Guidelines for Americans. (OR, 3f) indicates subjects who were classified as overreached.

Figure 2.3. Performance and Profile of Mood States in CON, AD, OR subjects.



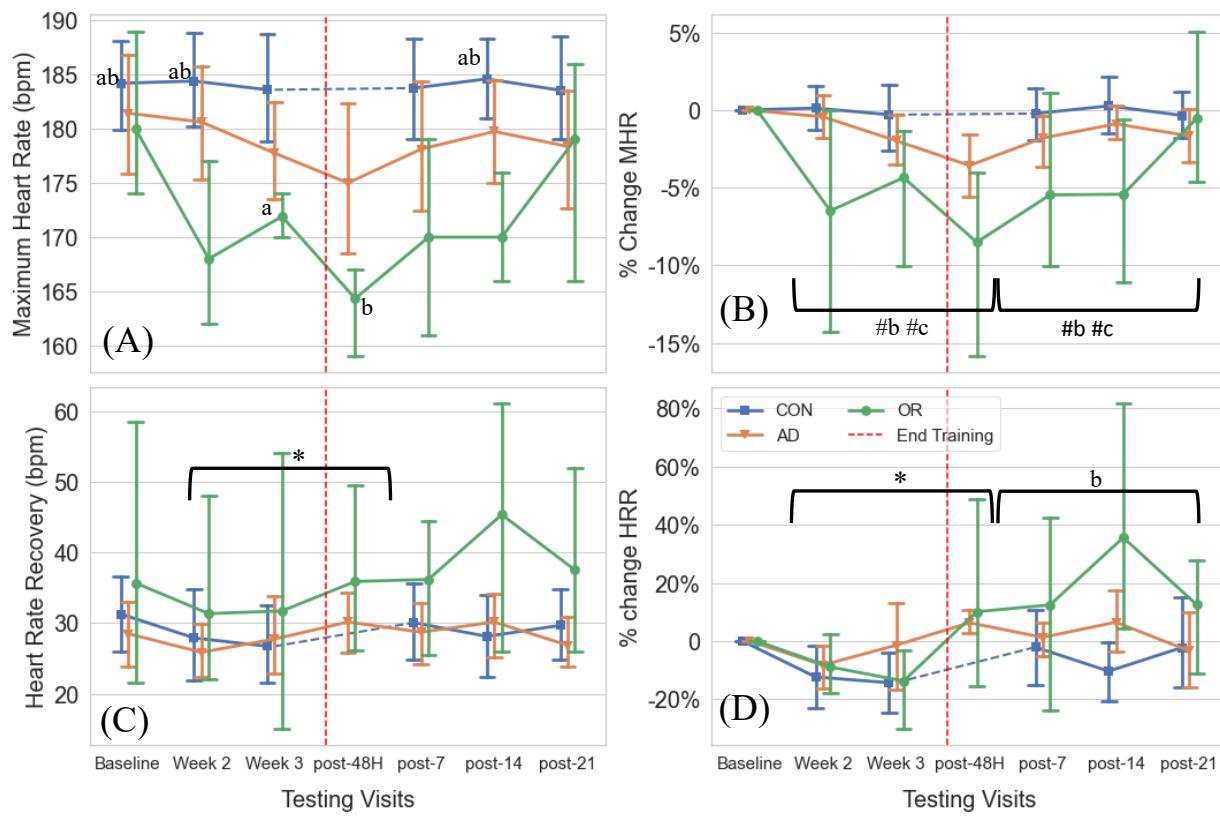
(A) Average ($\pm 95\%$ CI) percent change in performance (PWL) across time in CON, control; AD, adapted; OR, overreached groups. (B) Average ($\pm 95\%$ CI) Profile of Mood States Scores across time in CON, AD, and OR groups. Dashed circles P< 0.05 significant group-time(visit) interaction.

Figure 2.4. Average aerobic capacity and peak lactate in CON, AD, and OR subjects.



(A) Average ($\pm 95\%$ CI) peak aerobic capacity ($\dot{V}O_{2\text{Peak}}$) in CON, control; AD, adapted; OR, overreached groups. (B) Average ($\pm 95\%$ CI) percent change in aerobic capacity from baseline. (C) Average ($\pm 95\%$ CI) peak lactate(mmol/l). (D) Average ($\pm 95\%$ CI) percent change peak lactate from baseline. * $P < 0.05$ with bracket, significant main effect (phase) from baseline in all groups. Lowercase letter with brackets, $P < 0.05$ group-time(phase) interaction (a=CON, AD; b=CON, OR, c=AD, OR). *with letter, group differences occur during same phase; #with letter, group differences occur between different phases.

Figure 2.5. Maximum heart rate and heart rate recovery in CON, AD, and OR subjects.



(A) Average (\pm 95% CI) maximum heart rate (MHR) across time in controls, Con; adapted, AD; overreached, OR. (B) percent change in MHR across time. (C) Average (\pm 95% CI) change in heart rate recovery (HRR) across time. (D) Percent change in HRR across time. * $P < 0.05$ with bracket, significant main effect (phase) from baseline among all groups. Lowercase (a,b) $P < 0.05$ significant group-time(visit) interaction between corresponding letters. Lowercase letter with bracket, $P < 0.05$ significant group-time(phase) interaction (a=CON, AD; b=CON, OR; c=AD, OR). *with letter, group differences occur during same phase; #with letter, group differences occur between different phases.

CHAPTER THREE: SLEEP PATTERNS OF RECREATIONALLY ACTIVE ADULTS
THROUGHOUT A THREE-WEEK HIGH-INTENSITY
OVERREACHING TRAINING PROTOCOL

Introduction:

The American Academy of Sleep Medicine recommends that adults obtain between seven to nine hours of sleep per night for optimal health and performance,¹ yet approximately one third of adults in the United States fail to meet these guidelines.² Though the explanations for, “why we need sleep” remain unclear, it is undeniable that sleep is vital to overall health and well-being. Suboptimal sleep patterns including sleep deprivation and restricted sleep can lead to impaired cognitive,³ immunological,^{4,5} metabolic,^{3,6,7} and hormonal functions.^{6,8,9} Consistent suboptimal sleep patterns (e.g., consistently sleeping four to six hours per night) leads to the accumulation of sleep debt, which can erode cognitive performance and neurobehavioral health to the same magnitude as two nights of total sleep deprivation.¹⁰ Alarmingly, it can take up to four days to recover from one hour of sleep debt and up to nine days to eliminate the debt completely.¹¹

Sleep is a vital component of recovery following exercise given its restorative effects on physiological and psychological well-being^{12,13} and is considered one of the best tools of recovery for athletes.¹⁴ Optimizing training adaptations requires a meticulous balance between training stimuli and recovery. Athletes commonly undergo periods of intensified training with the intention of improving athletic performance by inducing a physiological response known as functional overreach (FOR). Inducing FOR is generally thought to be necessary for promoting

meaningful physiological adaptations and performance supercompensation.^{15–17} However, when training demands are persistently met with insufficient recovery, athletes may experience a prolonged maladaptive response known as non-functional overreach (NFOR).¹⁵ Ultimately, if the training-recovery imbalance is not addressed, NFOR may progress into overtraining syndrome (OTS).^{17,18} These three states of training maladaptation (FOR, NFOR, OTS) comprise the training-overtraining continuum. Symptoms of overtraining include decreased athletic performance and a number of psychophysiological disturbances including increased incidence of illness, worsened mood/behavioral health, and autonomic dysfunction.¹⁵ As an athlete progresses towards OTS, the severity of symptoms may increase; however, there are no clear boundaries besides the time course of performance recovery separating FOR, NFOR, and OTS. Recovery from FOR typically requires several days to weeks, while symptoms of NFOR and OTS can persist for months or years, respectively.^{15,19}

Sleep disturbances are often reported among athletes experiencing overtraining.^{15,18,20} However, it is unclear whether impaired sleep contributes to the progression of overtraining or is merely a symptom.^{21,22} Additionally, the severity of overtraining is influenced by training load, sleep, and immune function, which have bidirectional relationships with one another; however, the exact nature of how each contributes to overtraining is unclear.^{21,23–25} The body of literature regarding sleep in athletes is limited, particularly when concerning overtrained athletes.²² Moreover, previous sleep research in athletes has seldom included appropriate controls^{26,27} and has often relied on subjective reports rather than objective measures of sleep.^{22,26,28} Currently, there are no specific sleep recommendations for athletes, but given the heightened psychological and physiological stresses inherent to participation in sports, it is plausible that athletes may

require more sleep than the general population to allow for adequate recovery from the demands of training and competition.²⁹ In 2012, Leeder et al.²⁷ quantified sleep in athletes using wrist actigraphy and compared their sleep measures to non-athletic controls. Their data suggested that, while athletes and non-athletes obtained similar amounts of sleep, athletes had worse quality of sleep. Survey data has also found lower sleep quality in Olympic-level athletes compared to non-athletes, with no group differences in sleep duration.²⁶ Likewise, in an exercise study designed to induce overreach (OR) in elite athletes, those who experienced FOR after intensified training exhibited decreases in sleep duration and sleep efficiency, compared to non-overreached athletes who underwent the same training volume.²³

The purpose of this study was to determine whether recreationally active adults could exhibit symptoms of overtraining following a standardized training protocol, and aimed to address two questions: 1) How does a high-intensity, novel training stimulus affect sleep in healthy, recreationally active adults? 2) Do individuals experiencing overtraining exhibit different sleep characteristics compared to non-overtrained individuals undergoing the same training volumes? Wrist actigraphy and paired sleep surveys were used to characterize sleep parameters in a group of recreationally active adults who completed a three-week high-intensity training protocol (TR) designed to induce OR and in a control group (CON). We hypothesized that sleep parameters in the TR group, including sleep quality and duration (i.e., total sleep time), would worsen in TR subjects during the training protocol, compared to CON subjects. Sleep parameters were also analyzed among TR subjects classified as OR following the training protocol, compared to those classified as non-overreached (adapted [AD]). We hypothesized that subjects classified as OR would exhibit worse sleep quality and duration (total sleep time)

compared to AD subjects. As few studies have objectively looked at sleep in athletes undergoing periods of intensified training,^{21–23,30} this study will draw on sleep literature regarding both athletes and non-athletes.

Materials and Methods:

Participants. Twenty-four recreationally active adults (19 females, 5 males) volunteered to participate in this study. Upon recruitment, subjects were randomly assigned to a training group ([TR], n=12) or a control group ([CON], n=12). Three subjects (2 CON, 1 TR; 3 females) withdrew from the study due to scheduling conflicts. Additionally, one subject failed to regularly report their sleep data. Consequently, these subjects' data were excluded, and the final sample size was n=20 (9 CON, 11 TR). The study was approved by the Washington State University (WSU) Institutional Review Board (#18860) and was conducted in accordance with the Declaration of Helsinki. Before study participation, subjects completed health screening questionnaires and lung function testing to assess overall health.

Experimental Design. TR subjects underwent a three-week high-intensity training protocol exercising six days per week under laboratory conditions. An overview of the training protocol is shown in **Table 2.1B**. All training sessions were performed in the exercise physiology research lab at the WSU-Spokane campus and consisted of a mix of long-duration, interval, and sprint-like training sessions using a cycle ergometer. Workloads for all training sessions were calculated as a percentage of each individual's peak workload (PWL) achieved during weekly

performance testing. TR subjects performed a total of seven performance tests: one per week during the three-week training protocol (training phase), one 48-hours post-training, and one per week for three weeks following the end of the training protocol (recovery phase). CON subjects completed the same performance testing procedures as TR once per week for six consecutive weeks. CON participants were instructed to maintain their normal sleep and dietary habits for the duration of observation and were allowed to maintain their habitual exercise routines during the six weeks of observation.

Sleep parameters. Sleep-wake behavior was monitored using an Actiwatch Spectrum Plus (Philips Respironics, Bend, OR, USA) wristband worn on the nondominant wrist. The Actiwatch is a light weight, water-resistant, wrist-worn tri-axial actigraph designed to provide an empirical measurement of movement. The Actiwatch then applies algorithms and activity counts to provide a reliable and valid method for monitoring sleep.^{31,32} Compared to polysomnography, actigraphy has effectively monitored sleep duration and sleep efficiency in sleep disordered patients³² and has shown reasonable reliability and validity in individuals with normal sleep patterns.³¹ In this study, actigraphy data were collected using one minute epoch lengths and a medium wake threshold (40 or more activity counts per epoch). The sleep interval detection algorithm was used to determine sleep onset and sleep end, configured to 10 immobile minutes for each. Sleep surveys were filled out the morning after each sleep. Participants reported sleep and wake times and rated their sleepiness, fatigue, and sleep quality for all sleep periods. A sleep period was defined as the time a participant attempted to sleep and had been awake at least 10 minutes from a previous sleep period. Actigraph data was compared with sleep survey data

collected from participants to ensure that sleep periods were captured correctly in the actigraphy software (Philips Actiware 6). In-bed start and end times were manually adjusted using a standardized in-lab protocol in order to improve the accuracy of all sleep measures. Definitions for all sleep parameters are described in **Table 3.2**.

Illness Symptoms. Participants reported their upper respiratory tract illness (URTI) symptoms using the Wisconsin Upper Respiratory Symptom Score (WURSS-11).^{33,34} This survey was completed daily by TR subjects and once weekly (i.e., performance testing days) by CON subjects. Participants self-reported the days they considered themselves to be ill. Illness symptoms, rated on a seven-point scale, included runny nose, plugged nose, sneezing, sore throat, scratchy throat, cough, and feeling tired. Participants were not restricted from taking medications but were instructed to report any medications taken through their daily survey forms.

Performance Testing. All participants underwent weekly performance testing using a magnetically braked cycle ergometer (Ergoselect 200, Ergoline) to determine maximal oxygen uptake ($\dot{V}O_{2\text{PeakM}}$) and cycling performance (PWL). The initial performance test conducted by each participant served as the baseline reference for performance changes during all subsequent weekly performance tests. For all lab visits, subjects wore a face mask (Hans Rudolph, Kansas City, MO) for breath collection. Expired oxygen and carbon dioxide concentrations were continuously measured breath-by-breath (ParvoMedics TrueOne 2400, Salt Lake City, UT) and recorded in 15 second averages.

The performance test was a graded exercise test (GXT) until volitional exhaustion. Warm-up included cycling for five minutes at 25% PWL from the baseline performance test performed during intake screening. The GXT began immediately following warm-up with starting workload set at 75W (females) or 100W (males). Workload increased every two minutes by 30W (females) or 45W (males) until volitional exhaustion, or until participants were unable to maintain a cadence greater than 60rpm. Subjects then proceeded through a five-minute cooldown using the same power output as the warm-up. After a five-minute intermission off the bike, participants completed a second performance test at a constant, supramaximal workload. This second round was used to confirm $\dot{V}O_{2\text{peak}}$, as suggested by Pool and Jones.³⁵ The second round included a two-minute warm-up and a constant workload time-to-exhaustion cycling test. Resistance was set at 110% of each participant's PWL achieved during the GXT.

Maximal effort was determined by observing a plateau in the $\dot{V}O_{2\text{Peak}}$ data. If a plateau in $\dot{V}O_2$ was not observed during the GXT, maximal effort was determined by identifying at least two secondary criteria commonly used to determine maximal effort. Secondary criteria, as described by Howley et al.,³⁶ included a participants reaching a respiratory exchange ratio of at least 1.10, a peak heart rate over 90% of the predicted maximal HR, or a peak lactate (LA^-_{peak}) of at least 8 mmol/L. If a participant did not meet two of these three criteria, maximal effort was considered achieved if a participant's $\dot{V}O_{2\text{Peak}}$ from the two exercise rounds differed by less than 0.15 L.

Mood State. Weekly, on the day of performance testing, participants completed a short-form version of the Profile of Mood States (POMS),³⁷ a Likert scale questionnaire that provides

measures of vigor, depression, fatigue, anger, anxiety, and confusion. Vigor, the only “good” mood subscale, reduces POMS scores whereas the other five subscales aggregate and increase total POMS scores. As such, a higher POMS score indicates greater mood disturbances, whereas a lower score reflect a better mood state. The short-form POMS includes 37 items from the original POMS.

Assessment of Overreaching. Following the training protocol, TR participants were divided into AD and OR subgroups according to their performance response during the recovery phase. In line with previous research,^{23,38–40} the smallest worthwhile change (SWC) was used to determine a performance threshold to indicate OR (OR threshold). SWC was calculated as 0.3 times the coefficient of variation (CV) of PWL from all performance tests CON subjects completed during the study. TR subjects who exhibited a decrease in PWL beyond the OR threshold during at least one weekly performance test during the recovery phase, and accompanied by an overall increase in POMS, were classified as OR. The remaining subjects in the TR group who maintained or increased performance after training were considered adapted (AD).

Statistical Analysis. Data analysis was conducted using Scikit-learn (1.3.2),⁴¹ Statsmodels (0.13.2),⁴² SciPy (11.1.4),⁴³ and Pingouin (0.5.4)⁴⁴ libraries in Python. Levene’s test was used to assess homogeneity of variance and QQ plots and residual plots were inspected to determine if data was normally distributed among groups. Mixed-effects regression models were used to investigate the impact of group status (CON *versus* TR), phase (training or recovery), and time

(days of study participation). A single model was fitted for all sleep and illness outcome measures. As outlined in Van Dongen et al.,⁴⁵ mixed-effects regression models, compared to traditional repeat-measures analysis of variance (ANOVA), are capable of handling longitudinal measures when there is a specific temporal arrangement (i.e., order and interval). For ordinal sleep measures (e.g., sleep quality), mixed-effect logistic regression models were used. Mixed-effects regression incorporates random effects, representing individual-specific variance in a given outcome measure and effectively account for both interindividual and temporal variance within the data.^{46,47} These models are well-suited to handle unequal sample sizes, address heteroscedasticity in cumulative sleep measures, and support empirical Bayes estimation of individual subject parameters (random effects).⁴⁸ F-tests were performed on significant regression model effects and interactions ($\alpha \leq 0.05$). For outcomes with significant F values, Games-Howell post hoc tests were performed to determine when significant group differences occurred. Regression model fixed effects (β) and p-values are reported in text. All other data presented in-text are reported as mean \pm standard deviation (SD), unless otherwise specified.

Missing Data. Instances of missing sleep data were imputed using multiple imputation chained equations (MICE).⁴⁹ Multiple imputation is a modern and principled technique for dealing with missing data. It considers the circumstances surrounding missing data and provides more reliable model estimates compared listwise or pairwise deletion.⁵⁰ As outlined in Azur et al.,⁵¹ MICE is a robust technique used in research to systemically address missing data. Its iterative approach facilitates the imputation by considering interdependencies among variables, enhancing analytical precision, and mimicking of the variability and uncertainty found in real-world data.

As sleep patterns are personal to each individual and influenced by habitual lifestyles throughout a given week (e.g., work and social obligations),⁵² individual subject and day of the week were used as covariates in the imputation model.

Results:

Baseline characteristics for CON and TR groups are shown in **Table 3.3**; there were no group differences.

Actigraphy Sleep Measures in CON and TR Groups. The results of two-group mixed-effects regression and F-tests for all sleep parameters are shown in **Table S.4**. Average sleep duration throughout both phases was similar between CON and TR subjects (467 ± 69 and 442 ± 80 minutes per day, respectively). Additionally, average total sleep time across all days was not significantly different between the CON and TR group (421 ± 62 and 401 ± 72 minutes per day for CON and TR, respectively; **Figure 3.1A**). However, there was a larger variance in total sleep time per day throughout both phases in the TR group compared to CON (Levene's W=4.78, P<0.05). TR subjects accumulated more sleep debt over time throughout both phases, compared to CON, ($\beta=-23.664$, P=0.001; **Figure 3.2**). 64% (7/11 subjects) of TR subjects had progressive sleep debt whereas 33% (3/9 subjects) of CON subjects demonstrated sleep debt over time. By the end of the study, average sleep debt in these seven TR subjects was 27.58 ± 12.91 hours.

Survey-based Sleep Measures—CON and TR Groups (Table 3.4). Sleep quality improved over time throughout both phases in both groups ($\beta=-0.035$, $P=0.019$). From study onset to the end of the study, bedtime sleepiness increased in TR subjects ($\beta=0.058$, $P=0.027$); however, bedtime sleepiness began to decrease and return to pre-study levels during the recovery phase ($\beta=-0.118$, $P=0.004$; **Figure 3.3B**). Sleepiness upon waking also increased in TR subjects throughout both phases ($\beta=0.071$, $P=0.019$; **Figure 3.3B**).

Performance Testing and Incidence of Overreaching. Average PWL in CON and TR groups increased $7.73\% \pm 6.60\%$ and $5.23\% \pm 6.41\%$ from baseline values, respectively, at the end of the recovery phase ($\beta=0.066$, $P<0.001$). There were no group differences in PWL. In CON subjects, the average PWL for all performance tests was $222.13 \pm 7.44W$, which set the OR threshold to a 1.00% (0.3^*CV) decrease in performance from baseline.

After the training phase, three TR subjects were classified as OR for exhibiting a decline in performance beyond the OR threshold. The average nadir performance in OR subjects was $-10.38\% \pm 5.55\%$ from baseline values, which occurred at different timepoints during the recovery phase. Compared to CON subjects, PWL in OR subjects decreased over time ($\beta= -0.048$, $P = 0.003$) regardless of phase (**Figure 3.4**). At the end of the recovery phase, PWL in OR had returned to baseline levels ($-0.19 \pm 4.10\%$ from baseline values), whereas CON and AD subjects had improved their overall performance by $7.73\% \pm 6.60\%$ and $7.26\% \pm 6.05\%$ ($P<0.05$) from baseline, respectively. There were no significant between-group differences in POMS scores during any specific phase or week; however, the pooled average of POMS scores

across all visits were higher in OR compared to CON subjects (Welch's ANOVA $F(1,50.71)=10.21$, $P<0.001$, $\eta^2=0.188$; Games-Howell, adjusted $P<0.001$). Average POMS scores in OR subjects increased from 18.3 ± 22.4 at baseline and peaked at 42.3 ± 16.5 during the recovery phase, whereas average POMS scores in CON and AD groups remained near baseline values throughout the study (CON $\text{POMS}_{\text{baseline}}= 3.3 \pm 15.7$, $\text{POMS}_{\text{peak}}=13.2 \pm 12.3$; AD $\text{POMS}_{\text{baseline}}= 7.6 \pm 2.7$, $\text{POMS}_{\text{peak}}= 15.9 \pm 20.4$).

