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Examining the Effects of Exercise-Induced, Physical Stress Overtraining on Stress Biomarkers in Adolescent, C57BL/6 Mice

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EXAMINING THE EFFECTS OF EXERCISE-INDUCED, PHYSICAL STRESS OVERTRAINING ON STRESS BIOMARKERS IN ADOLESCENT, C57BL/6 MICE

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APPROVAL OF THESIS

We, the faculty supervising the work of Curtis "Scotty" Davis, affirm that the thesis, *Examining The Effects of Exercise Induced, Physical Stress Overtraining on Stress Biomarkers in Adolescent, C57bl/6 Mice*, meets the high academic standards for original scholarship and creative work established by the Department of Exercise Science and the College of Health Professions. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Robert O. Powell, PhD   Committee Chairperson   Date 11/04/2022

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LIST OF ABBREVIATIONS AND ACRONYMS

OTS – overtraining syndrome

OR – overreaching

FOR – functional overreaching

NFOR – non-functional overreaching

IGF-1 – insulin-like growth factor 1

GH – growth hormone

ELISA – enzyme linked immunosorbent assay

MRI – magnetic reasoning imaging

NS – not significant

MANOVA – multivariate analysis of variance

ANOVA – analysis of variance

GAS – general adaptation syndrome

FITT – frequency, intensity, time, type

SAID – specific adaptation to imposed demands

EV – exhaustion velocity

ILT – incremental load test

SD – standard deviation

ABSTRACT

Exercise has long been established as a therapeutic modality to improve health and related physical fitness, sports performance, and injury/risk reduction in both adults and adolescents (Haff & Triplett, 2015; Kaminsky, 2010). Nonetheless, there is a lack of research investigating the negative effects of exercise overstress (i.e., overtraining syndrome) in these populations, particularly adolescents (Brenner & American Academy of Pediatrics Council on Sports Medicine and Fitness, 2007; Matos & Winsley, 2007). The objective of this study was to examine hormone biochemical markers, anthropometric markers, and behavioral traits, which are associated with overtraining syndrome, in adolescent C57BL/6 mice. A total of 24 mice were randomly assigned to three groups in this study (sedentary group, moderately-trained exercise group, and overtrained exercise group; n=8 mice per group) during an eight-week intervention. Primary assessments were conducted at baseline, mid, and post-intervention and included the hormones: corticosterone and IGF-1 as well as body composition measures using MRI. Behavioral measures included weekly food consumption using a digital scale, edge time and center time using open field apparatus and Any-maze software. Baseline values for hormone concentrations, anthropometrics (body composition) and behavior of each group were the same, respectively with the exception of precent lean mass which displayed a significant difference between Overtrained and Sedentary groups (p= 0.034059). Following the intervention, there was a significant difference in plasma corticosterone concentrations across time between Overtrained and Sedentary groups (p=0.0022) as well as Overtrained and Moderate Trained groups (p=0.0098). The Overtrained group displayed the highest levels of corticosterone concentrations in ng/ml (153.09 ± 99.92) among all groups post-study time point (8 weeks). IGF-1 results showed no significant differences among groups across time (p=0.9754); however, time was

significant, and all groups decreased in IGF-1 levels across time (p=0.0168). Food consumption results showed significantly different group interactions (p<0.0001) with the Overtrained group displaying the greatest amount of food consumed among all groups between weeks four-eight (132g-183g). In conclusion, the results of this study contribute to the literature regarding the exercise stress response in adolescent mice with physical overstress demonstrating greater negative responses to overtraining variables. Further research is necessary to better understand overtraining syndrome in adolescents, what biomarkers need assessed, and their implications to determine clinical relevance to physicians, coaches, parents and exercise specialists alike to recognize and reduce overtraining syndrome.

CHAPTER 1

INTRODUCTION

Exercise and Physical Activity have long proven effective outcomes in improving quality of life, severity of diseases, and athletic performance (Haff & Triplett, 2015; Kaminsky, 2010). However, there is a paucity of literature observing negative physiological overstress due to excessive amounts of exercise, particularly in adolescents (Brenner & American Academy of Pediatrics Council on Sports Medicine and Fitness, 2007; Matos & Winsley, 2007).

Exercise and Physical Activity improve physical fitness and quality of life by providing appropriate stress to the body's organ systems such as the cardiorespiratory system, musculoskeletal system and endocrine system (Kaminsky, 2010). Conditions like osteoporosis and type-2 diabetes mellitus are among many diseases whose negative health parameters can be mitigated by properly implemented exercise and physical activity (Kaminsky, 2010). Under specific training load and modalities, the body will adapt accordingly and is classically termed Specific Adaptations to Imposed Demands (SAID) (Haff & Triplett, 2015). The basis of the SAID principle lays the foundation for exercise programs to specifically induce favorable adaptations, in both general or clinical populations, to improve quality of life or prevent/mitigate common physiological diseases and for athletes to improve athletic performance (Haff & Triplett, 2015; Kaminsky, 2010). However, if exercise programming is not appropriately designed where there is an inability to physiologically recover via rest then what is known as overtraining syndrome can occur (Cadegiani & Kater, 2019; Haff & Triplett, 2015; Kreher & Shwartz, 2012; Saw et al., 2016).