Actigraphy Sleep Parameters in Control, Adapted, and Overreached Groups. The results of three-group (CON, AD, OR) mixed-effects regression and F-tests for all sleep parameters are shown in **Table S.6**. Average sleep duration across all days was similar among CON, AD, and OR groups (467 ± 69 , 443 ± 78 , and 441 ± 83 minutes, respectively, $P>0.05$). However, sleep duration decreased over time in OR subjects throughout both training and recovery phases ($\beta=-3.387$, $P=0.042$). Throughout both phases, average daily total sleep time was similar among CON, AD, and OR groups (421 ± 62 , 401 ± 70 , and 403 ± 76 minutes per day, respectively; **Figure 3.5A**). AD subjects accumulated more sleep debt over time, throughout both phases, compared to CON $\beta=-25.948$, $P<0.001$; **Figure 3.6**). By the end of the study, AD and OR groups had accumulated an average sleep debt of 12.9 ± 22.2 and 11.8 ± 33.0 hours, respectively. In contrast, the CON group accrued 0.30 ± 13.6 hours of sleep gain by the end of the study.

Survey-based Sleep Parameters in Control, Adapted, and Overreached Groups. Average sleep quality improved over time in all three groups throughout both phases ($\beta=-0.035$,

$P=0.031$); there were no differences in sleep quality among groups. Self-reported bedtime sleepiness increased over time in OR subjects throughout the entire study ($\beta=0.097$, $P=0.013$). However, during the recovery phase, the rate of bedtime sleepiness decreased from levels shown during the training phase in AD ($\beta=-0.110$, $P=0.012$) and OR groups ($\beta=-0.141$, $P=0.013$; **Figure 3.7B-C**). Sleepiness upon wake increased in AD subjects ($\beta=0.066$, $P=0.045$) throughout both phases, and decreased in OR subjects during the recovery phase compared to reported sleepiness at the onset of the study ($\beta=-0.139$, $P=0.03$; **Figure 3.7C**).

Upper Respiratory Tract Illness. Average WURSS-11 scores are shown in **Figure 3.8**. OR subjects experienced an increase in URTI symptoms during both the training and recovery phase, compared to CON and AD subjects ($\beta=0.364$, $P=0.030$). During the training phase, average WURSS-11 scores in OR subjects was 12.8 ± 13.4 compared to 2.2 ± 2.8 and 3.9 ± 4.3 in CON and AD subjects respectively ($P<0.05$). Additionally, OR subjects continued to exhibit higher respiratory illness symptoms during the recovery phase (11.0 ± 8.8) compared to CON and AD subjects (2.96 ± 7.3 and 4.4 ± 6.5 , respectively; $P<0.05$).

Missing Data. Missing data points were assumed to be missing at random. There were 775 possible observations for sleep outcome measure (36 days of participation for 9 CON subjects; 41 days for 11 TR subjects). There were 47 (6%) instances of missing actigraphy measures and 155 (20%) missing sleep survey reports among all participants. Subsequently missing sleep data were imputed using the describe methods (MICE). TR subjects completed the WURSS-11 daily,

whereas CON subjects filled out surveys once per week, on the day of performance testing. Between CON and TR groups, there were 54 and 451 possible WURSS-11 survey observations, respectively. There were two (3.7%) missing WURSS-11 surveys among CON subjects and 39 (8.6%) missing among TR subjects. WURSS-11 data were not imputed prior to regression analysis.

Discussion:

The primary objective of this randomized controlled study was to investigate differences in sleep parameters, as measured by actigraphy and survey responses, between two groups of healthy recreationally active adults. Sleep quality and total sleep time were similar between groups throughout the study; however, CON subjects slept an average of seven hours per night (7.02 ± 1.03 hours), whereas TR subjects habitually slept less than seven hours per night (6.68 ± 1.20 hours; **Figure 3.1**). Additionally, findings from this study suggest that sleep pattern variability may be impacted by periods of high-intensity exercise, independent of OR status. These results build upon the limited body of sleep literature on athletes which suggests that, like the broader adult population,² elite athletes often struggle to obtain adequate sleep;^{27,53–58} however few studies have compared sleep in athletes with controls^{26,27,58,59} or used objective measures to monitor sleep.^{23,24,27,30} Moreover, OR subjects exhibited increased URTI symptoms throughout the entire study, compared to CON and AD. The increase of URTI symptoms, despite an absence in sleep differences between AD and OR subjects, suggests the immune system response plays a role in the overreaching process.

TR subjects, compared to CON subjects, were more likely to accumulate sleep debt throughout the study. This was driven by TR subjects consistently sleeping less than seven hours per night, the minimum recommended for optimal health.⁶⁰ Sleep debt in TR participants continued to accumulate throughout the recovery phase after the training protocol had ended. However, there was a slight plateau in sleep debt in the first week of the recovery phase, indicating that TR subjects increased their total sleep time to seven hours (**Figure 3.2**). The first week of recovery was the only time in which participants were not allowed to engage in exercise either through the training protocol or during their free time. However, once TR participants were allowed to resume habitual exercise training (week two of recovery), they continued to accumulate sleep debt. The slight change in sleep pattern observed during the cessation of training could have been a byproduct of participants having more free time for other activities, which presumably allowed for more sleep time. However, the exact mechanisms behind this sleep trend are unclear. There are no specific guidelines regarding how much sleep athletes should obtain compared to non-athletes. However, it is generally believed that athletes and other highly active individuals (e.g., military personnel) may require more sleep than non-athletes to cope with the inherent demands of training and competition.²⁹ In non-athletes, chronic sleep restriction (5-7 hours per night) progressively erodes neurocognitive performance including alertness reaction time, memory, and decision making.^{45,61-63} Neurocognitive deficits can require up to nine days of adequate sleep to resolve, depending on the severity of sleep debt.¹¹ Conversely, studies have found that extending sleep duration has shown to improve athletic performance.^{64,65}

A new finding in this study was that total sleep time was more variable in TR subjects than CON subjects. Thus, in addition to the progressive increase in sleep debt during the training and recovery phases in TR subjects, they also exhibited greater volatility in their day-to-day sleep. To our knowledge, sleep pattern variability has not been examined in athletes besides self-reported sleep/wake time patterns (i.e., chronotypes).^{26,59} This gap may stem from variations in the methodologies employed across the existing sleep literature. For example, previous studies of sleep in athletes would have missed sleep pattern variability if the study were relatively short in duration (e.g., lasting less than two weeks).^{20,66,67} Additionally, other studies might have overlooked sleep pattern variability by making the decision to aggregate and compare sleep among different sleep periods using multi-daily or weekly averages.^{23,24} A recent systematic review regarding sleep timing/consistency and health in adults found that greater sleep variability was associated with adverse health outcomes, though the quality of evidence ranged from moderate to very low.⁶⁸ Still, further investigation into sleep consistency in athletes is warranted as research has associated irregular sleep patterns with higher levels of inflammation,⁶⁸ lower academic performance,⁶⁹ and lower perceptions of well-being.⁷⁰

We hypothesized that sleep quality would decline in TR subjects after the training protocol on the basis that sleep quality has been found to decline in athletes after they underwent intensified training periods.^{23,24} Conversely, we found that sleep quality in TR subjects improved over time alongside that of CON subjects. This was unexpected given that TR subjects also reported higher levels of sleepiness at bedtime and upon waking throughout the study. (**Figure 3.7**). Three TR subjects were categorized as OR, based on a reduction in PWL during the training or recovery phases. Compared to CON subjects, OR subjects exhibited increased

perceived sleepiness at bedtime which persisted throughout the training and recovery phases. There were no differences in sleep duration between the OR and AD groups and both groups exhibited a decrease in bedtime sleepiness during recovery phase itself. This suggests a gradual reduction of perceived exhaustion after the exercise training protocol ended. These findings align with previous overtraining research in athletes, which has shown that subjective measures of stress and well-being, compared to objective markers, are generally more sensitive to changes in training volume.⁷¹ Similarly, in a cross-sectional study, Bender et al.²⁶ found that Olympic athletes, compared to non-athlete controls, self-reported poorer sleep, as evidenced by degraded sleep quality, challenges falling asleep, and increased frequency of sleep disturbances. These sleep differences were found in the absence of a difference in self-reported sleep duration between groups, leading researchers to suggest that elite athletes experience problems with subjective sleep quality rather than subjective sleep duration.

The apparent disconnect between subjective sleep measures (i.e., improved sleep quality with concomitant increases to bedtime sleepiness) warrants further investigation. Several meta-analysis and systematic reviews regarding sleep health have consistently reported that regular physical activity can improve sleep quality in addition to total sleep time, sleep efficiency, and sleep onset latency;^{72–76} however, these reviews rarely included studies of sleep in athletes. Moreover, the effects of physical activity on sleep health were found to be moderated by gender, age, health status, and baseline physical activity levels as well as the mode and intensity of exercise.^{72,74} As athletes typically undergo much larger volumes of training compared to non-athletes,^{77–79} the consensus findings of these reviews may not apply to athletes.

In the present study, TR subjects classified as OR exhibited increased URTI symptoms during the training phase, which persisted throughout the recovery phase. In contrast, URTI symptoms in AD and CON groups remained consistent. The significant and persistent increases to URTI symptoms despite a lack of differences in sleep duration between AD and OR subjects suggests that high-intensity training may negatively impact immune system function, which in turn may drive the overtraining process. Few studies have examined sleep in overtrained athletes or in athletes undergoing intensified training periods;^{21,23,30} however, sleep disturbances are often reported in overtrained athletes.^{15,18,20} As such, it is unclear whether sleep disturbances contribute to the overtraining process or are a symptom.^{21,22} In well-trained triathletes, Hausswirth et al.²³ found that after intensified training, FOR athletes reported more frequent bouts of respiratory illness and demonstrated decreases to sleep duration (-7.9%), sleep efficiency (-1.6%), and time immobile (-7.6%), compared to acutely fatigued athletes who underwent a similar training load.²³ The results of this study corroborates the findings in the present investigation and further supports the notion that the immune system plays a role in the overtraining process.

Limitations. This study had several limitations. First, while we included a control group and randomized subjects between groups, the sample size was relatively small. Additionally, only one male participant was randomized into the TR group, which prevented the comparisons of between-sex differences in sleep parameters among the groups. With a larger sample size, more subtle differences in sleep might have been observed, particularly between OR and AD groups. Additionally, we did not monitor a reference period of sleep prior to onset of the training protocol. Observing participants' habitual sleep patterns prior to the onset of the training protocol

would provide a better indication of whether the observed differences in sleep patterns between CON and TR groups were the result of a training protocol, or simply due to differences habitual sleep patterns between the groups. Similarly, without a reference period of sleep prior to active study participation, we were unable to determine whether participants began the study with a sleep debt or sleep gain. It is recommended that adults consistently obtain seven to nine hours of sleep per night for health⁶⁰ and research suggests that at least eight hours of sleep is required to avoid the cumulative effects of sleep debt.^{11,45} Knowing the habitual sleep patterns of our participants prior to participation would have allowed us to better detect both optimal and abnormal patterns of sleep duration and quality and allow us to calculate sleep debt based on individual sleep needs. As such, it is unlikely that basal sleep needs in our subjects was less than seven hours (i.e., the operational threshold to begin accumulating sleep debt), particularly with the increased training demands placed upon our TR group. TR subjects were instructed to avoid exercise beyond the training protocol until the second full week of the recovery phase and CON subjects were instructed to only perform habitual exercise that would maintain current fitness levels. However, It is possible participants were non-compliant with these instructions. Although caffeine intake was reported by participants, we did not control for caffeine intake or timing during the study. The effects of caffeine on sleep have been studied extensively and have been shown to impact sleep duration, sleep efficiency, and perceived sleep quality.⁸⁰ Controlling for caffeine intake might have resulted in a more precise understanding of the training protocol's effect on sleep duration and perceptions of sleep quality, sleepiness, and fatigue between groups. In the training protocol, the participants who were considered recreationally active performed exercised approximately seven hours per week during the training protocol. As training volumes

of endurance athletes often exceed 30h/week,^{77–79} the findings of this present investigation may have limited applicability to elite-level athletes.

Conclusions. Few studies have objectively measured sleep in athletes. Using wrist actigraphy and sleep surveys, we compared objective measures of sleep duration and subjective measures of sleep quality in a group of randomized recreationally active adults undergoing an overreach training protocol to those of healthy controls. Our findings suggest that chronic high-intensity exercise can impact an individual's ability to consistently obtain sufficient sleep, increases the likelihood of accumulating sleep debt, and may lead to heightened perceived sleepiness at bedtime that persists upon awakening. Furthermore, the heightened respiratory illness symptoms found in OR subjects, despite having similar quantitative and qualitative measures of sleep as AD subjects, suggests that the immune response plays a key role in the development of overtraining. Future research with larger sample sizes is warranted to better elucidate the relationships among sleep, immune function, and the progression of overtraining. Additionally, future longitudinal studies of sleep in athletes should continue to investigate sleep pattern variability to better understand how this may influence training adaptations and the progression of overtraining.

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Table 3.1. Three-week Cycle Training Protocol.

Week 1 (visits 1 to 7)	Week 2 (visits 8 to 13)	Week 3 (days 14 to 19)
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 @ 75% PWL	5x5:31 @ 75% PWL
3-min active recovery	3-min active recovery	3-min active recovery
2x20-min @ 65% PWL 5-min active recovery	2x25-min @ 65% PWL 5-min active recovery	2x30-min @ 65% PWL 5-min active recovery
12x45s @ 130% PWL 2-min active recovery	12x50s @ 130% PWL 2-min active recovery	12x55s @ 130% PWL 2-min active recovery
50-min Lactate ride $\geq 3\text{mmol*L}^{-1}$	55-min Lactate $\geq 3\text{mmol*L}^{-1}$	60-min Lactate ride $\geq 3\text{mmol*L}^{-1}$
Rest Day	Rest Day	Rest Day
Workloads for all training sessions were based on a percentage of the peak workload (PWL) achieved during baseline performance testing. In cases where an individual outperformed their PWL during weekly performance testing, workloads during all training sessions were set to a percentage of that individual's highest PWL achieved during any performance test.		

Table 3.2. Sleep Variable Definitions

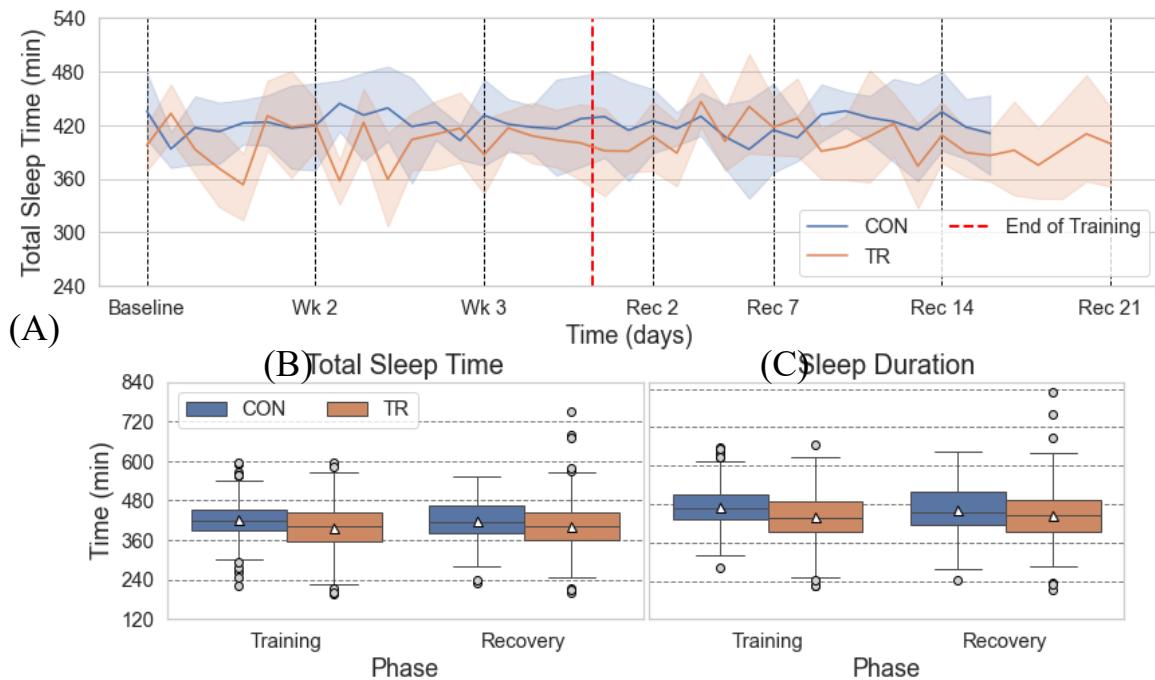
Sleep Duration (min)	The duration of time from when a sleep period started to when a sleep period ended, over the course of a 24-hour day sleep period (total sleep time + wake after sleep onset). Calculated via actigraphy. A 24-hour day was defined as noon to noon.
Total Sleep Time (min)	The amount of time participants were actually asleep during a sleep duration (sleep duration – wake after sleep onset). Calculated via actigraphy.
Wake after Sleep Onset (min)	The amount of time that a person is awake after having initially fallen asleep (sleep duration – total sleep time).
Sleep Efficiency (%)	Total sleep time expressed as a percentage of total sleep duration.
Sleepiness	Rating of sleepiness (1 “extremely alert” to 9 “extremely sleepy”) reported at both sleep and wake times; Karolinska Sleepiness Scale. ⁸¹
Delta Sleepiness	Self-reported sleepiness upon waking – sleepiness at bedtime.
Fatigue	Rating of fatigue (1 “extremely alert” to 7 “completely exhausted”) reported at both sleep and wake times; Samn-Perelli Fatigue Scale. ⁸²
Delta Fatigue	Fatigue upon waking – fatigue at bedtime. reported upon waking from each sleep period.
Sleep Quality	Rating of overall sleep quality (1 “extremely good” to 7 “extremely poor”) reported upon waking.
Sleep Debt	A cumulative effect of an ongoing or extended period of insufficient sleep. Calculated as the cumulative difference in the amount of sleep (total sleep time) in a 24-hour period below the recommended minimum of 7-hours of sleep per night.
Sleep Gain	The cumulative difference in the amount of sleep in a 24-hour period above the recommended minimum of 7-hours of sleep per night.

Table 3.3. Baseline characteristics of CON and TR subjects.

Subjects	n (gender)	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_{2\text{Peak}}$ (ml/kg/min)	% Predicted $\dot{V}O_{2\text{Peak}}$ (ml/kg/min)	PWL _{max} (W)
CON	9 (6f)	29.2 ± 9.1	166.4 ± 10.6	66.5 ± 11.0	40.9 ± 9.8	100.4 ± 21.5%	213.7 ± 50.6
TR	11 (9f)	28.4 ± 8.1	168.8 ± 8.4	72.8 ± 17.3	36.2 ± 6.2	105.3 ± 29.3%	199.4 ± 40.5

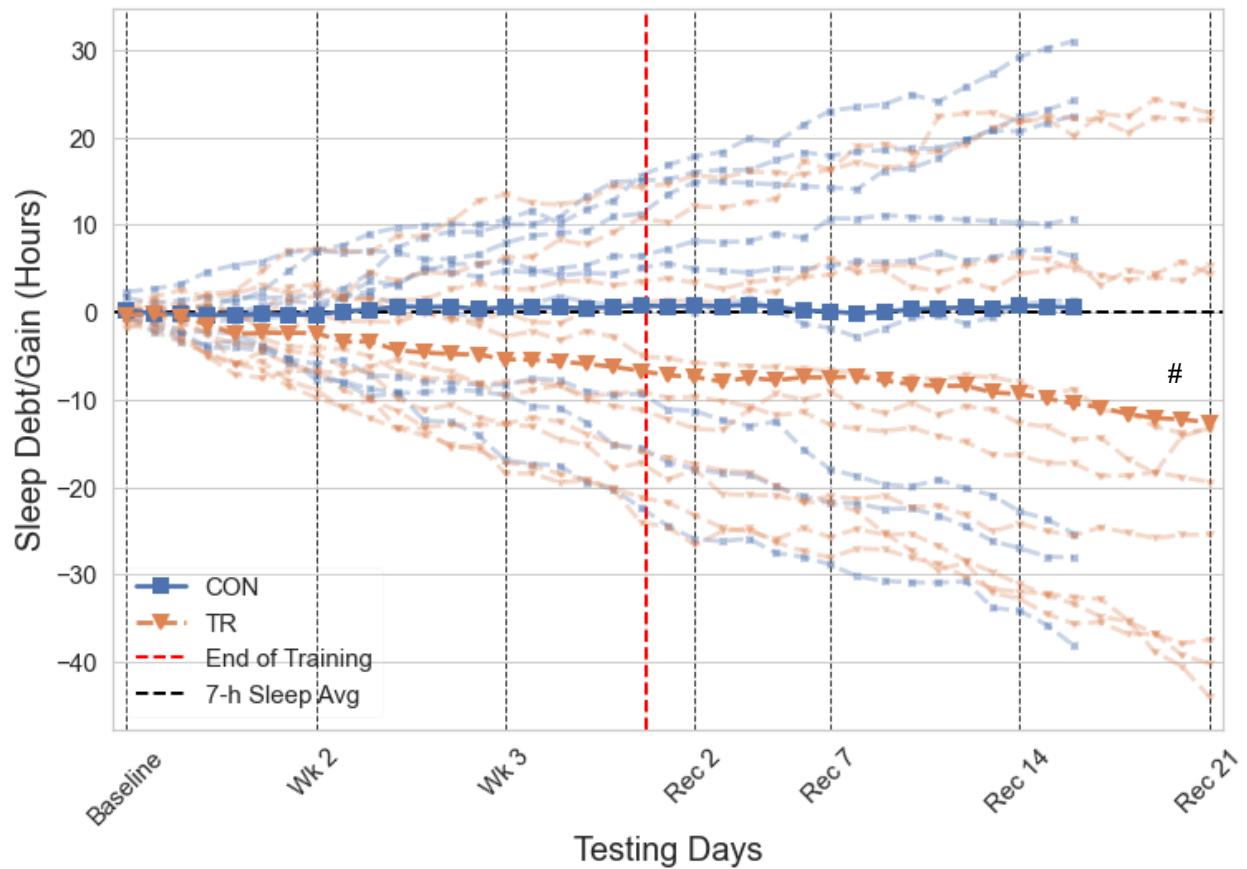
All values are reported from the baseline visit. Values are presented as mean ± SD. There were no between groups differences ($P > 0.05$). Predicted $\dot{V}O_{2\text{Peak}}$ was based on each participant's age, height, weight, and sex.⁸³

Figure 3.1. Total Sleep Time and Sleep Duration in CON and TR subjects.



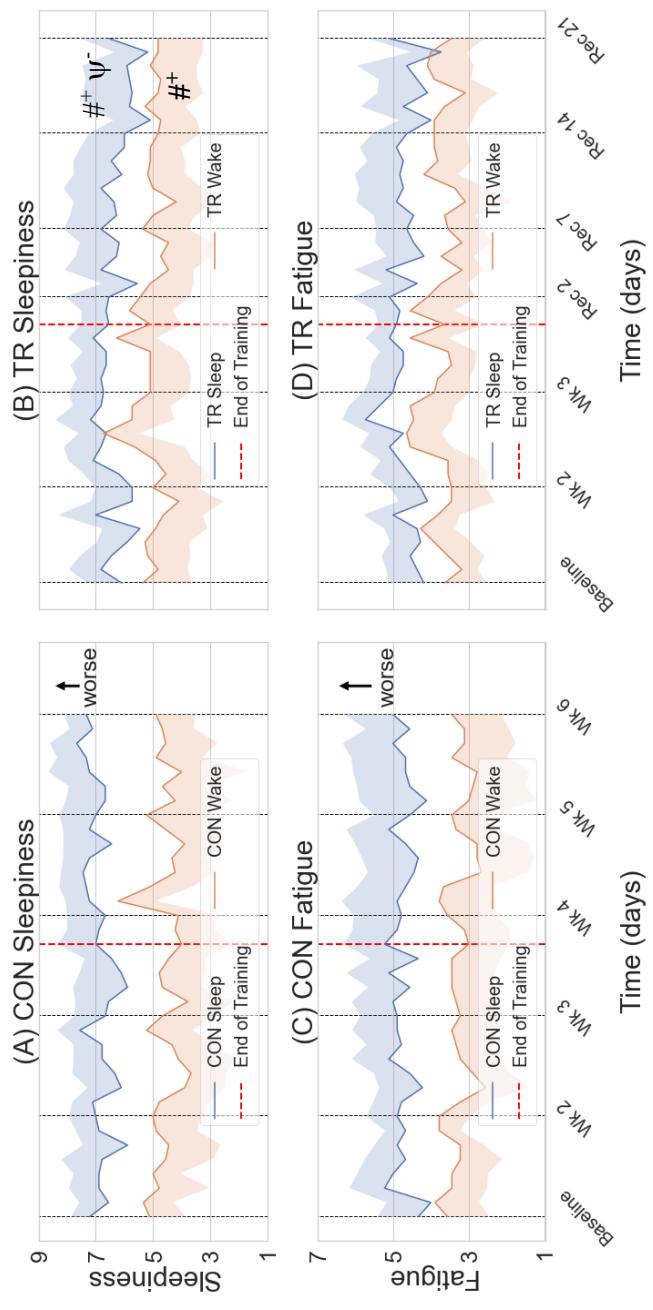
(A) Average (\pm 95% CI) Total Sleep Time across time in control (CON) and training (TR) subjects. Black dashed lines indicate performance testing visits. (B-C) Total Sleep Time and Sleep Duration boxplot comparisons between phases in CON and TR subjects. Compared to CON subjects, TR subjects had more day-to-day variability in Total Sleep Time compared to CON (Levene's $W= 4.78.$, $P<0.05$). White triangles are mean values; horizontal lines in box plots are median group values.

Figure 3.2. Cumulative sleep debt in CON and TR subjects.



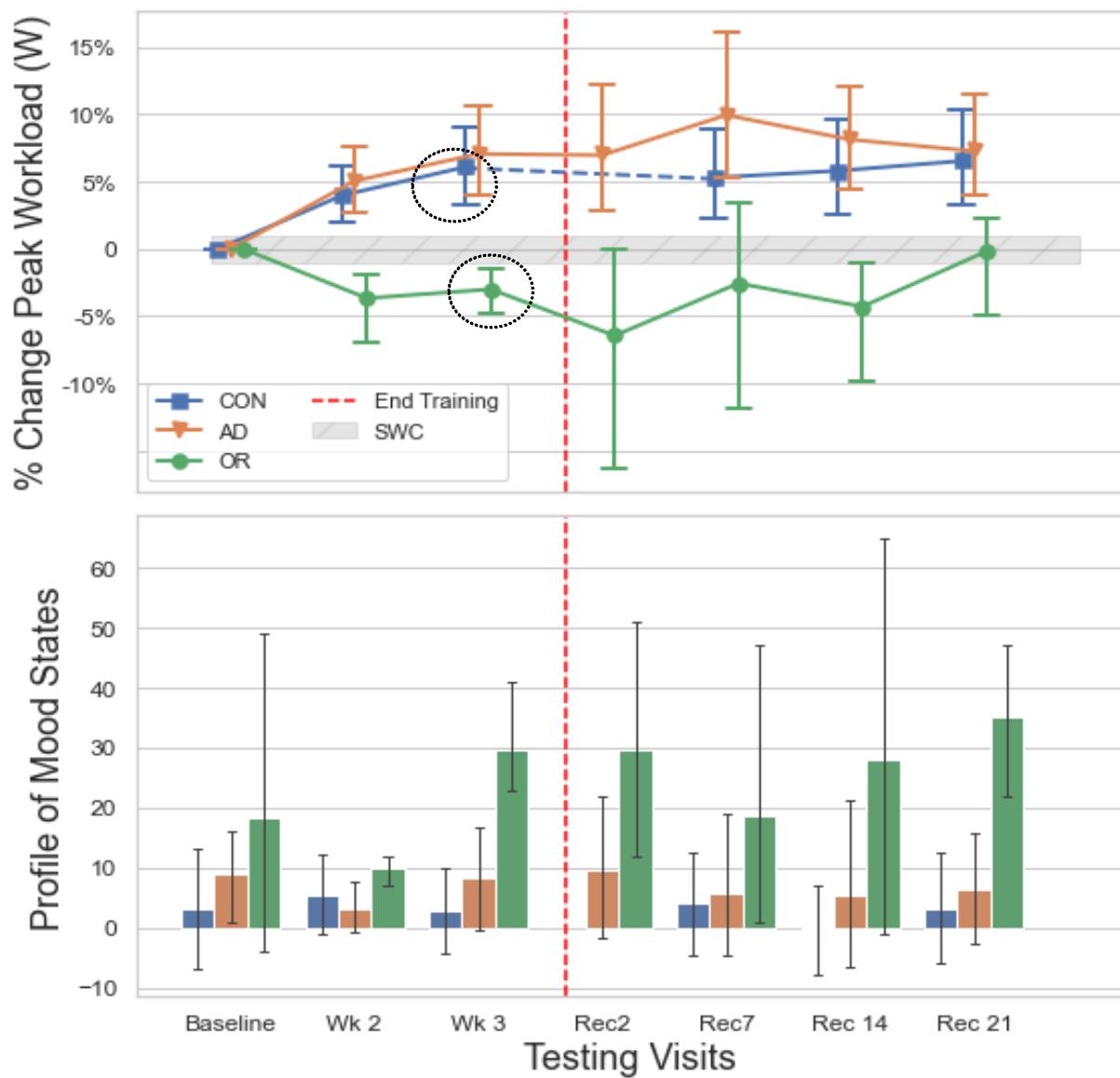
Cumulative sleep debt for control (CON) and training (TR) groups. The rate of sleep debt accumulated over time was higher in TR subjects ($\beta = -23.664$, $P=0.001$). # $P<0.05$ group-time interaction (regardless of phase). Vertical black dashed lines indicate performance testing visits.

Figure 3.3. Self-reported sleepiness and fatigue at bedtime and upon wake in CON and TR subjects.



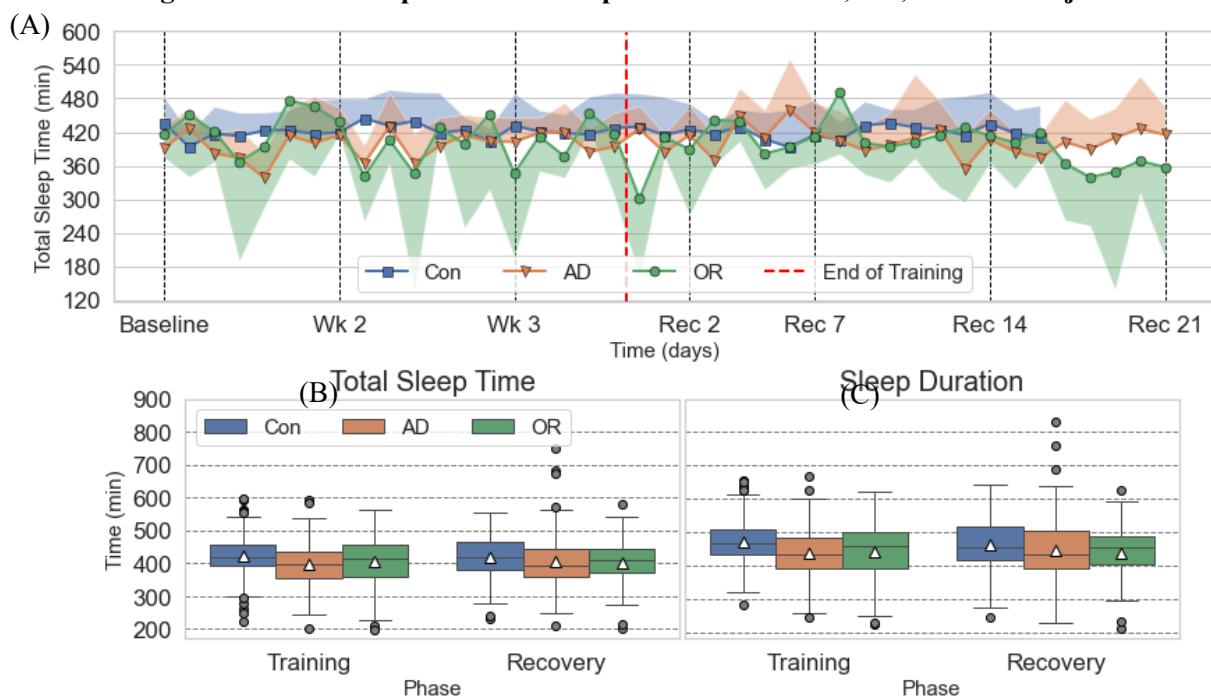
Average ($\pm 95\%$ CI) self-reported sleepiness at bedtime and wake for control (CON) and training (TR) subjects, (A–B, respectively). Average ($\pm 95\%$ CI) self-reported fatigue at bedtime and wake for CON and TR subjects, (C–D, respectively). # $P < 0.05$ group-time interaction (regardless of phase). Vertical black dashed lines indicate performance testing visits. Ψ $P < 0.05$ group-time-phase interaction (regardless of phase).

Figure 3.4. Performance Testing and Profile of Mood States in CON, AD, and OR subjects.



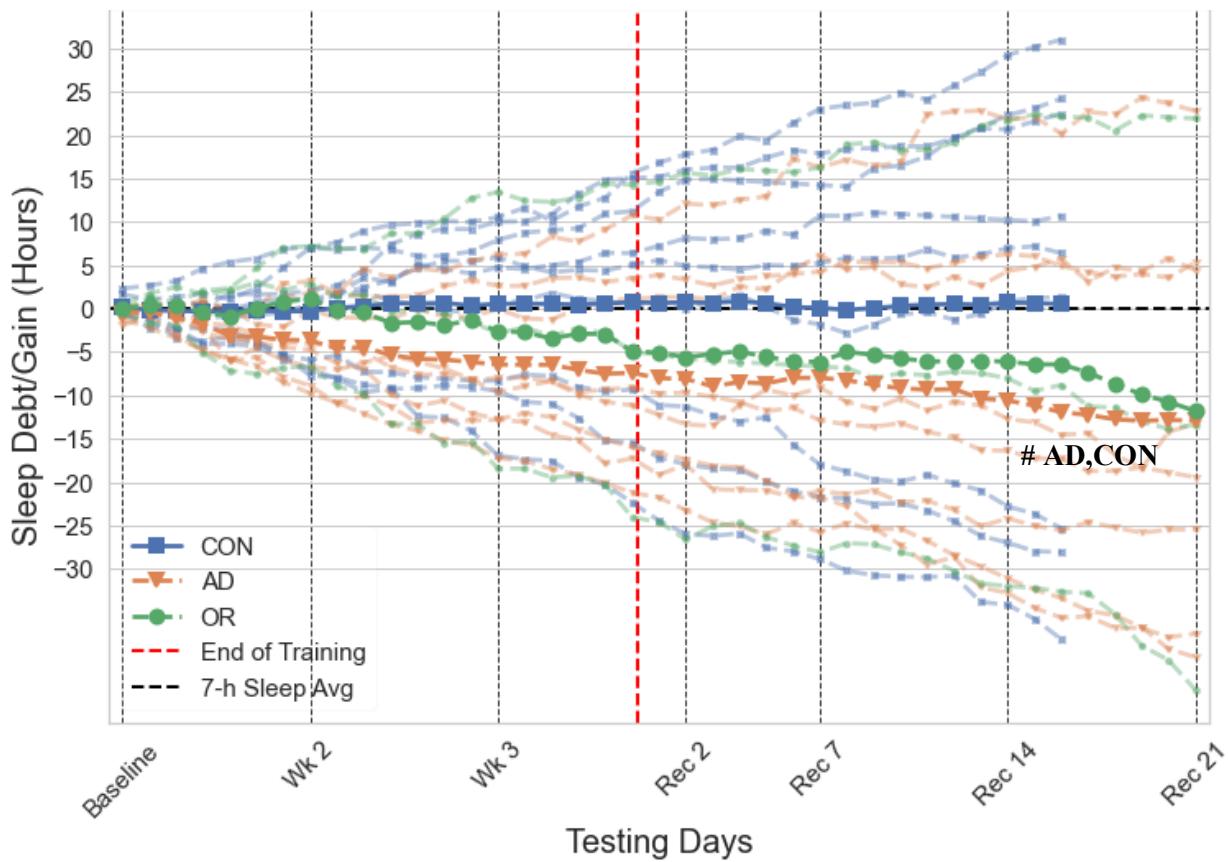
(A) Average change in peak workload (PWL) and (B) POMS total scores from baseline over time in three groups. CON, control; AD, adapted; OR, overreached. Dashed circle, $P<0.05$ group-time interaction at the given time points ($P<0.05$). Grey region represents the OR threshold (smallest worthwhile change).

Figure 3.5. Total Sleep Time and Sleep Duration in CON, AD, and OR subjects.



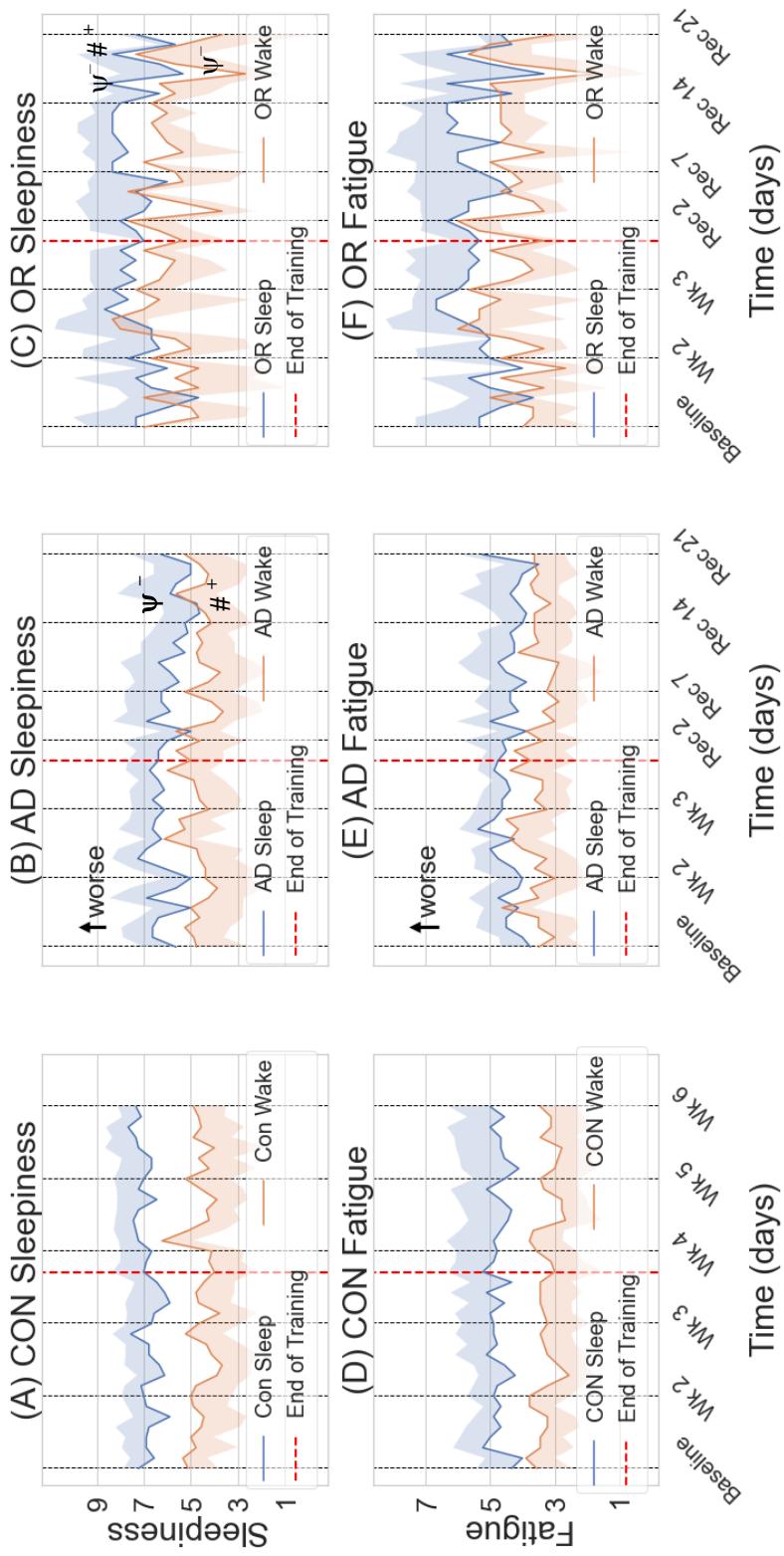
(A) Total Sleep Time across time, (B) total sleep time boxplots between phases, (C) Sleep Duration between phases. CON, control group; AD, adapted; OR, overreached. AD subjects had more day-to-day variability in total sleep time (Levene's $F=5.78$, $P=0.003$), throughout both phases (Games-Howell; $T=-3.61$, $P=0.004$). Sleep duration decreased over time in OR subject across both phases ($\beta=03.387$, $P=0.042$). Vertical black dashed lines indicate performance testing visits. White triangles in boxplots represent mean values.

Figure 3.6. Cumulative sleep debt across time in CON, AD, and OR subjects.



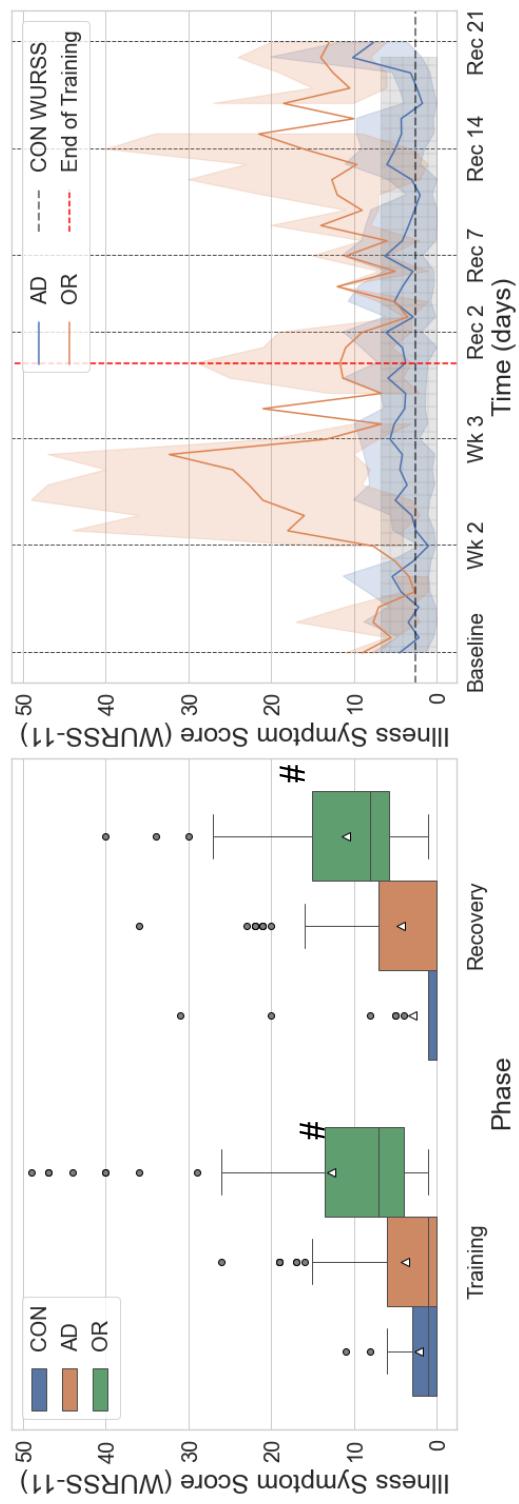
Cumulative sleep debt across time for control (CON), adapted (AD), and overreached (OR) subjects. The rate of sleep debt accumulated over time was greater in AD subjects across both phases ($\beta=-25.948$, $P=0.001$ compared to CON subjects. # $P<0.05$ group-time interaction (regardless of phase). Vertical black dashed lines indicate performance testing visits.

Figure 3.7. Sleepiness and fatigue for bedtime and upon wake in CON, AD, and OR subjects.



Average (\pm 95% CI) self-reported sleepiness at bedtime and wake for control (CON), adapted (AD), and overreached (OR) subjects (A-C, respectively). Average (\pm 95% CI) self-reported fatigue at bedtime and wake score for CON, AD, and OR subjects (D-F, respectively). # $P < 0.05$ group-time interaction (regardless of phase). Ψ $P < 0.05$ group-time-phase interaction. Vertical black dashed lines indicate performance testing visits.

Figure 3.8. Upper respiratory tract illness symptoms in CON, AD, and OR subjects.



Average (\pm 95% CI) upper respiratory tract illness symptom scores from the Wisconsin Upper Respiratory Symptoms Score-11 (WURSS-11). CON, control; AD, adapted; OR, overreached. # $P < 0.05$ group-time interaction (regardless of phase). Horizontal dashed line and shaded regions indicates average (\pm 95% CI) WURSS-11 scores in CON. Vertical dashed black lines indicate performance testing visits.

CHAPTER FOUR: GLOBAL PROTEOME RESPONSES IN RECREATIONALLY ACTIVE
ADULTS FOLLOWING A 3-WEEK OVERREACHING PROGRAM: A PILOT
INVESTIGATION

Introduction.

Optimizing training adaptations requires a meticulous balance between training stimuli and recovery. Athletes commonly undergo periods of intensified training with the intention of improving athletic performance by inducing a physiological response known as *functional* overreach (FOR). Inducing FOR is generally thought to be necessary for promoting meaningful physiological adaptations and performance supercompensation.^{1–3} However, when training demands are persistently met with insufficient recovery, athletes may experience a more prolonged maladaptive response known as *non-functional* overreach (NFOR),¹ resulting in performance decrements lasting several weeks to months. Eventually, if the training-recovery imbalance is not addressed, this maladaptive training response may escalate beyond NFOR to manifest as overtraining syndrome (OTS).^{3,4} Collectively, FOR, NFOR, and OTS comprise the training-overtraining continuum.^{1,5} Symptoms of overtraining include decreased athletic performance and a number of psychophysiological disturbances including increased incidence of illness, worsened mood/behavioral health, and autonomic dysfunction.¹ As an athlete progresses towards OTS, the severity of symptoms may increase; however, there are no clear boundaries besides the time course of performance recovery separating FOR, NFOR, and OTS. Recovery from FOR typically requires several days to weeks, while symptoms of NFOR and OTS can persist for months or years, respectively.^{1,6}

The pathophysiological mechanisms of overtraining remain unknown and there remains an absence of sensitive objective diagnostic criteria to identify overtraining or predict its impending occurrence. Contemporary evidence suggests that subjective measures are more reliable indicators of overtraining status as compared with objective measures such as plasma hormones, cytokines, and workload monitoring.⁷⁻⁹ In the last decade, advances in technology and bioinformatics have made the simultaneous analysis of large quantities of proteins (i.e., the proteome) more feasible. Proteins are the main components of cellular metabolic pathways, and large-scale studies of protein structure and function have proven useful for identifying candidate proteins for studying pathological processes and monitoring drug therapy responses.¹⁰

Large-scale top-down proteomics has the potential to identify upstream biomarkers of overtraining; however, few studies have used large scale proteomics in exercise-based, human research. These studies have focused proteome activity related to the immune response and inflammation,¹¹⁻¹⁶ lipid clearance, and glucose-insulin transport¹⁷ during short-term protocols of exhaustive exercise (i.e., lasting less than a week) or ultra-endurance events.^{14,15} Few studies have examined the impact of prolonged chronic high-intensity endurance training on the proteome.¹⁷⁻¹⁹ Recently, Knab et al.¹⁸ used a targeted panel of proteins previously linked to overreaching (OR)²⁰ as markers of training distress and illness in collegiate swimmers during their competitive swim season. These authors found several immune-related proteins to be upregulated during periods of illness and high training distress.¹⁸

Few studies have examined the influence of high-intensity exercise on the proteome beyond short-term training interventions. Additionally, exercise-based proteomics studies have focused on high-level athletes, thereby limiting the scope of their findings to broader

populations. As such, it is unclear how periods of chronic high-intensity exercise may influence the proteome and the subsequent physiological training adaptations that follow. No previous study has used large-scale proteomics to determine the biological mechanisms of overtraining beyond highly trained athletes, nor have these studies implemented exercise protocols lasting more than a few days to induce a state of OR. Thus, the purpose of this pilot study was to examine the proteome's response to chronic high-intensity exercise in recreationally active adult females following a three-week training protocol under laboratory conditions. We hypothesized that, similar to high-level athletes experiencing FOR/NFOR, recreationally active participants undergoing chronic, high-intensity exercise would exhibit altered proteome expressions associated with increased acute-phase immune system activity and training distress following the training protocol. If successful, these proteins could serve as potential upstream biomarkers of overtraining progression in future overtraining research.

Materials and Methods.

Participants. Seven recreationally active adult females volunteered to participate in this study. These participants were part of a larger investigation seeking to examine the progression of overtraining under laboratory conditions. The study was approved by the Washington State University (WSU) Institutional Review Board (#18860) and was conducted in accordance with the Declaration of Helsinki. Before study participation, subjects completed health screening questionnaires and lung function testing to assess overall health. Participants started the study at random time points in their menstrual cycle.

Experimental Design. Participants underwent a three-week high-intensity training protocol exercising six days per week under laboratory conditions. An overview of the training protocol is shown in **Table 2.1C**. All training sessions were performed in the exercise physiology research lab at the WSU-Spokane campus and consisted of a mix of long-duration, interval, and sprint-like training sessions using a cycle ergometer. All participants reported that they did not regularly engage in cycling exercise in the six months prior to study participation. Workloads for all training sessions were calculated as a percentage of each individual's peak workload (PWL) achieved during performance testing. Exercise performance and proteomic analysis (described below) were assessed during the initial training visit (Baseline [BL]), 48-hours post-training phase (MID), and after a three-week recovery phase (END).

Lab-based Measurements.

Performance Testing Procedures and Peak Oxygen Uptake ($\dot{V}O_{2\text{peak}}$). All exercise and performance testing was done using a magnetically braked cycle ergometer (Ergoselect 200, Ergoline GmbH, Bitz, Germany) to determine $\dot{V}O_{2\text{peak}}$ and cycling PWL. An initial performance test was conducted during the screening visit to familiarize participants with the study protocols and to establish general cardiorespiratory fitness levels of each participant. The second performance test at the onset of the training phase (BL) served as the reference period for changes in outcome measures during all subsequent performance tests. For all lab visits, subjects wore a face mask (Hans Rudolph, Kansas City, MO, USA) for exhaled breath collection. Expired gas volume and oxygen and carbon dioxide concentrations were continuously measured breath-

by-breath (ParvoMedics TrueOne 2400, Salt Lake City, UT, USA) and recorded in 15 second averages.

A graded exercise test (GXT) until volitional exhaustion served as the performance test. Warm-up included cycling for five minutes at 25% PWL from the GXT first performed during the intake visit. The GXT began immediately following warm-up with starting workload set at 75W (females) or 100W (males). Workload increased every two minutes by 30W (females) or 45W (males) until volitional exhaustion, or until participants were unable to maintain a cadence greater than 60rpm. Subjects then proceeded through a five-minute cooldown using the same power output as the warm-up. After a five-minute intermission off the bike, participants completed a second performance test at a constant, supramaximal workload. This second round was used to confirm $\dot{V}O_{2\text{peak}}$, as suggested by Pool and Jones.²¹ The second round included a two-minute warm-up and a constant workload time-to-exhaustion cycling test. Resistance was set at 110% of each participant's PWL achieved during the GXT.

Maximal effort was determined by observing a plateau in the $\dot{V}O_{2\text{Peak}}$ data. If a plateau in $\dot{V}O_2$ was not observed during the GXT, maximal effort was determined by identifying at least two secondary criteria commonly used to determine maximal effort. Secondary criteria, as described by Howley et al.,²² included participants reaching a respiratory exchange ratio of at least 1.10, a peak heart rate over 90% of the predicted maximal HR, or a peak lactate (LA^-_{Peak}) of at least 8 mmol/L. If a participant did not meet two of these three criteria, maximal effort was considered achieved if a participant's $\dot{V}O_{2\text{Peak}}$ from the two exercise rounds differed by less than 0.15 L.

Profile of Mood States. Weekly, on the day of performance testing, participants completed a short-form version of the Profile of Mood States (POMS).²³ The original POMS²⁴ is a 65-item Likert scale questionnaire that assesses vigor, depression, fatigue, anger, anxiety, and confusion. The short-form POMS consists of 37 items from the original POMS. Survey data were collected and managed using REDCap electronic data capture tools hosted at Washington State University.^{25,26}

Upper Respiratory Tract Illness Symptoms. Each day, participants reported their upper respiratory tract illness symptoms using the Wisconsin Upper Respiratory Symptom Score (WURSS-11).^{27,28} Illness symptoms, rated on a seven-point scale, included runny nose, plugged nose, sneezing, sore throat, scratchy throat, cough, and feeling tired. Participants were not restricted from taking medications but were instructed to report any medications taken through their survey forms.

Metabolic Equivalents of Task (MET-min) Per Week. To assess the volume of physical activity in the training protocol, metabolic equivalents of task (MET-min) were calculated as follows:

$$\text{MET-min} = \left(\sum \frac{\dot{V}\text{O}_2 \text{ during exercise}}{\text{resting } \dot{V}\text{O}_2} \right) \times \text{exercise duration (minutes)}$$

For each participant, MET-min per week was calculated by summing the calculated MET-min for all exercise sessions throughout each week of the three weeks during the training protocol. During all exercise training sessions, breath-by-breath gas exchange was collected using a

facemask (Hans Rudolph, Kansas City, MO, USA) and metabolic cart (TrueOne 2400, ParvoMedics) and recorded in 15 second averages.

Dried Blood Spot Collection. Dried blood spots (DBS) samples were collected via finger prick onto standard blood spot cards (Whatman protein saver cards, Sigma-Aldrich, St. Louis, MO, USA). DBS Samples were collected at BL, MID, and END timepoints. DBS Samples were collected at the beginning of performance testing visits after participants had rested for five minutes in a seated position, prior to exercise. For all DBS samples, researchers guided each participant's finger to prevent physical contact with the DBS card. Researchers massaged blood flow in the hand towards the fingertip to release a drop of blood approximately the size of a raindrop on the center of each blood collection circle. Collected DBS samples were dried under ambient conditions and stored at -80°C in a sealed bag with desiccant until all samples were collected and ready for processing.

Sample Preparation For Proteomics Analysis By Liquid Chromatography-Mass Spectrometry. One 6 mm wide punch was made in the center of a DBS sample. From the DBS sample punch, proteins were resolubilized in 6 M urea, 50mM ammonium bicarbonate, and 0.1 mM dithiothreitol for 30 minutes at 37°C while shaking continuously. Samples were then stored at -80°C until they were transported to the Tissue Imaging, Metabolomics and Proteomics Laboratory at the WSU-Pullman campus for proteomics analysis.

From the processed DBS samples, 40 µg of protein were reduced, alkylated, and subjected to trypsin digestion using 1.5 µg Pierce Lys-C/trypsin protease mix mass spectrometry (MS) grade (Thermo Scientific Cat. #A40009, Waltham, MA, USA) as per the manufacturer's instructions. Samples were first allowed to digest under high salt conditions in a volume of 80 µL, and then diluted to 1 M urea with 50 mM ammonium bicarbonate to activate trypsin and incubated at 37°C overnight. Peptides were purified using G-Biosciences (St. Louis, MO, USA) C18 spin columns (Cat. #786-931), as per the manufacturer's instructions. Dry residues were dissolved in a small volume of 3% acetonitrile in water with 0.1% formic acid. Peptide concentrations were determined using Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific Cat. #23275). For each sample, 300 ng of peptides were used for proteomics analysis by liquid chromatography-mass spectrometry (HPLC).

Proteomics Analysis by Liquid Chromatography-Mass Spectrometry. Analyses are performed using an ultra-HPLC system (Easy nanoLC 1000, Thermo Scientific) coupled to a Fusion Orbitrap Tribrid mass spectrometer (Thermo Scientific) in data-dependent acquisition (DDA) mode. Protein samples were loaded on the column in trapping mode with 3 µL injected onto the column corresponding to 300 ng total protein. The pre-column used was Acclaim PepMap 100 (75 µm x 20 mm, 3 µm particle size, Thermo Scientific Cat. #164535). The analytical column used was an ACQUITY UPLC M-Class Peptide BEH C18 column (75 µm i.d. x 100 mm, 1.7 µm particle size, Waters Corporation, Cat. #186007481, Milford, MA, USA). The flow rate during the gradient was kept at 350 nL per minute with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) as mobile phase and the gradient

conditions were: 3% to 35% B for 120 minutes, 35% to 90% in 5 minutes, and held at 90% for 20 minutes. Both columns were equilibrated with 10 µL solvent A prior to sample loading.

A Fusion Orbitrap Tribrid mass spectrometer (Thermo Scientific) was used for peptide MS/MS analysis. Samples were introduced using the Nanospray Flex source ion source (Thermo Scientific, Cat. ES 071). Positive electrospray mode voltage was 2100 V and ion transfer tube was set to 275°C. Survey scans of peptide precursors were performed with a mass range of m/z 200-1800 at 120K full width half-maximum (FWHM) resolution. The instrument was set to run at top speed mode with three second cycles for the survey and tandem MS/MS scans. After the survey scan, tandem MS/MS was performed on the most abundant precursors exhibiting a charge state from 2 to 6 of greater than 3000 intensity by isolating the precursors in the quadrupole at a window of 1.6 *m/z*. Dynamic exclusion parameters were to exclude after 1 time for 60 seconds with 10 ppm mass tolerance. Higher energy collisional dissociation (HCD) fragmentation was applied at 30% collision energy and resulting fragments were detected using the ion trap at a rapid rate. Quality control samples of commercial digests of Pierce bovine serum albumin ([BSA], Thermo Scientific, Cat. #88341) and Pierce HeLa cells (Thermo Scientific, Cat. #88329) were analyzed prior to experimental runs and had to meet set thresholds of >75% coverage (BSA) and >2700 protein IDs (HeLa). Raw data was processed using Proteome Discoverer (v2.2) and searched against the human proteome (downloaded from UniProt on December 28, 2020) using the SEQUEST HT engine.²⁹

Statistical Analysis.

Performance Testing and Mood States. The Shapiro-Wilk test was utilized to assess the normality of the data. A repeat-measures analysis of variance (RM ANOVA) was used to compare differences in performance outcomes (PWL, $\dot{V}O_{2\text{peak}}$), POMS scores and WURSS scores among BL, MID, and END timepoints. In cases where outcome measures did not exhibit a normal distribution, the nonparametric Friedman test was employed. Daily WURSS scores were averaged during the training and recovery phases to correspond with MID and END timepoints, respectively, during analysis.

Proteomic Analysis. The relative abundance of proteins across all timepoints was normalized by calculating log₂ fold-change ratios with abundance values at BL serving as the reference period. Not all proteins were identified at all three timepoints in all samples. As such, the Friedman test was used to compare protein abundance for all proteins shared among the three timepoints, while Wilcoxon signed-rank tests were used to compare protein abundance for all proteins shared between BL and MID timepoints, and between BL and END timepoints. In line with previous systems biology approaches,^{30,31} unadjusted *P*-values (*p*≤0.1) were used to determine significant fold change. All data analysis was conducted using Statsmodels (v0.13.2),³² SciPy (v11.1.4),³³ and Pingouin (0.5.4)³⁴ libraries in Python.

Protein-Protein Interaction Network Analysis. Proteins with significant changes in abundance across time were mapped onto STRING v12 (search tool for the retrieval of interacting genes and proteins) to build and investigate protein-protein interaction (PPI) networks. STRING v12 is

a database of known and predicted physical functional protein associations based on data mining, genomic context, high-throughput experimentation, co-expression, and previous knowledge (<https://string-db.org/>).³⁵

Results:

Lab-based Exercise Measures. Subject characteristics are shown in **Table 4.2**. During the training protocol, participants performed an average of 1216 ± 131 , 1391 ± 146 , and 1551 ± 245 MET-min of physical activity during each week of the training protocol (**Figure 4.1**). Performance outcomes (PWL, $\dot{V}O_{2\text{peak}}$), POMS scores, and WURSS scores were compared across time and are shown in **Table 4.3 and Figure 4.2**. These outcome measures did not significantly change from BL during MID or END timepoints ($P>0.05$).

Proteomics Analysis. A total of 380 unique proteins were identified from the proteomics procedures. 206 proteins were identified among all 21 samples (seven subjects, three timepoints), resulting in a recovery rate of 54.2%. The recovery rate between BL and MID timepoints and BL and END timepoints was 55% (209 proteins) and 58% (219 proteins), respectively. A total of 38 proteins were up- or downregulated from BL at MID or END timepoints (**Table 4.4**). All upregulated proteins ($n=35$) from the three tests were grouped together to construct an upregulated protein-protein interaction (PPI) network. Six proteins were downregulated from BL at either MID (P43487, P07195, P22061), END (P07451, P55072) or both (Q9BTM1)

timepoints. As such, there was an insufficient number of downregulated proteins to construct a downregulated PPI network.

Upregulated Proteins from Baseline to 48-hours Post-Training. All proteins upregulated from BL to either MID or END timepoints are shown in **Table 4.4** and **Figure 4.3**. Of these 38 proteins, 19 were immune-related and entered into STRING. The mean log2-fold change for upregulated immune-related proteins was 0.456 ± 0.213 at MID and 0.572 ± 0.306 at END, with an average local cluster coefficient of 0.706 ($P<0.001$). Reactome Pathway terms from STRING supported an increase in proteins related to the innate immune system, fibrin clot formation, regulation of complement system, platelet degranulation, and neutrophil degranulation (**Table 4.5, Figure 4.4**).

Discussion.

The primary aim of this pilot investigation was to identify potential upstream biomarkers of overtraining progression using proteomic analysis. Using DBS sampling, A total of 380 unique proteins were identified among seven moderately fit females who underwent a three-week high-intensity aerobic training protocol under laboratory conditions. STRING PPI networks found multiple proteins ($n=19$) related to the acute-phase immune response, including complement system activation, neutrophil degranulation, and platelet degranulation. Many of these proteins were upregulated both immediately post-training (MID) and after three weeks of recovery (END). These findings suggest that chronic high-intensity exercise may cause a prolonged

increase in the acute-phase immune response and supports findings from previous exercise-based proteomics studies that found altered acute-phase response-related proteins in overreached athletes.^{15,20,36}

S100-A4 was the most upregulated protein following the training protocol at both MID and END timepoints. To our knowledge, previous exercise-based proteomic research has not identified this protein in relation to overtraining. Upregulated S100-A4 has been linked with cardiac muscle tissue repair following cardiac injury (myocardial infarction)^{37,38} and was found upregulated in rats after exhaustive exercise.³⁹ S100 proteins interact with other proteins to modulate various biological functions including inflammation, innate immunity, tissue damage, and wound healing.⁴⁰ Other S100 proteins (S100-A8, S100A9, S100A12) are abundantly expressed by neutrophils.⁴¹ Neutrophils are the most abundant leukocyte in circulation and are the first line of defense against pathogens.⁴² Neutrophil activity and other aspects of innate immunity can be altered for hours to days following prolonged and intensive endurance exercise.⁴³ While considered a part of the innate immune system, neutrophils are continuously recruited to sites of chronic inflammation, which can lead to tissue damage during an exaggerated inflammatory response.⁴⁴ As neutrophils have a circulating half-life of 6-8 hours,⁴⁵ it is plausible that the upregulation of neutrophil-related proteins observed at both MID and END timepoints is indicative of chronic inflammation as a training response from the exercise training protocol (**Table 4.5, Figure 4.4**). As we did not directly observe neutrophil activity between MID and END timepoints, it is also plausible that participants were experiencing separate instances of upregulated innate immune activity during these timepoints. However, there were no

significant increases in WURSS-11 scores from BL at MID or END timepoints to indicate possible upper respiratory illness.

In the present study, multiple proteins related to the complement system (complement C3, complement C6, plasma protease C1 inhibitor) were upregulated following the training protocol. These findings support previous research that found upregulated complement proteins in high-level athletes experiencing OR, or following extreme athletic events.^{15,16,20,36} The complement system is a fundamental aspect of the innate immune system and functions as a cascade of proteases that act upon each other in an enzymatic fashion.⁴⁶ Protease cascades of the three complement pathways (classical, lectin, alternative) each result in the cleavage of complement C3,^{46,47} whose cleavage promotes chemotaxis, opsonization of target cells (e.g., pathogens) for phagocytotic cells, and lysis of target cells via the membrane attack complex (complement components C5b, C6, C7, C8, C9).⁴⁸ The effects of exercise on complement proteins are poorly understood. This is partly due to the complexities of complement regulation, the vast number of complement proteins (there are more than 40 complement proteins), and the heterogeneity of exercise stimuli used when observing the complement system cascade in relation to exercise.⁴⁶ Activation of all three complement pathways, and subsequently a pro-inflammatory response, can occur following acute exercise and last up to four days, depending on the extent of muscle damage.^{46,49,50} Previous research has found that an acute bout of exercise (e.g., running or cycling 30 to 60 minutes) was insufficient to increase various complement proteins.⁵¹⁻⁵⁵ Conversely, longer-duration exercise (e.g., ultra-endurance) or intense resistance training has been shown to upregulate complement components up to 72-hours after exercise.^{16,56,57} The time course of complement upregulation may provide an explanation for the

upregulated complement proteins found at MID, but would not explain the upregulated complement C3 observed at END in the present study. As such, persistent upregulation of complement proteins is also suggestive of a chronic acute-phase inflammatory response following chronic high-intensity exercise.

The current Physical Activity Guidelines for Americans recommends at least 500 MET-min of physical activity (150 minutes of moderate-to-vigorous aerobic activity) per week for optimal health,⁵⁸ and it has been estimated that maximal cardiovascular health benefits are obtained at exercise volumes approximately three to four times these recommendations.^{59,60} As such, the lack of a positive training response in aerobic capacity or performance, even after the three-week recovery phase, was unexpected. There is some evidence to suggest that athletes experiencing OR may exhibit attenuated improvements in aerobic capacity compared to non-OR (acutely fatigue) counterparts following intensified training;^{61–63} however, we did not distinguish AD from OR participants in this study given the small sample size. Nevertheless, the lack of a training response after three-weeks of high-intensity aerobic training may suggest an overtraining response in the absence of a detectable decline in exercise performance.

Limitations. This study had several limitations. An unadjusted p-value of 0.1 to determine significant changes in protein abundance, which carries an increased risk of type I error. Systems biology research often adjusts the alpha level to account for the false discovery rate (e.g., Benjamini-Hochberg method);⁶⁴ however, some researchers advise against correcting for multiple comparisons with proteomics as it is still a large-scale technology and reproducibility of results is limited.⁶⁵ As such, while not correcting for multiple comparisons may increase the risk

of type I error, doing so has a high risk of type II error.⁶⁶ Still, the results of this study should be interpreted with caution considering the small sample size of this pilot investigation, and should be validated against future proteomics studies using larger sample sizes. Different approaches to proteomic analysis can dramatically impact how many proteins are identified, which can make it challenging to compare results across studies.⁶⁷ For example, we identified 380 proteins using DDA proteomics analysis, which is in line with proteomics studies using DDA techniques;^{68–71} however, other proteomics studies using data independent acquisition have been able to identify several hundred to nearly two thousand proteins in their analyses.^{36,72} The use of DBS sampling for multiomics procedures is minimally invasive and simple to collect; however, hematocrit can influence the spread of whole blood and thus the protein concentration among different DBS samples.⁷³ DBS sample collection, protein extraction, and proteomic analysis took place in different labs. MS-based proteomics involves multiple steps from collection, protein extraction, and proteome analysis.^{74,75} Additionally, this study required the collaboration of multiple laboratories, which may have further affected the protein recovery and limited protein identification.

Conclusion. This study investigated the proteome in seven recreationally active females, who underwent a three-week high-intensity training protocol. To our knowledge, this study is the first study to examine how chronic high-intensity exercise may influence the proteome using large-scale proteomics. Multiple proteins related to the innate immune system (complement activation, neutrophil degranulation, platelet degranulation, and fibrin clot formation) were upregulated immediately following the training phase (MID) and three weeks after the cessation of training

(END). Together, the findings of this study suggest that excessive or recurring high-intensity exercise may generate a chronic inflammatory response and supports the findings of previous exercise-based proteomics research, which has found similar evidence of immune dysfunction following exhaustive exercise or ultra-endurance events. Future studies with larger samples sizes are warranted to further investigate the effects of prolonged or excessive exercise/physical activity on the immune system in a variety of populations.

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Table 4.1. Three-week Cycle Training Protocol.

Week 1 (visits 2 to 7)	Week 2 (visits 8 to 13)	Week 3 (visits 14 to 19)
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 @ 75% PWL	5x5:31 @ 75% PWL
3-min active recovery	3-min active recovery	3-min active recovery
2x20-min @ 65% PWL	2x25-min @ 65% PWL	2x30-min @ 65% PWL
5-min active recovery	5-min active recovery	5-min active recovery
12x45s @ 130% PWL	12x50s @ 130% PWL	12x55s @ 130% PWL
2-min active recovery	2-min active recovery	2-min active recovery
50-min Lactate ride $\geq 3\text{mmol*L}^{-1}$	55-min Lactate $\geq 3\text{mmol*L}^{-1}$	60-min Lactate ride $\geq 3\text{mmol*L}^{-1}$
Rest Day	Rest Day	Rest Day

Table 4.2. Subject characteristics at Baseline (BL).

Subject	Age (years)	Height (cm)	Weight (kg)	VO ₂ Peak (ml/kg/min)	VO ₂ Peak ⁷⁶ (% Predicted)
A	21	178	71.39	33.79	88.78%
B	31	160	56.97	36.8	96.36%
C	40	183	99.40	36.81	161.24%
D	46	178	92.72	33.09	147.20%
E	22	17	58.30	41.98	101.57%
F	25	156	46.14	43.13	99.01%
G	21	166	83.02	33.13	95.17%
Group (mean ± SD)	29.4 (10.0)	170 (58.8)	72.6 (19.9)	36.7 (4.1)	107.0% (26.9%)

Predicted VO₂Peak was based on subject characteristics at baseline including age, sex, height, and weight.⁷⁶

Table 4.3 Performance Outcomes, POMS scores, and WURSS scores at BL, MID, and END.

Outcome Measure	BL	MID	END	P-value
PWL (W)	200.8 ± 34.5	206.5 ± 33.8	211.7 ± 34.0	0.290
% PWL from Baseline	---	$103.6\% \pm 11.8\%$	$105.8\% \pm 8.1\%$	0.323
VO2Peak (ml/kg/min)	36.93 ± 4.15	38.64 ± 8.23	39.87 ± 6.94	0.446
% VO2Peak from Baseline	---	$103.9\% \pm 12.5\%$	$107.6\% \pm 9.8\%$	0.335
POMS	16.4 ± 16.1	15.6 ± 20.0	14.3 ± 19.1	0.879
WURSS	5.6 ± 4.6	6.5 ± 8.7	5.5 ± 9.1	1.000

WURSS scores at MID and END represent average WURSS scores during the Training and Recovery phases, respectively.

Table 4.4. Description of upregulated and downregulated proteins.

Gene	UniProt Protein	Description	Basic Function	MID END
HP	P00738	Haptoglobin	Hepatic recycling of heme iron; antioxidant; antibacterial; modulates acute phase immune response.	↑↑
PLG	P00747	Plasminogen	Proteolytic factor tissue remodeling, tumor invasion, and inflammation.	↑↑
A2M	P01023	Alpha-2-macroglobulin	Inhibits all four classes of proteinases.	↑-
C3	P01024	Complement C3	Central role in the activation of complement system cascade by all pathways (classical, lectin, alternative); chemoattractant for neutrophils in chronic inflammation.	-↑
IGHV3-30	P01768	Immunoglobulin heavy variable 3-30	Variable domain of immunoglobulin heavy chains that participates in the antigen recognition.	↑↑
SPTA1	P02549	Spectrin alpha chain, erythrocytic 1	Major constituent of cytoskeletal network underlying erythrocyte plasma membrane.	-↑
SLC4A1	P02730	Band 3 anion transport protein	erythrocyte flexibility and stability. Functions as a transporter inorganic anions across erythrocyte membrane.	↑-
FN1	P02751	Fibronectin	Various processes; cell adhesion/motility, opsonization, wound healing, and maintenance of cell shape.	-↑
GC	P02774	Vitamin D-binding protein	Vitamin D transport/storage; scavenging of extracellular G-actin; proinflammatory, enhances C5 alpha response for neutrophils and macrophage activation.	↑-
ALDOA	P04075	Fructose-bisphosphate aldolase A	Plays a key role in glycolysis and gluconeogenesis; possible scaffolding protein.	↑↑
APOB	P04114	Apolipoprotein B-100	Major protein constituent of chylomicrons; functions as a recognition signal for the cellular binding and internalization of LDL particles.	↑↑
CAPNS1	P04632	Calpain small subunit 1	cytoskeletal remodeling and signal transduction; embryonic development.	↑-
SERPING1	P05155	Plasma protease C1 inhibitor	Controls activation of C1 complex in complement system; roles in complement activation, blood	↑-

			coagulation, fibrinolysis and generation of kinins.	
SERPINA7	P05543	Thyroxine-binding globulin	Major thyroid hormone transport protein in serum.	↑-
SERPIND1	P05546	Heparin cofactor 2	Regulates thrombin and coagulation process during inflammation and wound healing.	↑-
LDGB	P07195	L-lactate dehydrogenase B chain	Simultaneously Interconverts pyruvate and lactate with concomitant interconversion of NADH and NAD+.	↓↑
CAPN1	P07384	Calpain-1 catalytic subunit	Ca+ regulated protease. Catalyzes substrates involved in cytoskeletal remodeling and signal transduction	↑↑
TUBB	P07437	Tubulin beta chain	Major constituent of microtubules.	↑↑
CA3	P07451	Carbonic anhydrase 3	Bidirectional conversion of carbon dioxide (CO2) and water (H2O) into bicarbonate (HCO3) and H+.	→↓
SERPINF2	P08697	Alpha-2-antiplasmin	Serine protease inhibitor. Major targets are plasmin and trypsin.	↑↑
C4B	P0C0L5	Complement C4-B	Non-enzymatic component C3, C5 convertases; essential for complement activation.	↑-
C6	P13671	Complement component C6	Constituent of the membrane attack complex of complement system; forms pores in target cells.	↑↑
APEH	P13798	Acylamino-acid-releasing enzyme	Catalyzes the hydrolysis of the N-terminal peptide bond of an N-acetylated peptide.	→↑
EPB42	P16452	Erythrocyte membrane protein band 4.2	Regulates ion channel activity and transmembrane ion transport; regulates ASIC2 and ASIC3 channel activity.	↑↑
ITIH2	P19823	Inter-alpha-trypsin inhibitor heavy chain H2	Carrier of hyaluronan in serum or as a binding protein between hyaluronan and other matrix protein.	↑-
PCMT1	P22061	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	Assists in the repair and/or degradation of damaged proteins.	↓-
CPN2	P22792	Carboxypeptidase n subunit 2	Binds and stabilizes the catalytic subunit at 37°C and keeps it in circulation.	↑↑
S100A4	P26447	Protein S100-A4	Ca+ binding protein; role in motility, angiogenesis, cell differentiation, autophagy, and apoptosis.	↑↑

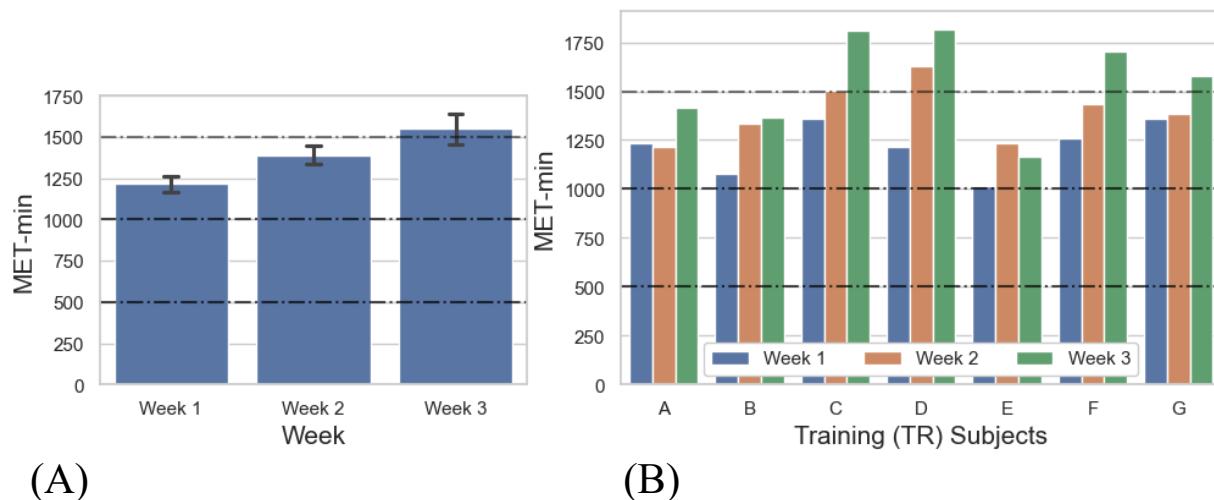
STOM	P27105	Erythrocyte band 7 integral membrane protein	Erythrocyte membrane protein band 4.2; probable role in regulation of erythrocyte shape and mechanical properties.	-↑
GLRX	P35754	Glutaredoxin-1	Glutathione-disulfide oxidoreductase activity in the presence of NADPH and glutathione reductase.	↑↑
SNCA	P37840	Alpha-synuclein	Roles in synaptic activity.	↑↑
RANBP1	P43487	Ran-specific GTPase-activating protein	Required for normal mitotic spindle assembly.	↓↑
ARHGDIIB	P52566	Rho GDP-dissociation inhibitor 2	Regulates GDP/GTP exchange reaction of Rho proteins; Regulates reorganization of actin cytoskeleton mediated by Rho family members.	-↑
VCP	P55072	Transitional endoplasmic reticulum ATPase	Necessary for the fragmentation of Golgi stacks during mitosis and mitosis; essential for maturation autophagosomes.	-↓
PRPS1	P60891	Ribose-phosphate pyrophosphokinase 1	Essential for nucleotide synthesis.	↑↑
DMTN	Q08495	Dematin	Plays a role in maintaining functional integrity of PKA-activated erythrocyte shape and membrane mechanical properties; modulates actin dynamics in fibroblasts; tumor suppressor.	↑-
ITIH4	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	Type II acute-phase protein involved in inflammatory responses to trauma.	↑-
H2AJ	Q9BTM1	Histone H2A.J	Core component of nucleosome; central role in transcription regulation, DNA repair, DNA replication and chromosomal stability.	↓↓
Proteins identified as either up- or downregulated from BL values (n=41; P<0.1). Arrows indicate whether protein was up- or downregulated at MID and END timepoints, respectively. Dash indicates protein not identified at MID or END timepoint, respectively. Protein information and descriptions retrieved from UniProt Knowledgebase (http://www.uniprot.org). ⁷⁷				

Table 4.5. Upregulated immune-related proteins from BL.

Gene	UniProt Protein	Description	MID Log2-FC	END Log2-FC	P-value
SERPING1	P05155	Plasma protease C1 inhibitor	0.994	---	0.047
CPN2	P22792	Carboxypeptidase n subunit 2	0.644	0.727	0.066
ALDOA	P04075	Fructose-bisphosphate aldolase A	0.572	0.538	0.066
TUBB	P07437	Tubulin beta chain	0.547	0.324	0.050
PLG	P00747	Plasminogen	0.523	0.293	0.004
C6	P13671	Complement component C6	0.494	0.483	0.066
APOB	P04114	Apolipoprotein B-100	0.461	1.405	0.005
IGHV3-30	P01768	Immunoglobulin heavy variable 3-30	0.428	0.333	0.050
HP	P00738	Haptoglobin	0.427	0.937	0.050
SERPINF2	P08697	Alpha-2-antiplasmin	0.360	0.835	0.050
A2M	P01023	Alpha-2-macroglobulin	0.326	---	0.078
SERPIND1	P05546	Heparin cofactor 2	0.288	---	0.031
C4B	P0C0L5	Complement C4-B	0.199	---	0.078
CAPN1	P07384	Calpain-1 catalytic subunit	0.117	0.252	0.066
C3	P01024	Complement C3	---	0.419	0.078
FN1	P02751	Fibronectin	---	0.442	0.031

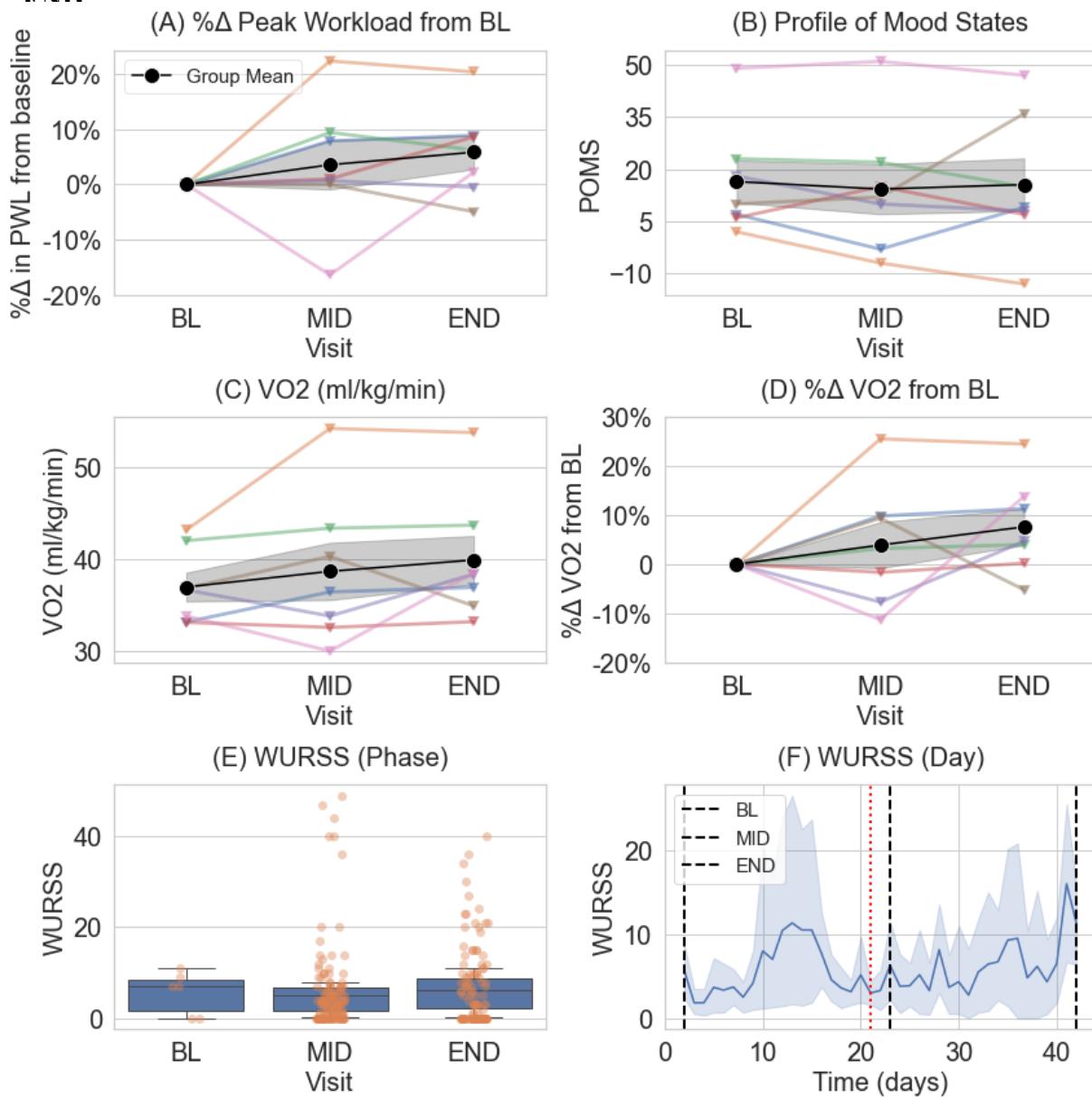
APEH	P13798	Acylamino-acid-releasing enzyme	---	0.685	0.078
STOM	P27105	Erythrocyte band 7 integral membrane protein	---	0.506	0.031
ITIH4	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	---	0.406	0.078

Figure 4.1. MET-min per week of exercise from training protocol.



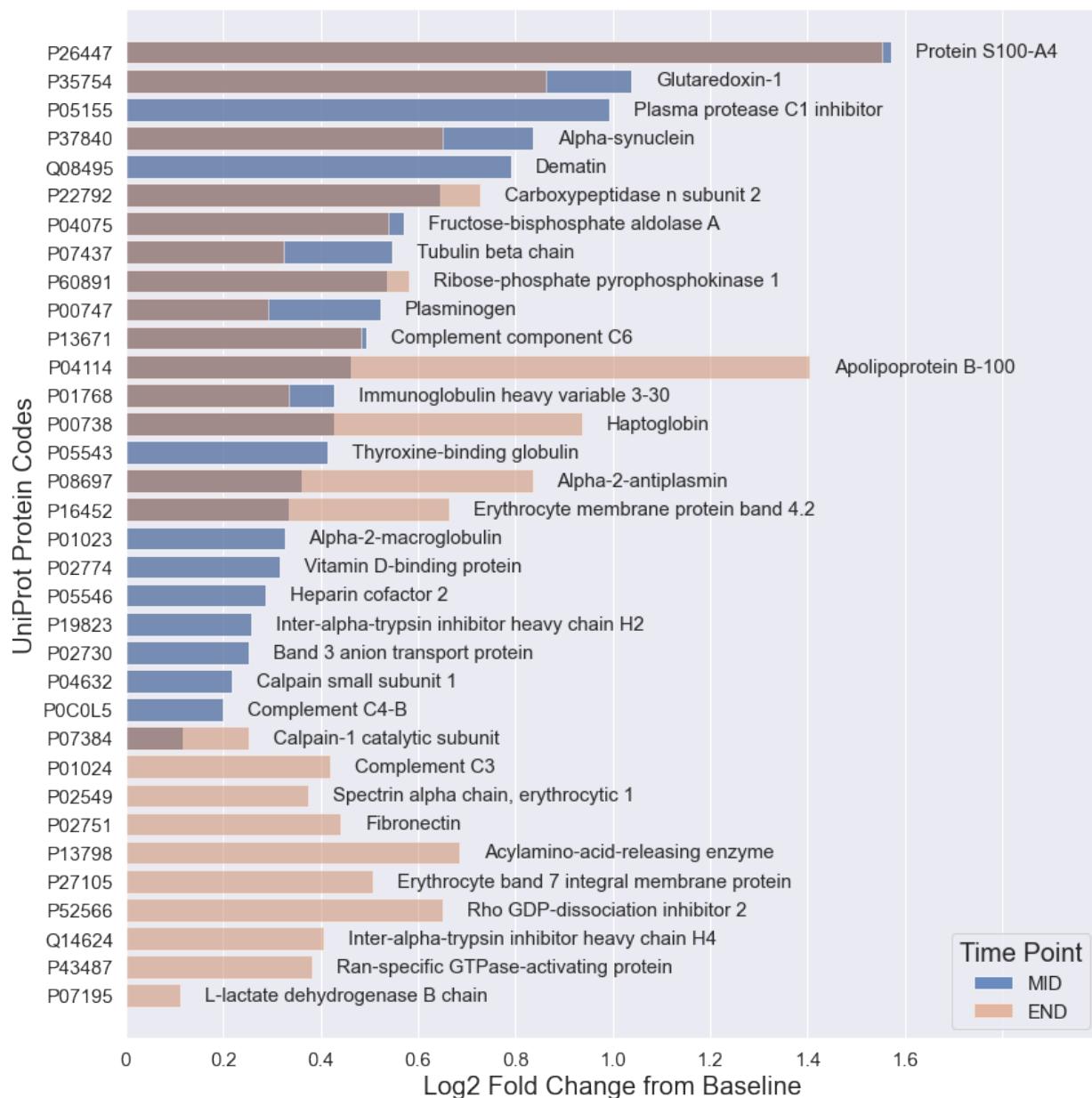
(A) Average (\pm SEM) exercise in MET-min performed by the training (TR) subjects per week of the three week training protocol. (B) Individual MET-min per week for each subject. Values were obtained by measuring gas exchange during rest and throughout all exercise sessions. 500 MET-min per week is equivalent to 150 minutes of moderate-to-vigorous physical activity per week as recommended by the Physical Activity Guidelines for Americans.

Figure 4.2. Peak Workload, $\dot{V}O_{2\text{Peak}}$, POMS, and WURSS scores at BL, MID, and END



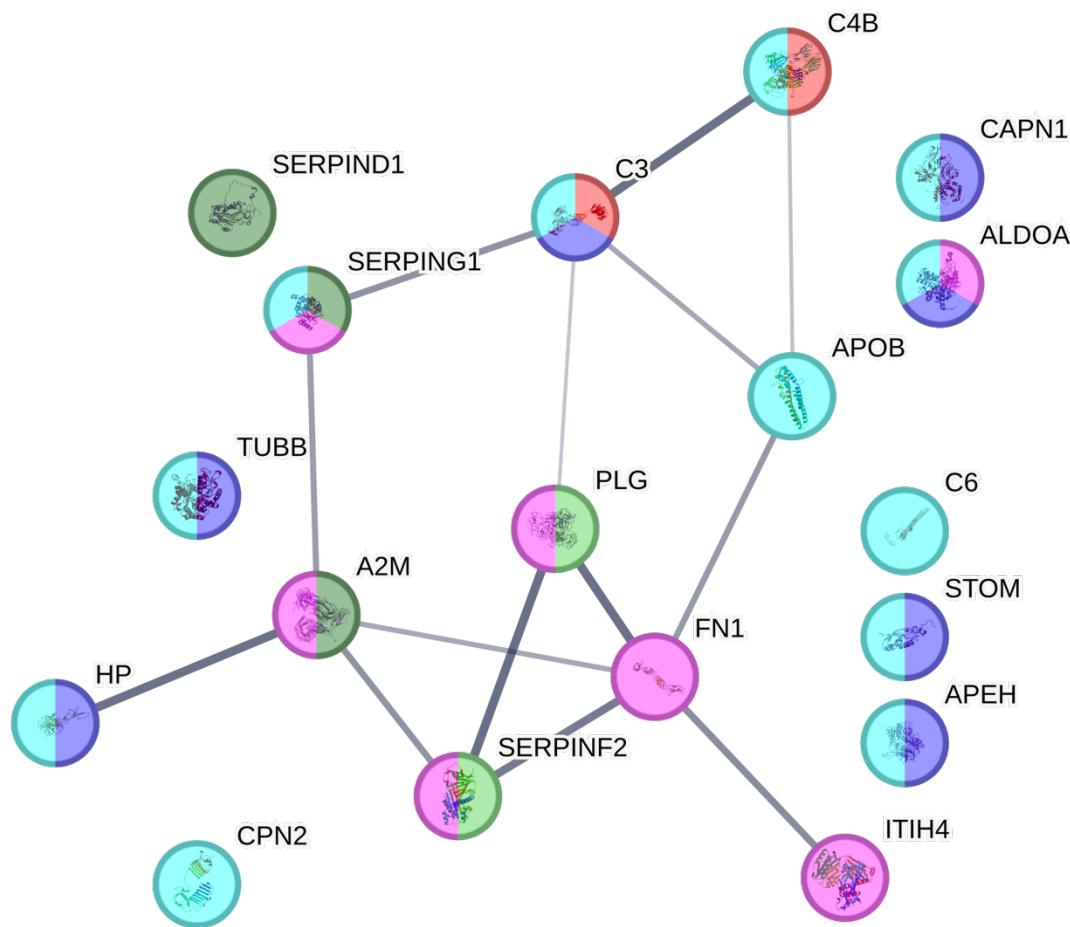
(A) Individual subject and group mean (\pm SEM) performance (PWL) across time. (B) Individual subject and group mean (\pm SEM) % change in PWL from BL. (C) Individual subject and group mean $\dot{V}O_{2\text{Peak}}$ (ml/kg/min) across time. (D) Individual subject and group mean (\pm SEM) % change in $\dot{V}O_{2\text{Peak}}$ (ml/kg/min). (E) Mean (\pm SEM) upper respiratory illness symptoms (WURSS) during the three-week training and recovery phases. (F) Daily average (\pm SEM) WURSS scores across the length of the entire study. Red dotted line indicates end of training protocol. On graphs A-D, colored lines represent individual participant data.

Figure 4.3. Upregulated proteins from BL at MID and END timepoints.



All upregulated proteins from baseline at MID and END timepoints (n=37). Proteins are sorted from largest to smallest log2-fold change at MID timepoint.

Figure 4.4. STRING PPI network for upregulated immune system-related proteins.



STRING protein-protein interaction (PPI) network for upregulated immune system-related proteins ($n=18$). GO terms for biological process were coded as follows: Teal, innate immune system; Red, regulation of complement cascade; Green/dark green, fibrin clot formation; Pink, platelet degranulation; Purple, neutrophil degranulation. Acronyms represent upregulated protein genes (see Table 3.5 for descriptions). Thickness of the network line (edges) indicates the strength of data support. One immunoglobulin was not listed in STRING and therefore not depicted in the PPI network (IGHV3-30).

CHAPTER FIVE: GENERAL DISCUSSION

A chain is no stronger than its weakest link.¹

Exercise-Associated Adaptive Response. It is clear that mediation of the exercise-associated adaptive response depends on a number of training-related factors including: type of training performed (e.g., resistance training, aerobic exercise), exercise modality (e.g., running or cycling), exercise intensity, duration, frequency, recovery time, work-to-rest ratios, duration of the entire training program, and overall training volume.² Additionally, age, genetics, sex, diet, sleep, the time of day in which exercise is performed (circadian rhythm), and the previous training history of the individual, have also shown to modulate the training response.²⁻⁶

Overtraining Hormesis—Pathophysiological mechanisms.

Glycogen Depletion Hypothesis. Hormesis is associated with a paradoxical biphasic dose-response. In chapter two of this dissertation, both OR and AD subjects demonstrated progressive decreases to LA_{Peak} throughout the training phase, which returned to near baseline levels during the recovery phase (**Figure 2.4A-B**). A rightward shift in blood LA⁻ responses during submaximal and maximal exercises intensities, attributable to increased LA⁻ clearance via oxidation,^{7,8} is generally associated with a positive training response (i.e., increased aerobic capacity);⁹⁻¹¹ however, depleted glycogen stores can also produce a rightward shift in blood LA⁻ during exercise,^{12,13} and a ‘blunted’ LA⁻ response to exercise is a common physiological finding in overtrained athletes.¹⁴⁻¹⁸ As such, it has been theorized that the reduction in LA_{Peak} observed in

overtraining literature could be attributed to reduced glycogen stores;^{12,13} however, previous research has not found evidence to support this theory.^{18–20}

A primary concept within the paradigm of nutrient-related training responses in skeletal muscle is that substrate availability mediates the cellular response to contractile activity.²¹ As such, it is common to implement exercise and dietary strategies in athletics to optimize muscle glycogen content and delay its depletion.²² Glycogenolysis provides key substrates for glycolysis and subsequently oxidative phosphorylation in skeletal muscle, and low glycogen content is a key determinant of muscle fatigue.^{23–25} LA⁻, a byproduct of glycogenolysis, is an important signaling molecule that can mediate exercise-induced adaptations related to mitochondrial biogenesis²⁶ and has been referred to a ‘pseudo-hormone’ or “lactormone” given its paracrine-, autocrine-, and endocrine-like effects.^{27,28} Research has associated depleted intramyofibrillar glycogen content with reductions in Ca²⁺ released from the sarcoplasmic reticulum during fatiguing exercise.^{29,30} Additionally, performing exercise with initially lower glycogen stores may attenuate muscle protein synthesis³¹ and exacerbate muscle protein degradation.^{32–34} Conversely, there is mounting evidence to suggest that exercising with low muscle glycogen stores can also induce beneficial metabolic mitochondrial training adaptations. For example, Hansen et al.³⁵ found higher resting muscle glycogen and citrate synthase levels in subjects who performed half of their repeat bouts of exercise with low muscle glycogen stores compared to those who performed all their repeat bouts with low glycogen stores. In skeletal muscle, other putative mediators associated with mitochondrial biogenesis^{36,37} and improved mitochondrial enzymatic activity^{35,38,39} have been found after exercise with low glycogen stores. Accordingly,

the term ‘mitohormesis’ has been coined whereby mild and transient mitochondrial stress induces beneficial responses in a cell, tissue, or organism.⁴⁰

From a hormesis perspective, exercising with low levels of muscle glycogen can generate a positive adaptive response by modulating the LA⁻ response during training, which imposes metabolic stress on multiple biological systems including mitochondrial function. At an appropriate dose of exercise, this response can be beneficial and lead to enhanced oxidative capacity and endurance performance; however, chronic or excessive metabolic stress, via exercising at low glycogen beyond the tolerance of a biological system may lead to attenuated or even adverse responses to training. Conversely, excessive insulin secretion, in response to overnutrition, can lead to insulin resistance and type 2 diabetes and may be a similar maladaptive response at the other end of the nutrient-training response continuum.⁴¹ Without a full accounting of carbohydrate intake, muscle glycogen content, rates of LA⁻ appearance and clearance, and mitochondrial function in relation to exercise performance and aerobic capacity, it is challenging to fully understand if a rightward shift of LA⁻ levels in response to exercise is indicative of a positive or maladaptive training response. Nevertheless, some research has specifically linked the overtraining process with mitochondrial dysfunction and impaired muscle oxidative capacity.⁴²⁻⁴⁴ In the present study, we were limited to measuring mixed capillary LA⁻ during exercise and did not account for dietary intake, muscle glycogen content, or mitochondrial function. Thus, given the modest improvements to $\dot{V}O_{2\text{Peak}}$ found in AD and OR subjects (~6%), it is plausible to conclude that the reduction of LA_{Peak} observed with training despite TR subjects undergoing three weeks of high-intensity aerobic training can be attributed to a maladaptive training response.

Autonomic Nervous System Dysfunction. The autonomic nervous system (ANS) controls cardiovascular function through sympathetic and parasympathetic modulation.⁴⁵ As the sympathetic-parasympathetic balance is affected by changes in training load, autonomic HR regulation has been a popular non-invasive means of determining ANS function.^{46,47} In the present study, OR subjects exhibited progressive decreases to MHR (**Figure 2.5B-C**) in conjunction with progressive decreases to LA_{Peak}. In OR subjects, MHR returned to near baseline levels during the recovery phase with a concomitant increase in HRR (**Figure 2.5D**). Concomitant decreases in MHR and blood LA⁻ have successfully been used to determine overreaching status (OR *versus* non-OR) in well-trained triathletes,¹⁴ and autonomic dysfunction has been suggested as a potential mechanism of overtraining.^{16,48} Decreases in MHR and LA_{Peak} can indicate a downregulation of the sympathetic nervous system or increased parasympathetic to sympathetic balance during OT.^{14,49-51} Specifically, reductions to MHR after intensified training can be attributable to reduced sympathetic tone, increased parasympathetic tone, decreased tissue responsiveness to catecholamines, reduced changes in adrenergic receptor activity, or may simply be the result of exercise intolerance related to other contributing factors of fatigue.^{15,16} From a hormesis perspective, it is plausible that beyond a certain threshold, the repetitive or excessive metabolic stress associated with chronic high-intensity training (and inadequate recovery) may overwhelm one of these aspects from either branch of the autonomic nervous system and cause a transient dysfunction of the autonomic response to exercise. As HR measures are reflective of both PNS and SNS activity,⁴⁶ this provides an explanation for the

mixed, often conflicting autonomic HR responses that have been reported during intensified training throughout the overtraining literature.^{46,47,52}

Immune System Dysfunction. The relationship between physical activity and illness has already been described as a hormetic ‘J’-shaped dose-response where both low levels and high levels of physical activity are associated with increased risk of illness.^{53,54} In chapter three of this dissertation, OR subjects exhibited a prolonged increase in URTI symptoms during both the training and recovery phases. Similarly, in chapter four, multiple immune-related proteins associated with certain aspects of the innate immune system and acute-phase response were upregulated both immediately following the training phase (MID) and following the three-week recovery phase (END). The majority of investigators in the field of exercise immunology support the viewpoint that the immune system reflects the magnitude of physiological stress experienced by the exercise.^{55–60} Acute bouts of moderate- and vigorous-intensity aerobic exercise (<60 minutes) enhances the recirculation of immunoglobulins, anti-inflammatory cytokines, neutrophils, natural killer cells, and lymphocytes, which are all integral towards maintaining immunity and overall metabolic health.^{61–67} Conversely, the associated physiological, metabolic, and psychological stresses from prolonged and intensive exercise have been linked to immune dysfunction, inflammation, oxidative stress, and muscle damage.^{58,68–71} Accordingly, from a hormetic perspective, physical inactivity (e.g., sedentarism) is associated with an increased risk of illness, moderate levels of exercise results in an adaptive protective response against infection, and prolonged or excessive exercise can cause perturbations to the immune response and its

associated signaling pathways thereby increasing the risk for illness to occur (see ‘open window’ theory^{53,56,72}) (**Figure 1**).

Sleep, Nutrition, and Exercise-induced hormesis. Overtraining is defined as a maladaptive training response caused by excessive training-related stress and inadequate recovery.^{16,73} Accordingly, recovery primarily refers to obtaining adequate sleep and nutrition (energy availability/nutrient balance).^{74–77} Both sleep and nutrition are essential in maintaining every biological process and are considered vital to the recovery process following exercise and sport;^{74,78,79} however, most overtraining research has focused on studying the overtraining response through modulation of exercise volumes and have often overlooked key aspects of recovery such as sleep and nutrition. Insufficient sleep can lead to perturbations in cognitive,⁸⁰ immunological,^{81,82} metabolic^{80,83,84} and hormonal functions,^{83,85,86} and low energy availability (LEA) can impair performance and negatively affect various physiological processes including immune function, blood glucose regulation, mental health, decrease performance, and increase injury risk.^{76,87} As such, there are many overlapping symptoms between OTS and relative energy deficiency in sport (RED-S), which is primarily caused by LEA.⁷⁶

In hormesis theory, homeostasis is a dynamic process with firm biological endpoints and adaptive functional endpoints.^{88,89} Biological endpoints signify the point at which a system collapses (e.g., ‘genetic potential’), whereas functional endpoints represent a system’s current ability to tolerate a stressful stimulus and is determined by the accumulation of adaptive stress-responses.⁸⁹ Additionally, the adaptive zone between functional and biological endpoints narrows as training adaptations accumulate over time.⁸⁹ Under this concept, obtaining sufficient

sleep and nutrition maintains the adaptive zone between functional and biological endpoints by acting as pressure release valves, thereby allowing the adaptive training process to continue. Thus, insufficient recovery (sleep and nutrition) further constrains the adaptive zone during periods of intensified training, which can result in injury/illness or a maladaptive overtraining response. As an example, insufficient sleep can cause perturbations to the sympathetic-parasympathetic balance of the autonomic nervous system. In non-rapid eye movement sleep (NREMS), an increase in parasympathetic activity is associated with a reduction in sympathetic activity. When the normal expression of NREMS is prevented, as in patients with insomnia or frequent sleep disturbances, the reduction of sympathetic activity is also prevented. In these patients, sympathetic activity remains high during both sleep and when they are awake.^{90,91} Additionally, chronically restricted sleep has a direct impact on immune function.⁹² Accordingly, insufficient sleep can impair normal immune system and autonomic functions, both of which have previously been theorized as potential pathophysiological mechanisms of overtraining.

In the present investigation, TR subjects expressed more volatile sleep patterns and consistently slept less than the recommended seven hours per night.⁹³ As a result, TR subjects accumulated nearly 12 hours of sleep debt on average by the end of the study. These insufficient sleep needs likely influenced the physiological adaptations following training and led to a modest improvement in aerobic capacity (~6%) that was comparable to the improvements in aerobic capacity shown in CON subjects. Additionally, OR subjects exhibited increased URTI symptoms throughout both the training and recovery phases, compared to AD and CON subjects. Conversely, maintaining optimal nutrition and sleep during periods of intensified training would potentially mitigate the risk of overtraining during periods of intense training. As an example,

well-trained cyclists were observed for signs of overtraining during a 21-day cycling tour covering 3211 km.⁹⁴ Participants were fed ad libitum by a catering service and were provided as much commercially available supplemental foods as desired. Despite a $418\% \pm 142\%$ increase in training volume (time) compared to the 60 days prior to the study, time trial performance did not change. There were no overt changes in overtraining parameters (testosterone, cortisol, HR response, POMS scores, $P>0.05$).⁹⁴

Proteomics, Multiomics, and Future Directions for Overtraining and Hormesis Research.

Over the past decade, large-scale initiatives such as the Molecular Transducers of Physical Activity Consortium (MoTrPAC)^{95,96} have begun to employ systems biology and multiomics approaches to unravel the health benefits of exercise at the biomolecular level. These approaches hold great promise for determining the upstream mechanisms of the exercise response and researchers already have started using these techniques to investigate the pathophysiological mechanisms of overtraining progression.^{97–100} In chapter four of this dissertation, large-scale proteomics was performed on a subgroup of TR subjects immediately before and after the training protocol, and after three weeks of recovery (BL, MID, END, respectively). This pilot investigation detected an increase in multiple proteins related to the innate immune response during MID and END timepoints, which may suggest a chronic inflammatory response following high-intensity training. While the results of this pilot investigation (among others) highlight the potential for “omics” approaches to identify early upstream markers of an overtraining response to exercise, these approaches are far from refined. Over 10,000 proteins have been identified in the human proteome.¹⁰¹ In the present investigation, 380 proteins were identified using DBS

sampling, albeit not all proteins were found in all persons, whereas other studies have identified several hundred to a few thousand proteins using proteomics, depending on the sampling techniques (i.e., tissue/fluid type) and LC-MS/MS techniques (DIA- or DDA-MS approaches).^{98–100,102,103} As such, there is a large variability in proteomics methodology and 100% coverage of the proteome using bottom-up MS approaches is not attainable.¹⁰¹ Together, these factors make the interpretation of proteomics research challenging; even more so when interpreting proteomics results in tandem with multiomics techniques or alongside more traditional methods of analysis in exercise physiology (e.g., hormonal assays, autonomic HR measures, gas exchange during exercise, etc.).

In a recent review, Armstrong et al.¹⁰⁴ have referred to overtraining as a complex systems phenomenon. In nature, complex systems have numerous integrated and interdependent functional components that generate variable responses. Importantly, the outcomes of complex systems cannot be completely characterized or consistently predicted by a discrete number of variables alone.¹⁰⁴ Previous overtraining research has focused on a select number of physiological variables (e.g., HPA-axis, autonomic nervous system, testosterone-cortisol ratios); however, it is becoming increasingly evident that observing a single or series of markers is insufficient to capture the holistic physiological response to stress.^{16,104–107} Given the highly variable symptoms associated with the overtraining response, Armstrong et al.¹⁰⁴ also suggested that there are likely multiple pathophysiological mechanisms ('subtypes') of overtraining. Furthermore, these authors suggested the use of big data and predictive statistical modeling (i.e., machine learning/neural networks) would be an advantageous approach to predict overtraining responses.¹⁰⁴ It is also plausible that these statistical approaches could integrate multiomics data

and data from wearable fitness technologies, which are becoming increasingly popular and provide 24-h continuous monitoring of certain physiological variables (e.g., HRV).^{108,109} Nevertheless, the theory of overtraining as a complex systems phenomenon is in line with the exercise-induced hormesis theory discussed within this dissertation. As such, two individuals (A and B) undergoing periods of intensified training may experience a different pathophysiological mechanism of overtraining, based on the status of their respective training- and non-training-related factors. Person A may exhibit an overtraining phenotype consistent with symptoms of autonomic dysfunction and glycogen depletion (e.g., decreased MHR and LA⁻ responses during maximal exercise), whereas person B may demonstrate an overtraining phenotype characteristic of immune system dysfunction (e.g., URTI and diminished neutrophil counts). The underlying concepts between exercise-induced hormesis theory and overtraining as a complex system phenomenon provide a working framework that can encompass the previously theorized pathophysiological mechanisms of overtraining, and can explain the highly interindividual symptoms associated with the overtraining process. As systems biology approaches become more refined and more widely available, concepts like precision medicine,¹¹⁰ precision exercise,¹¹¹ and precision nutrition¹¹² will become a reality. Accordingly, a better understanding of the complex biomolecular responses to exercise will follow, allowing recreationally active individuals, athletes, coaches, and researchers alike to optimize the training response and mitigate the risk of injury or overtraining.

Conclusion. The primary focus of this dissertation was to determine whether symptoms of overtraining could be identified in a group of healthy, moderately fit (i.e., non-elite) individuals

using a three-week high-intensity training protocol performed under laboratory conditions. OR participants exhibited concomitant and progressive decreases to MHR and LA_{Peak} throughout training, compared to CON subjects. OR subjects also exhibited an increase in HRR during the recovery phase. However, AD participants experienced similar decreases to LA_{Peak}, indicating that a reduction in LA_{Peak} is not a specific marker of overtraining, but of intensified training itself. In Both OR and AD groups, MHR and LA_{Peak} returned to near baseline levels during the three-week recovery phase. Together, these findings provide evidence that autonomic perturbations, caused by chronic high-intensity training, contributed to the overtraining response observed.

During this study, sleep duration and quality were observed using a combination of wrist actigraphy and sleep surveys. Compared to CON subjects, TR subjects exhibited more volatile sleep patterns, frequently slept less than the recommended 7-h per night, and were more likely to accumulate sleep debt. Despite the differences in sleep duration and sleep debt, TR participants reported a similar increase to sleep quality as their CON counterparts while simultaneously experiencing heightened perceptions of sleepiness at bedtime and upon waking. These findings suggest that subjective measures of sleep quality may not accurately reflect sleep needs and support the notion that athletes sleep less than non-athlete counterparts, despite the propensity for athletes undergo higher levels of physical activity inherent to their sport. No differences in sleep duration (TST) or sleep quality between AD and OR groups were observed; however, compared to CON and AD subjects, OR subjects exhibited significantly higher URTI symptoms during both the training phase and recovery phase. These findings suggest that high-intensity training can lead to more volatile sleep patterns and cause an accumulation of sleep debt, regardless of

overtraining status. Furthermore, these results suggest that immune dysfunction may be a contributing mechanism to the overtraining response observed.

A large-scale proteomic analysis using DBS sampling was performed in a subset of TR participants at baseline, 48-hours post-training, and three weeks post-training to investigate the impact of prolonged, high-intensity exercise on the proteome. In line with our initial hypothesis, the results of this pilot investigation found multiple upregulated proteins related to the innate immune system, pertaining to complement activation, neutrophil degranulation, platelet degranulation, and fibrin clot formation. These results were found both immediately following the training phase (MID) and after three weeks of recovery (END), which suggests a chronic inflammatory response from prolonged exercise (i.e., immune dysfunction). However, we did not separate OR and AD subjects in this pilot investigation and thus, were unable to attribute these findings to overreach.

The pathophysiological mechanisms of overtraining remain poorly understood; however, the results of this study provide evidence that the overtraining response to high levels of physical activity is not a phenomenon unique to elite-level athletes. Furthermore, hormesis theory provides a potential unifying framework to support previous theories behind the pathophysiological mechanisms of overtraining (i.e., autonomic dysfunction and immune system dysfunction). Exercise-induced hormesis theory can serve as a ‘unifying theory’ to explain the interindividual symptoms associated with overtraining and provides an explanation as to why exercise-stress can result in both positive and adverse physiological responses. Future well-controlled investigations with larger sample sizes observing overtraining progression in a variety of active populations are warranted. Modern approaches to investigating the personalized exercise training response (e.g., systems biology, wearable fitness technology, AI/ML) are

promising tools which can be used in future research to gain a better understanding of the complex adaptive response to training. Together, these technologies have the potential to elucidate the pathophysiological mechanisms behind the (over)training response to exercise at the biomolecular level. In closing, when it comes to ‘Exercise is Medicine’, perhaps the dose *does* make the poison.

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APPENDIX

APPENDIX A: SUPPLEMENTARY TABLES

Table S.1. Maximal effort exercise testing criteria met during weekly exercise testing.

Subject	Baseline	Week 2	Week 3	Post-48h	Post-7	Post-14	Post-21
TR_1	A	A	A	B/C	A	A	B
TR_2	B/C	A	B/C	A	B/C	A	A
TR_3	A	A	A	A	A	A	A
TR_4	A	A	A	A	A	B/C	A
TR_5	A	B/C	A	A	A	C	B/C
TR_6	B/C	A	A	A	A	C	B/C
TR_7	A	A	A	A	A	A	C
TR_8	A	B/C	A	B/C	A	A	A
TR_9	B/C	A	A	A	A	A	A
TR_10	A	A	A	A	A	A	A
TR_11	A	A	A	A	A	A	A
CON_1	A	A	B/C	---	A	B/C	B
CON_2	B/C	A	A	---	A	A	A
CON_3	A	A	A	---	A	A	A
CON_4	A	A	A	---	A	A	A
CON_5	B/C	B/C	A	---	A	A	A
CON_6	B/C	B/C	A	---	A	A	B/C
CON_7	A	B/C	A	---	B/C	A	A
CON_8	A	A	A	---	A	A	A
CON_9	A	A	A	---	A	B/C	A
CON_19	A	A	A	---	B/C	A	A
Priority of testing criteria to determine maximal effort during exercise testing were as follows: (A) observed plateau in $\dot{V}O_2$ data during exercise testing (collected in 15s averages). (B) two of the three criteria: heart rate $\geq 90\%$ age-predicted max heart rate, 2) respiratory exchange ratio ≥ 1.10 , 3) peak lactate concentration ≥ 8.0 mmol/L (measured two minutes after cessation of exercise). (C) $\dot{V}O_{2\text{Peak}}$ values from round one (GXT) and round 2 (supramaximal) exercise tests were within 0.15 L of one another.							

Table S.2. Mixed-effect regression model results for chapter II, CON, AD, OR subjects.

Fixed-effect	Fixed-effect (β)	95% confidence intervals	F-statistic) (df 1,128)	P-value
Peak Workload (W)				
Intercept	211.688	-186.095, 237.280	---	---
Group(AD)	1.656	-36.733, 40.046	0.97	0.933
Group(OR)	-49.521	-102.797, 3.755	3.32	0.071
Phase(Training)	11.068	5.627, 16.509	15.90	<0.001
Phase(recovery)	13.500	8.370, 18.630	26.60	<0.001
Group(AD):phase(Training)	1.526	-6.380, 9.431	0.14	0.706
Group(OR): phase(Training)	-18.901	-29.733, -8.070	11.70	0.001
Group(AD):phase(recovery)	3.031	-4.664, 10.726	0.60	0.441
Group(OR):phase(recovery)	-17.306	-27.984, -6.627	10.09	0.002
% Change Peak Workload				
Intercept	1.000	0.967, 1.033	---	---
Group(AD)	0.000	-0.049, 0.049	0.00	1.000
Group(OR)	0.000	-0.069, 0.069	0.00	1.000
Phase(Training)	0.054	0.027, 0.082	14.77	<0.001
Phase(recovery)	0.066	0.040, 0.092	24.57	<0.001
Group(AD):phase(Training)	0.009	-0.031, 0.049	0.20	0.655
Group(OR): phase(Training)	-0.098	-0.153, -0.043	12.15	0.001
Group(AD):phase(recovery)	0.018	-0.021, 0.058	0.84	0.360
Group(OR):phase(recovery)	-0.089	-0.144, -0.035	10.42	0.002
Profile of Mood States (POMS)				
Intercept	3.300	-6.310, 12.910	---	---
Group(AD)	5.575	-8.840, 19.990	0.57	0.450
Group(OR)	15.033	-4.971, 35.038	2.17	0.143
Phase(Training)	0.800	-6.076, 7.676	0.05	0.820
Phase(recovery)	-0.467	-6.949, 6.016	0.02	0.888
Group(AD):phase(Training)	-2.675	-12.665, 7.315	0.28	0.601
Group(OR): phase(Training)	3.978	-9.710, 17.666	0.32	0.570
Group(AD):phase(recovery)	-2.533	-12.257, 7.191	0.26	0.610
Group(OR):phase(recovery)	9.356	-4.139, 22.850	1.85	0.177
$\dot{V}O_{2\text{Peak}}$ (L/min)				
Intercept	2.663	2.349, 3.977	---	---
Group(AD)	0.112	-0.359, 0.583	0.22	0.642
Group(OR)	-0.633	-1.286, 0.020	3.61	0.060
Phase(Training)	0.101	0.010, 0.191	4.69	0.032
Phase(recovery)	0.150	0.065, 0.236	11.81	0.001
Group(AD):phase(Training)	-0.019	-0.151, 0.113	0.08	0.780
Group(OR): phase(Training)	-0.204	-0.385, -0.023	4.87	0.029
Group(AD):phase(recovery)	0.006	-0.122, 0.135	0.01	0.923
Group(OR):phase(recovery)	-0.154	-0.332, 0.025	2.85	0.094
% Change $\dot{V}O_{2\text{Peak}}$ (L/min)				
Intercept	1.000	0.958, 1.042	---	---
Group(AD)	0.000	-0.062, 0.062	0.00	1.000
Group(OR)	0.000	-0.087, 0.087	0.00	1.000
Phase(Training)	0.042	0.003, 0.081	4.49	0.036

Phase(recovery)	0.059	0.023, 0.096	10.12	0.002
Group(AD):phase(Training)	-0.005	-0.062, 0.051	0.03	0.857
Group(OR): phase(Training)	-0.093	-0.170, -0.016	5.57	0.020
Group(AD):phase(recovery)	0.006	-0.039, 0.061	0.05	0.820
Group(OR):phase(recovery)	-0.057	-0.133, 0.019	2.13	0.147
VO₂Peak (ml/kg/min)				
Intercept	39.770	34.643, 44.897	---	---
Group(AD)	-2.093	-9.782, 5.597	0.28	0.595
Group(OR)	-7.543	-18.215, 3.129	1.92	0.168
Phase(Training)	1.277	-0.229, 2.783	2.76	0.099
Phase(recovery)	1.803	0.383, 3.223	6.19	0.014
Group(AD):phase(Training)	-0.445	-2.633, 1.743	0.16	0.691
Group(OR): phase(Training)	-2.217	-5.215, 0.781	2.10	0.150
Group(AD):phase(recovery)	0.214	-1.916, 2.344	0.04	0.844
Group(OR):phase(recovery)	-2.000	-4.956, 0.956	1.76	0.187
% Change VO₂Peak (ml/kg/min)				
Intercept	1.000	0.955, 1.045	---	---
Group(AD)	0.000	-0.068, 0.068	0.00	1.000
Group(OR)	0.000	-0.095, 0.095	0.00	1.000
Phase(Training)	0.039	-0.002, 0.079	3.54	0.062
Phase(recovery)	0.050	0.012, 0.088	6.70	0.011
Group(AD):phase(Training)	-0.015	-0.073, 0.044	0.25	0.621
Group(OR): phase(Training)	-0.072	-0.152, 0.008	3.09	0.081
Group(AD):phase(recovery)	0.005	-0.052, 0.062	0.03	0.856
Group(OR):phase(recovery)	-0.046	-0.125, 0.033	1.29	0.258
Maximum Heart Rate				
Intercept	184.200	179.161, 189.239	---	---
Group(AD)	-2.762	-10.322, 4.797	0.51	0.475
Group(OR)	-4.200	-14.690, 6.290	0.62	0.434
Phase(Training)	-0.200	-3.277, 2.877	0.02	0.899
Phase(recovery)	-0.233	-3.135, 2.668	0.02	0.875
Group(AD):phase(Training)	-3.446	-7.917, 1.025	2.28	0.133
Group(OR): phase(Training)	-11.720	-17.846, -5.594	14.06	<0.001
Group(AD):phase(recovery)	-2.454	-6.806, 1.898	1.22	0.271
Group(OR):phase(recovery)	-6.767	-12.806, -0.727	4.82	0.030
% Change Maximum Heart Rate				
Intercept	1.000	0.981, 1.019	---	---
Group(AD)	0.000	-0.029, 0.029	0.00	1.000
Group(OR)	0.000	-0.040, 0.040	0.00	1.000
Phase(Training)	-0.001	-0.018, 0.016	0.01	0.924
Phase(recovery)	-0.001	-0.017, 0.015	0.01	0.903
Group(AD):phase(Training)	-0.019	-0.043, 0.005	2.37	0.126
Group(OR): phase(Training)	-0.064	-0.097, -0.030	14.08	<0.001
Group(AD):phase(recovery)	-0.014	-0.037, 0.010	1.26	0.264
Group(OR):phase(recovery)	-0.037	-0.070, -0.004	4.92	0.028
Heart Rate Recovery				
Intercept	31.278	25.032, 37.523	---	---
Group(AD)	-2.778	-12.220, 6.664	0.33	0.565

Group(OR)	4.389	-8.102, 16.880	0.47	0.492
Phase(Training)	-3.975	-7.487, -0.463	4.92	0.029
Phase(recovery)	-2.000	-5.311, 1.311	1.40	0.239
Group(AD):phase(Training)	3.386	-1.755, 8.527	1.67	0.199
Group(OR): phase(Training)	1.283	-5.443, 8.008	0.14	0.709
Group(AD):phase(recovery)	2.071	-2.935, 7.078	0.66	0.419
Group(OR):phase(recovery)	6.000	-0.623, 12.623	3.15	0.070
% Change Heart Rate Recovery				
Intercept	1.000	0.883, 1.117	---	---
Group(AD)	0.000	-0.177, 0.177	0.00	1.000
Group(OR)	0.000	-0.235, 0.235	0.00	1.000
Phase(Training)	-0.133	-0.259, -0.008	4.34	0.039
Phase(recovery)	-0.049	-0.167, 0.069	0.66	0.419
Group(AD):phase(Training)	0.122	-0.061, 0.305	1.70	0.195
Group(OR): phase(Training)	0.091	-0.149, 0.331	0.56	0.457
Group(AD):phase(recovery)	0.063	-0.115, 0.242	0.48	0.488
Group(OR):phase(recovery)	0.251	0.014, 0.487	4.32	0.040
Peak Lactate				
Intercept	9.380	8.221, 10.539	---	---
Group(AD)	1.807	0.069, 3.546	4.15	0.044
Group(OR)	-0.580	-2.992, 1.832	0.22	0.638
Phase(Training)	0.648	-0.300, 1.596	1.80	0.182
Phase(recovery)	0.943	0.050, 1.837	4.28	0.041
Group(AD):phase(Training)	-1.319	-2.697, 0.058	3.52	0.063
Group(OR): phase(Training)	-2.159	-4.047, -0.272	5.03	0.027
Group(AD):phase(recovery)	-1.395	-2.736, -0.055	4.16	0.043
Group(OR):phase(recovery)	-2.643	-4.504, -0.783	7.75	0.006
% Change Peak Lactate				
Intercept	1.000	0.899, 1.101	---	---
Group(AD)	0.000	-0.152, 0.152	0.00	1.000
Group(OR)	0.000	-0.211, 0.211	0.00	1.000
Phase(Training)	0.081	-0.016, 0.179	2.66	0.106
Phase(recovery)	0.124	0.032, 0.216	6.93	0.010
Group(AD):phase(Training)	-0.145	-0.287, -0.002	3.97	0.049
Group(OR): phase(Training)	-0.253	-0.448, -0.058	6.47	0.012
Group(AD):phase(recovery)	-0.168	-0.306, -0.030	5.65	0.019
Group(OR):phase(recovery)	-0.322	-0.515, -0.130	10.82	0.001

Table S.3. Chapter II post-hoc power calculations and supplementary statistics calculations.

	Marginal R ²	Conditional R ²	ICC	n	F ²	Post-hoc B
Peak Workload	0.537	0.986	0.970	21	1.159	0.950
% Δ Peak Workload	0.491	0.761	0.529	21	0.966	0.907
̇VO_{2peak} (ml/kg/min)	0.482	0.970	0.942	21	0.931	0.896
% Δ ̇VO_{2peak}	0.329	0.649	0.477	21	0.491	0.621
Maximum Heart Rate	0.484	0.872	0.751	21	0.938	0.898
% Δ Maximum Heart Rate	0.409	0.704	0.499	21	0.693	0.782
Heart Rate Recovery	0.432	0.880	0.789	19	0.761	0.822
% Δ Heart Rate Recovery	0.241	0.423	0.239	19	0.318	0.430
Peak Lactate	0.450	0.755	0.554	21	0.818	0.851
% Δ Peak Lactate	0.384	0.617	0.379	21	0.623	0.733

Post-hoc power analyses were performed using G*Power. (<https://stats.oarc.ucla.edu/other/gpower/>)

Table S.4. Chapter III sleep parameter mixed-effect model regression results for CON and TR subjects.

Fixed-Effect	(β)	95% Confidence Intervals	F-statistic (DF 1,767)	P-value
Sleep Duration (min)				
Intercept	466.971	433.069, 500.073	---	---
Group(TR)	-29.787	-75.501, 15.926	1.63	0.202
Phase(Rec)	-10.112	-81.730, 61.507	0.08	0.782
Group(TR)*Phase(Rec)	73.839	-12.801, 160.480	2.79	0.095
Time	0.359	-1.280, 1.998	0.18	0.668
Time*Group(TR)	-0.078	-2.288, 2.132	0.00	0.945
Time*Phase(Rec)	-0.157	-2.975, 2.661	0.01	0.913
Time*Group(TR)*Phase(Rec)	-1.879	-5.348, 1.591	1.13	0.289
Total Sleep Time (min)				
Intercept	418.732	388.025, 449.439	---	---
Group(TR)	-21.233	-62.638, 20.172	1.01	0.315
Phase(Rec)	-13.162	-77.672, 51.348	0.16	0.689
Group(TR)*Phase(Rec)	55.058	-22.983, 133.098	1.91	0.167
Time	0.306	-1.170, 1.782	0.16	0.685
Time*Group(TR)	-0.104	-2.095, 1.887	0.01	0.919
Time*Phase(Rec)	-0.153	-2.385, 2.692	0.01	0.906
Time*Group(TR)*Phase(Rec)	-1.486	-4.611, 1.639	0.97	0.352
Wake After Sleep Onset (min)				
Intercept	48.239	39.093, 57.385	---	---
Group(TR)	-8.554	-20.887, 3.778	1.85	0.174
Phase(Rec)	3.050	-14.315, 20.415	0.12	0.731
Group(TR)*Phase(Rec)	18.782	-2.226, 39.789	3.07	0.80
Time	0.053	-0.344, 0.450	0.07	0.925
Time*Group(TR)	0.026	-0.510, 0.562	0.01	0.925
Time*Phase(Rec)	-0.311	-0.994, 0.373	0.79	0.373
Time*Group(TR)*Phase(Rec)	-0.393	-1.234, 0.449	0.84	0.361
Sleep Efficiency				
Intercept	89.825	88.001, 91.649	---	---
Group(TR)	1.165	-1.295, 3.624	0.86	0.354
Phase(Rec)	-0.679	-3.996, 2.638	0.16	0.688
Group(TR)*Phase(Rec)	-2.641	-6.655, 1.372	1.66	0.197
Time	-0.014	-0.090, 0.062	0.14	0.712
Time*Group(TR)	0.004	-0.098, 0.107	0.01	0.934
Time*Phase(Rec)	0.065	-0.065, 0.196	0.97	0.326
Time*Group(TR)*Phase(Rec)	0.042	-0.119, 0.202	0.26	0.612
Sleep Quality				
Intercept	3.724	3.082, 4.366	---	---
Group(TR)	-0.353	-1.219, 0.512	0.64	0.424
Phase(Rec)	-0.406	-1.787, 0.975	0.33	0.565
Group(TR)*Phase(Rec)	-0.010	-1.680, 1.661	0.00	0.991
Time	-0.035	-0.067, -0.003	4.69	0.031
Time*Group(TR)	-0.011	-0.031, 0.054	0.28	0.600

Time*Phase(Rec)	0.028	-0.026, 0.083	1.05	0.306
Time*Group(TR)*Phase(Rec)	-0.007	-0.074, 0.060	0.04	0.835
Sleepiness (Bedtime)				
Intercept	6.875	6.140, 7.610	---	---
Group(TR)	-0.824	-1.815, 0.167	2.65	0.104
Phase(Rec)	-0.368	-2.034, 1.298	0.19	0.665
Group(TR)*Phase(Rec)	1.751	-0.264, 3.767	2.90	0.089
Time	-0.018	-0.056, 0.021	0.82	0.365
Time*Group(TR)	0.058	0.007, 0.110	4.93	0.027
Time*Phase(Rec)	0.037	-0.028, 0.103	1.25	0.264
Time*Group(TR)*Phase(Rec)	-0.118	-0.199, 0.038	8.28	0.004
Sleepiness (Wake)				
Intercept	5.007	4.055, 5.960	---	---
Group(TR)	-0.177	-1.461, 1.107	0.07	0.788
Phase(Rec)	-0.338	-2.252, 1.575	0.12	0.729
Group(TR)*Phase(Rec)	0.891	-1.425, 3.206	0.57	0.451
Time	-0.038	-0.082, 0.006	2.87	0.091
Time*Group(TR)	0.071	0.012, 1.30	5.57	0.019
Time*Phase(Rec)	0.036	-0.039, 0.111	0.87	0.351
Time*Group(TR)*Phase(Rec)	-0.083	-0.175, 0.010	3.05	0.081
Delta Sleepiness				
Intercept	-01.868	-2.900, -0.835	---	---
Group(TR)	0.647	-0.745, 2.039	0.83	0.363
Phase(Rec)	0.029	-2.240, 2.299	0.00	0.980
Group(TR)*Phase(Rec)	-0.860	-3.606, 1.885	0.38	0.539
Time	-0.020	-0.072, 0.032	0.58	0.446
Time*Group(TR)	0.013	-0.057, 0.083	0.13	0.718
Time*Phase(Rec)	-0.002	-0.091, 0.088	0.00	0.973
Time*Group(TR)*Phase(Rec)	0.036	-0.074, 0.146	0.41	0.523
Fatigue (Bedtime)				
Intercept	4.573	3.905, 5.241	---	---
Group(TR)	-0.337	-1.237, 0.564	0.054	0.464
Phase(Rec)	0.088	-1.145, 1.322	0.02	0.888
Group(TR)*Phase(Rec)	0.866	-0.626, 2.358	1.29	0.256
Time	0.016	-0.012, 0.044	1.21	0.271
Time*Group(TR)	0.028	-0.010, 0.066	2.02	0.156
Time*Phase(Rec)	-0.015	-0.064, 0.033	0.38	0.539
Time*Group(TR)*Phase(Rec)	-0.047	-0.107, 0.013	2.36	0.125
Fatigue (Wake)				
Intercept	3.527	2.913, 4.141	---	---
Group(TR)	0.086	-0.742, 0.915	0.04	0.838
Phase(Rec)	0.001	-1.296, 1.297	0.00	0.999
Group(TR)*Phase(Rec)	0.138	-1.430, 1.706	0.03	0.863
Time	-0.016	-0.046, 0.014	1.10	0.295
Time*Group(TR)	0.038	-0.002, 0.078	3.55	0.060
Time*Phase(Rec)	0.004	-0.047, 0.055	0.02	0.875
Time*Group(TR)*Phase(Rec)	-0.028	-0.091, 0.035	0.76	0.385
Delta Fatigue				

Intercept	-1.046	-1.839, -0.253	---	---
Group(TR)	0.423	-0.647, 1.493	0.60	0.439
Phase(Rec)	-0.088	-1.682, 1.507	0.01	0.914
Group(TR)*Phase(Rec)	-0.728	-2.657, 1.201	0.55	0.460
Time	-0.032	-0.068, 0.005	2.91	0.089
Time*Group(TR)	0.011	-0.038, 0.060	0.19	0.666
Time*Phase(Rec)	0.019	-0.043, 0.082	0.36	0.546
Time*Group(TR)*Phase(Rec)	0.019	-0.058, 0.096	0.23	0.631
Sleep Debt (min)				
Intercept	-1.268	-31.975, 29.439	---	---
Group(TR)	-21.233	-62.638, 20.172	1.01	0.315
Phase(Rec)	-13.162	-77.672, 51.348	0.16	0.689
Group(TR)*Phase(Rec)	55.058	-22.983, 133.098	1.91	0.167
Time	0.306	-1.170, 1.782	0.16	0.685
Time*Group(TR)	-0.104	-2.095, 1.887	0.01	0.919
Time*Phase(Rec)	0.153	-2.385, 2.692	0.01	0.906
Time*Group(TR)*Phase(Rec)	-1.486	-4.611, 1.639	0.87	0.352
Cumulative Net Sleep (min)				
Intercept	-443.925	-960.802, 62.952	---	---
Group(TR)	40.108	-643.365, 723.581	0.01	0.086
Phase(Rec)	63.823	-397.015, 524.662	0.07	0.786
Group(TR)*Phase(Rec)	-98.121	-655.620, 459.379	0.12	0.730
Time	423.251	412.704, 433.797	6186.40	<0.001
Time*Group(TR)	-23.664	-37.885, -9.442	10.64	0.001
Time*Phase(Rec)	-3.661	-21.794, 14.472	0.16	0.692
Time*Group(TR)*Phase(Rec)	7.557	-14.769, 29.882	0.44	0.507
Cumulative Sleep Debt (min)				
Intercept	-23.925	-530.802, 482.952	---	---
Group(TR)	40.108	-643.365, 723.581	0.01	0.908
Phase(Rec)	63.823	-397.015, 524.662	0.07	0.786
Group(TR)*Phase(Rec)	-98.121	-655.620, 459.379	0.12	0.730
Time	3.251	-7.296, 13.797	0.36	0.546
Time*Group(TR)	-23.664	-37.885, -9.442	10.64	0.001
Time*Phase(Rec)	-3.661	-21.794, 14.472	0.16	0.692
Time*Group(TR)*Phase(Rec)	7.557	-14.769, 29.882	0.44	0.507

Table S.5. Chapter III post-hoc power calculations and supplementary statistics for CON and TR subjects.

	Marginal R ²	Conditional R ²	ICC	F ²	Estimated B
Sleep Duration	0.213	0.435	0.283	0.270	0.210
Total Sleep Time	0.208	0.435	0.287	0.263	0.205
Cumulative Sleep Debt	0.435	0.866	0.773	0.770	0.570
WASO	0.253	0.517	0.354	0.339	0.260
Sleep Efficiency	0.259	0.542	0.382	0.349	0.267
Sleep Quality	0.201	0.417	0.271	0.252	0.197
Bedtime Sleepiness	0.204	0.392	0.236	0.256	0.200
Waking Sleepiness	0.229	0.473	0.316	0.297	0.230
Bedtime Fatigue	0.249	0.529	0.372	0.332	0.253
Waking Fatigue	0.222	0.443	0.284	0.285	0.221

Post-hoc power analyses were performed using G*Power. (<https://stats.oarc.ucla.edu/other/gpower/>)

Table S.6 Chapter III sleep parameter mixed-effect regression model results for CON, AD, and OR subjects.

Fixed-Effect	(β)	95% Confidence Intervals	F-Statistic (Df 1,771)	P-Value
Sleep Duration (min)				
Intercept	466.971	432.493, 504.448	---	---
Group(TR)	-44.847	-95.106, 5.413	3.06	0.081
Group(OR)	10.371	-58.584, 79.326	0.09	0.768
Phase(Rec)	-10.112	-81.302, 61.079	0.08	0.781
Group(AD)*Phase(Rec)	62.872	-28.222, 153.966	1.83	0.177
Group(OR)*Phase(Rec)	103.085	-13.882, 220.052	2.98	0.085
Time	0.359	-1.270, 1.988	0.19	0.666
Time*Group(AD)	1.163	-1.212, 3.538	0.92	0.338
Time*Group(OR)	-3.387	-6.646, -0.129	4.15	0.042
Time*Phase(Rec)	-0.157	-2.958, 2.644	0.01	0.912
Time*Group(AD)*Phase(Rec)	-2.244	-5.906, 1.419	1.44	0.230
Time*Group(OR)*Phase(Rec)	-0.906	-5.670, 3.857	0.14	0.709
Total Sleep Time (min)				
Intercept	418.732	387.482, 449.981	---	---
Group(TR)	-34.471	-80.025, 11.083	2.20	0.138
Group(OR)	14.068	-48.431, 76.567	0.19	0.659
Phase(Rec)	-13.162	-77.384, 51.060	0.16	0.688
Group(AD)*Phase(Rec)	46.733	-35.444, 128.910	1.24	0.265
Group(OR)*Phase(Rec)	77.256	-28.261, 182.774	2.06	0.152
Time	0.306	-1.164, 1.776	0.17	0.683
Time*Group(AD)	0.870	-1.273, 3.013	0.63	0.426
Time*Group(OR)	-2.701	-5.640, 0.239	3.27	0.072
Time*Phase(Rec)	0.153	-2.374, 2.680	0.01	0.905
Time*Group(AD)*Phase(Rec)	-1.752	-5.056, 1.551	1.08	0.299
Time*Group(OR)*Phase(Rec)	-0.776	-5.073, 3.521	0.13	0.723
Wake After Sleep Onset (min)				
Intercept	48.239	38.957, 57.521	---	---
Group(TR)	-10.376	-23.907, 3.156	2.26	0.133
Group(OR)	-3.697	-22.262, 14.868	0.15	0.696
Phase(Rec)	3.050	-14.252, 20.353	0.12	0.730
Group(AD)*Phase(Rec)	16.139	-6.000, 38.279	2.04	0.153
Group(OR)*Phase(Rec)	25.829	-2.599, 54.257	3.17	0.075
Time	0.053	-0.343, 0.449	0.07	0.793
Time*Group(AD)	0.293	-0.284, 0.870	0.99	0.321
Time*Group(OR)	-0.687	-1.479, 0.105	2.89	0.090
Time*Phase(Rec)	-0.311	-0.992, 0.370	0.80	0.371
Time*Group(AD)*Phase(Rec)	-0.491	-1.381, 0.399	1.17	0.280
Time*Group(OR)*Phase(Rec)	-0.130	-1.288, 1.027	0.05	0.826
Sleep Efficiency				
Intercept	89.825	87.971, 91.679	---	---
Group(TR)	1.254	-1.449, 3.957	0.83	0.363
Group(OR)	0.925	-2.783, 4.634	0.24	0.625

Phase(Rec)	-0.679	-3.994, 2.636	0.16	0.688
Group(AD)*Phase(Rec)	-2.172	-6.414, 2.070	1.01	0.316
Group(OR)*Phase(Rec)	-3.893	-9.340, 1.554	1.96	0.162
Time	-0.014	-0.090, 0.062	0.14	0.711
Time*Group(AD)	-0.028	-0.138, 0.083	0.24	0.626
Time*Group(OR)	0.089	-0.062, 0.241	1.33	0.249
Time*Phase(Rec)	0.065	-0.065, 0.196	0.97	0.326
Time*Group(AD)*Phase(Rec)	0.052	-0.119, 0.222	0.35	0.554
Time*Group(OR)*Phase(Rec)	0.015	-0.207, 0.237	0.02	0.894
Sleep Quality				
Intercept	3.724	3.075, 4.373	---	---
Group(TR)	-0.321	-1.267, 0.625	0.44	0.506
Group(OR)	-0.439	-1.737, 0.859	0.44	0.508
Phase(Rec)	-4.06	-1.787, 0.975	0.33	0.565
Group(AD)*Phase(Rec)	0.109	-1.658, 1.876	0.01	0.904
Group(OR)*Phase(Rec)	-0.326	-2.595, 1.944	0.08	0.779
Time	-0.035	-0.067, -0.003	4.69	0.031
Time*Group(AD)	-0.001	-0.047, 0.045	0.00	0.960
Time*Group(OR)	-0.045	-0.018, 0.108	1.94	0.164
Time*Phase(Rec)	0.028	-0.026, 0.083	1.05	0.306
Time*Group(AD)*Phase(Rec)	-0.002	-0.073, 0.070	0.00	0.966
Time*Group(OR)*Phase(Rec)	-0.022	-0.114, 0.070	0.22	0.642
Sleepiness (Bedtime)				
Intercept	6.785	6.195, 7.555	---	---
Group(TR)	-0.888	-1.879, 0.104	3.08	0.080
Group(OR)	-0.652	-2.013, 0.708	0.88	0.348
Phase(Rec)	-0.368	-2.026, 1.290	0.19	0.664
Group(AD)*Phase(Rec)	1.533	-0.589, 3.654	2.00	0.157
Group(OR)*Phase(Rec)	2.334	-0.390, 5.058	2.82	0.094
Time	-0.018	-0.056, 0.020	0.83	0.363
Time*Group(AD)	0.044	-0.012, 0.099	2.40	0.121
Time*Group(OR)	0.097	0.021, 0.173	6.25	0.013
Time*Phase(Rec)	0.037	-0.028, 0.013	1.26	0.262
Time*Group(AD)*Phase(Rec)	-0.110	-0.195, -0.025	6.40	0.012
Time*Group(OR)*Phase(Rec)	-0.141	-0.252, -0.030	6.19	0.013
Sleepiness (Wake)				
Intercept	5.007	4.082, 5.932	---	---
Group(TR)	-0.470	-1.818, 0.878	0.47	0.495
Group(OR)	0.606	-1.244, 2.456	0.41	0.521
Phase(Rec)	-0.338	-2.254, 1.577	0.12	0.729
Group(AD)*Phase(Rec)	0.353	-2.098, 2.803	0.08	0.778
Group(OR)*Phase(Rec)	2.326	-0.821, 5.472	2.10	0.148
Time	-0.038	-0.082, 0.006	2.86	0.091
Time*Group(AD)	0.066	0.002, 0.129	4.05	0.045
Time*Group(OR)	0.086	-0.002, 0.174	3.69	0.055
Time*Phase(Rec)	0.036	-0.039, 0.111	0.87	0.351

Time*Group(AD)*Phase(Rec)	-0.061	-0.160, 0.037	1.50	0.222
Time*Group(OR)*Phase(Rec)	-0.139	-0.267, -0.011	4.52	0.034
Delta Sleepiness				
Intercept	-1.868	-2.918, -0.817	---	---
Group(TR)	0.418	-1.113, 1.949	0.29	0.593
Group(OR)	1.258	-0.842, 3.358	1.38	0.241
Phase(Rec)	0.029	-2.232, 2.291	0.00	0.980
Group(AD)*Phase(Rec)	-1.180	-4.074, 1.714	0.64	0.424
Group(OR)*Phase(Rec)	-0.008	-3.724, 3.708	0.00	0.997
Time	-0.020	-0.072, 0.032	0.59	0.444
Time*Group(AD)	0.022	-0.054, 0.097	0.32	0.571
Time*Group(OR)	-0.011	-0.114, 0.093	0.04	0.837
Time*Phase(Rec)	-0.002	-0.091, 0.087	0.00	0.973
Time*Group(AD)*Phase(Rec)	0.049	-0.068, 0.165	0.67	0.413
Time*Group(OR)*Phase(Rec)	0.002	-0.149, 0.153	0.00	0.981
Fatigue (Bedtime)				
Intercept	4.573	3.932, 5.214	---	---
Group(TR)	-0.497	-1.431, 0.438	1.09	0.298
Group(OR)	0.090	-1.192, 1.373	0.02	0.890
Phase(Rec)	0.088	-1.146, 1.322	0.02	0.889
Group(AD)*Phase(Rec)	0.537	-1.041, 2.116	0.45	0.505
Group(OR)*Phase(Rec)	1.742	-0.286, 3.769	2.84	0.093
Time	0.016	-0.012, 0.044	1.21	0.271
Time*Group(AD)	0.021	-0.020, 0.062	1.00	0.318
Time*Group(OR)	0.045	-0.011, 0.102	2.47	0.116
Time*Phase(Rec)	-0.015	-0.064, 0.033	0.38	0.539
Time*Group(AD)*Phase(Rec)	-0.034	-0.098, 0.029	1.13	0.288
Time*Group(OR)*Phase(Rec)	-0.080	-0.162, 0.003	3.58	0.059
Fatigue (Wake)				
Intercept	3.527	2.928, 4.126	---	---
Group(TR)	-0.045	-0.918, 0.828	0.01	0.919
Group(OR)	0.437	-0.760, 1.635	0.51	0.474
Phase(Rec)	0.001	-1.296, 1.297	0.00	0.999
Group(AD)*Phase(Rec)	-0.262	-1.921, 1.397	0.10	0.757
Group(OR)*Phase(Rec)	1.206	-0.924, 3.336	1.24	0.268
Time	-0.016	-0.046, 0.014	1.10	0.295
Time*Group(AD)	0.035	-0.008, 0.078	2.50	0.115
Time*Group(OR)	0.048	-0.011, 0.017	2.51	0.114
Time*Phase(Rec)	0.004	-0.047, 0.055	0.02	0.875
Time*Group(AD)*Phase(Rec)	-0.015	-0.082, 0.051	0.21	0.650
Time*Group(OR)*Phase(Rec)	-0.061	-0.148, 0.026	1.89	0.169
Delta Fatigue				
Intercept	-1.046	-1.854, -0.238	---	---
Group(TR)	0.451	-0.727, 1.629	0.56	0.453
Group(OR)	0.347	-1.269, 1.963	0.18	0.674
Phase(Rec)	-0.088	-1.685, 1.510	0.01	0.914
Group(AD)*Phase(Rec)	-0.800	-2.844, 1.244	0.59	0.443

Group(OR)*Phase(Rec)	-0.536	-3.161, 2.089	0.16	0.689
Time	-0.032	-0.068, 0.005	2.90	0.089
Time*Group(AD)	0.014	-0.039, 0.067	0.26	0.610
Time*Group(OR)	0.003	-0.070, 0.076	0.01	0.943
Time*Phase(Rec)	0.019	-0.044, 0.082	0.36	0.547
Time*Group(AD)*Phase(Rec)	0.019	-0.063, 0.101	0.21	0.651
Time*Group(OR)*Phase(Rec)	0.019	-0.088, 0.126	0.12	0.730
Sleep Debt (min)				
Intercept	-1.268	-32.518, 29.981	---	---
Group(TR)	-34.471	-80.025, 11.083	2.20	0.138
Group(OR)	14.068	-48.431, 76.567	0.19	0.659
Phase(Rec)	-13.162	-77.384, 51.060	0.16	0.688
Group(AD)*Phase(Rec)	46.733	-35.444, 128.910	1.24	0.265
Group(OR)*Phase(Rec)	77.256	-28.261, 182.774	2.06	0.152
Time	0.306	-1.164, 1.776	0.17	0.683
Time*Group(AD)	0.870	-1.273, 3.013	0.63	0.426
Time*Group(OR)	-2.701	-5.640, 0.239	3.24	0.072
Time*Phase(Rec)	0.153	-2.374, 2.680	0.01	0.905
Time*Group(AD)*Phase(Rec)	-1.752	-5.056, 1.551	1.08	0.299
Time*Group(OR)*Phase(Rec)	-0.776	-5.073, 3.521	0.13	0.723
Cumulative Net Sleep (min)				
Intercept	-443.925	-962.487, 74.638	---	---
Group(TR)	12.581	-743.347, 768.510	0.00	0.974
Group(OR)	113.511	-923.614, 1150.636	0.05	0.830
Phase(Rec)	63.823	-397.752, 525.398	0.07	0.786
Group(AD)*Phase(Rec)	-110.733	-701.351, 479.886	0.14	0.713
Group(OR)*Phase(Rec)	-64.489	-822.860, 693.883	0.03	0.868
Time	423.251	412.687, 433.814	6166.67	<0.001
Time*Group(AD)	-25.948	-41.347, -10.548	10.91	0.001
Time*Group(OR)	-17.575	-38.702, 3.553	2.66	0.103
Time*Phase(Rec)	-3.661	-21.823, 14.501	0.16	0.693
Time*Group(AD)*Phase(Rec)	9.381	-14.364, 33.126	0.60	0.439
Time*Group(OR)*Phase(Rec)	2.693	-28.190, 33.576	0.03	0.864
Cumulative Sleep Debt (min)				
Intercept	-23.925	-542.487, 494.638	---	---
Group(TR)	12.581	-743.347, 768.510	0.00	0.974
Group(OR)	113.511	-923.614, 1150.636	0.05	0.830
Phase(Rec)	63.823	-397.752, 525.398	0.07	0.786
Group(AD)*Phase(Rec)	-110.733	-701.351, 479.886	0.14	0.713
Group(OR)*Phase(Rec)	-64.489	-822.860, 693.883	0.03	0.868
Time	3.251	-7.313, 13.814	0.36	0.547
Time*Group(AD)	-25.948	-41.347, -10.548	10.91	0.001
Time*Group(OR)	-17.575	-38.702, 3.553	2.66	0.103
Time*Phase(Rec)	-3.661	-21.823, 14.501	0.16	0.693
Time*Group(AD)*Phase(Rec)	9.381	-14.364, 33.126	0.60	0.439
Time*Group(OR)*Phase(Rec)	2.693	-28.190, 33.576	0.03	0.864

Table S.7. Chapter III post-hoc power calculations and supplementary statistics for CON, AD, and OR subjects.

	Marginal R ²	Conditional R ²	ICC	F ²	Estimated B
Sleep Duration	0.219	0.452	0.298	0.281	0.183
Total Sleep Time	0.213	0.450	0.301	0.271	0.177
Cumulative Sleep Debt	0.426	0.869	0.771	0.742	0.452
WASO	0.256	0.528	0.366	0.343	0.218
Sleep Efficiency	0.258	0.550	0.394	0.348	0.220
Sleep Quality	0.202	0.424	0.278	0.254	0.168
Bedtime Sleepiness	0.223	0.371	0.190	0.288	0.186
Waking Sleepiness	0.236	0.462	0.296	0.309	0.198
Bedtime Fatigue	0.259	0.515	0.345	0.350	0.222
Waking Fatigue	0.229	0.434	0.267	0.296	0.191

Post-hoc power analyses were performed using G*Power. (<https://stats.oarc.ucla.edu/other/gpower/>)

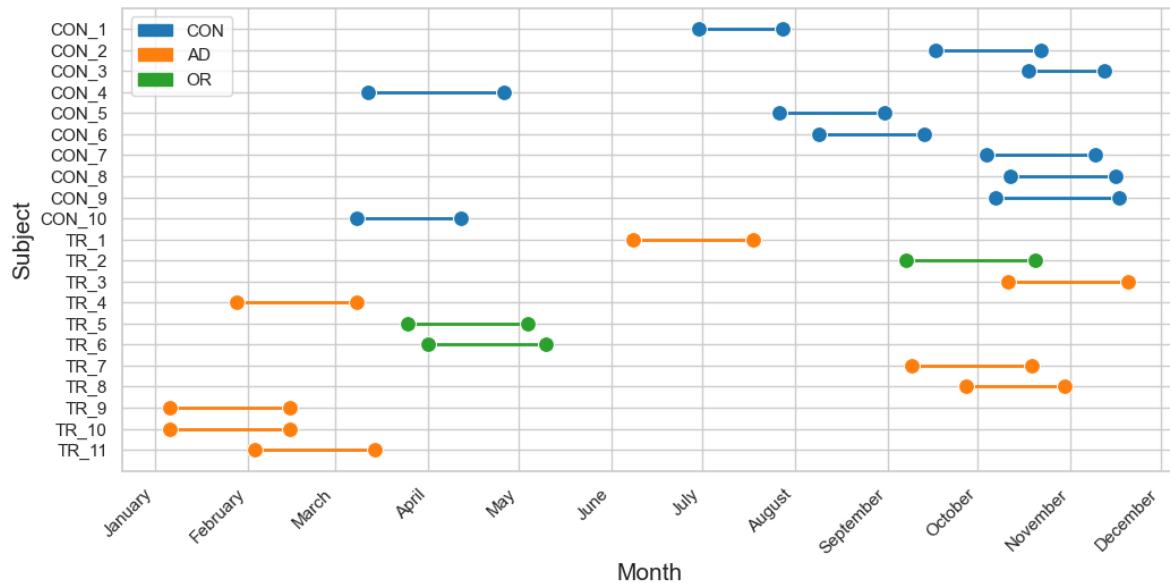
Table S.8. General description of subject physical activity history.

Subject	Group	Gender	Primary Physical Activities	Frequency of Physical Activity*
1	AD	M	Power Lifter	moderate
2	OR	F	GPP	light
3	AD	F	Mountaineer	moderate
4	AD	F	Distance Runner	moderate-heavy
5	OR	F	GPP	light
6	OR	F	Field Hockey; Runner	light-moderate
7	AD	F	GPP; hiking, spin classes, paddleboarding, yoga	moderate-heavy
8	AD	F	Mountaineer; Mix of recreational activities	moderate
9	AD	F	GPP; recreational Golf and Basketball	moderate
10	AD	F	GPP	light-moderate
11	AD	F	GPP; recreational soccer and pickleball	moderate
1	CON	M	GPP; snowboarding, cycling	light-moderate
2	CON	M	GPP	moderate
3	CON	F	runner; GPP (light)	moderate
4	CON	F	GPP, former soccer player	moderate
5	CON	F	GPP (HIIT, circuit training)	light-moderate
6	CON	M	GPP, manual labor	light
7	CON	F	GPP	light-moderate
8	CON	F	GPP	moderate
9	CON	M	Distance Runner	heavy
10	CON	F	Distance Runner	moderate-heavy

GPP= General Physical Preparedness (mix of cardio and resistance training). Frequency of Physical Activity sessions per week: Light (1-3 sessions per week), moderate (4-5 sessions per week), heavy (5+ sessions per week). Subjects with multiple categories reported a blend of physical activity frequency during their six-month physical activity history. HIIT= high-intensity interval training.

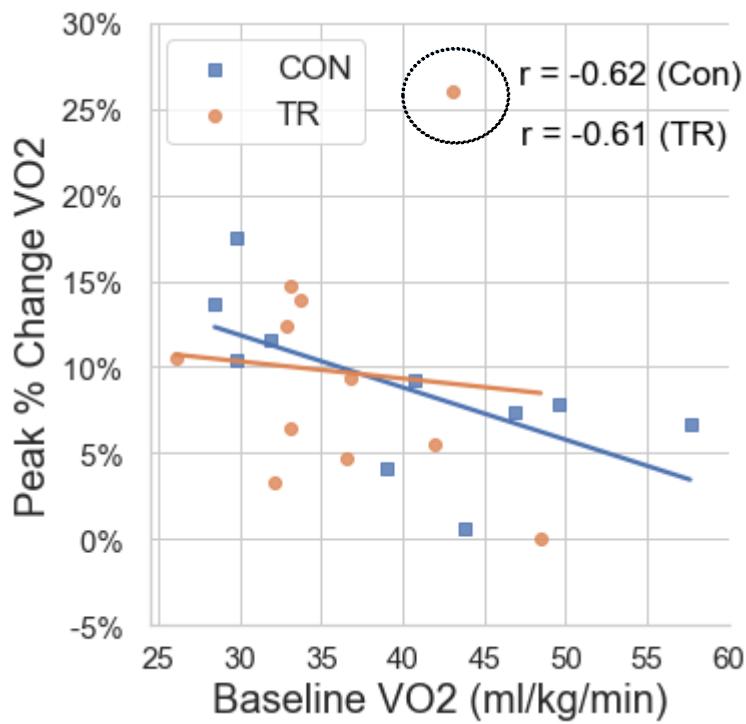
APPENDIX B: SUPPLEMENTARY FIGURES

Figure S.1. Timeline of study participation by month for CON, AD, and OR subjects.



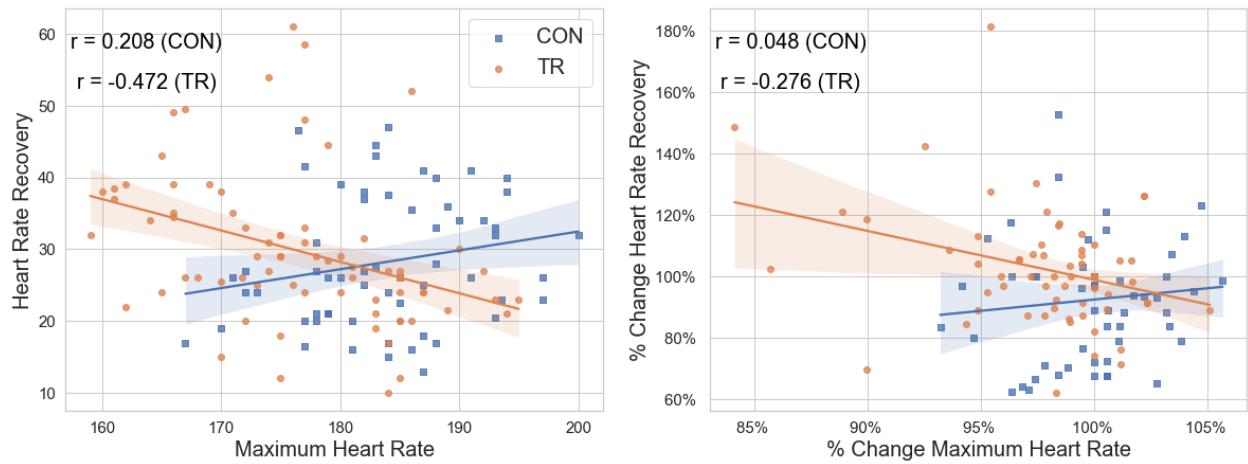
Study participant timelines for control (CON), adapted (AD), and overreached (OR) participants. Participants are organized by either control or training (TR) groups in the figure; whoever, participants were randomized into either group upon recruitment. Data collection was performed from June 2021 through April 2023.

Figure S.2. Correlation plot of baseline $\dot{V}O_{2\text{Peak}}$ and peak change in $\dot{V}O_{2\text{peak}}$ in CON and TR subjects.



Pearson's correlation coefficient (r) for data from control (CON) and training (TR) groups. TR datapoint with surrounding circle was identified as an outlier and removed during correlation calculations. Pearson $r=0.08$ for TR data when this datapoint remained in correlation calculation.

Figure S.3. Correlation plots of MHR and HRR in CON and TR subjects.



Pearson's correlation coefficient (r) for data from control (CON) and training (TR) groups.