Overtraining syndrome (OTS) can be defined as a physiological state in which an individual is unable to adapt/recover from training loads that result in decreases in performance as well as negative effects on health (Cadegiani & Kater, 2019; Kreher & Shwartz, 2012; Saw et al., 2016). OTS can affect anyone who encounters chronic, physical overstress, however its prevalence in athletes is most common. According to Meeusen et al. (2013), 60% of female elite runners and 64% male elite runners have been reported experiencing overtraining syndrome at least once within their career. Other findings report that OTS has been observed in 33% of non-elite runners, 34.6% in young athletes and 37% across 16 different high school sports (Meeusen et al., 2013). Additionally, the multifaceted and complex nature of OTS has proven difficult to determine direct causes, specific biomarker expression, as well as acute and chronic implications (Lewis et al., 2015; Meeusen et al., 2013). Therefore, more attention to OTS is required to determine its cause, prevalence, and overall negative side effects associated within this syndrome (Meeusen et al., 2013).

OTS leads to a multitude of symptoms (Kreher & Schwartz, 2012). OTS can vary on the individual, however common symptoms may include feelings of staleness, fatigue, and decreased performance in part or coupled with the inability to recover from exercise training. This reduces positive physiological adaptations and performance measures even with prolonged rest (Carfagno & Hendrix, 2014). Kreher & Schwartz (2012) explain the symptoms of overtraining include: fatigue, depression, bradycardia, "loss of motivation," insomnia, irritability, agitation, tachycardia, hypertension, restlessness, anorexia, weight loss, "lack of mental concentration," "heavy, sore, stiff muscles," anxiety, and "awakening unrefreshed" (pg.129). Indeed, OTS can have negative effects on an athlete's health. Therefore, finding strategies or evidence to identify OTS is beneficial to anyone who may be at risk of its development.

Currently, OTS has been determined within subjects through surveys, symptoms and most importantly, changes in physiological performance (Carrard et al., 2021). However, these methods primarily require subjective feelings and retrospective analysis. For instance, those who are experiencing OTS are likely to have disturbances in mood, underperformance and reduced vigor. Still, these symptoms /assessments of mood and vigor are not solely a result of OTS (Uusitalo, 2001). Subjective feelings of mood and performance could be a result of non-training factors like undiagnosed diseases, poor diet, and lack of sleep (Uusitalo, 2001). Therefore, using these methods alone may not be appropriate for diagnosing OTS. Common outcome measures of overtraining are defined as the inability to recover while sustaining a continual decrease in performance after 14-21 days of rest (Kreher & Schwartz, 2012). Such rest and recovery provide a detriment to the functional capacity and performance requirements of athletes and those necessitating occupational physical labor or physical activity recreation. The ability to analyze pre-symptomatic precursors of overtraining has given rise to multiple theories of different assessments that researchers use to predict and diagnose OTS (Lee et al., 2017).

Diagnosing OTS can be done through the analysis of biochemical/biological markers (Cadegiani & Kater, 2017; Cadegiani & Kater, 2019; Califf, 2018; Lee et al., 2017). Biomarkers can be defined as any biological substance that can be used to indicate a pathological condition or can change due to external or internal biological influences (Califf, 2018). Researchers have attempted to use biomarkers as a form of assessing the cause, prevalence, and diagnosis of overtraining in adults (Cadegiani & Kater, 2017; Cadegiani & Kater, 2019; Lee et al., 2017). Common biomarkers explored have been IGF-1, Growth Hormone (GH), Cortisol, and Testosterone (Cadegiani & Kater, 2019). Cortisol levels have shown to be an important marker in OTS, however only if they are assessed regularly over time (Gouarné et al., 2005).

A study done by Cadegiani and Kater (2019) found decreases in testosterone of overtrained adult males in comparison to non-overtrained adult males, however no significant differences in IGF-1 or testosterone-cortisol ratio was observed. Conversely, an earlier comprehensive review conducted by Cadegiani & Kater (2017) found 50% of studies reported alterations in IGF-1 in overtrained subjects while the other 50% of studies reported no significant changes in IGF-1. Although IGF-1 appears to have conflicting evidence in OTS, changes in its levels are dependent on initial or current circulating levels (Haff & Triplett, 2015).

Debate remains about which biomarkers are critical to the stress-induced state that overtraining places on the body. This has led to analyzing hormones as useful biomarkers (Carfagno & Hendrix, 2014; Cadegiani & Kater, 2019). With this, IGF-1 and Cortisol are among the most commonly used biomarkers to assess OTS. However, the aforementioned studies have been evaluated in adults and therefore, these results cannot be generalized to the adolescent population.

Adolescents like adults can improve health, strength and athletic performance as a response to exercise training (Matos & Winsley, 2007). However, hormonally and metabolically, adults and adolescents are different in the degree of change in physiological adaptations over time (Matos & Winsley, 2007). Therefore, overtraining syndrome analyses in adolescents must take into consideration their physiological differences from adults. The lack of research and assessments of overtraining in youth has considered the synopsis to most that children experience overtraining syndrome both at similar duration, symptoms and etiology to adults (Brenner & American Academy of Pediatrics Council on Sports Medicine and Fitness, 2007; Matos & Winsley, 2007).

In conclusion OTS is a serious pathophysiological state that not only detriments physical performance but health as well (Cadegiani & Kater, 2019; Carfagno & Hendrix, 2014; Hug et al., 2003; Kreher & Shwartz, 2012; Meeusen et al., 2013). Although the extent of how common overtraining is in athletes, many researchers have identified that overtraining is a real syndrome due to the inability of individuals to adapt and recover from training induced stressors. (Cadegiani & Kater, 2019; Carfagno & Hendrix, 2014; Kreher & Shwartz, 2012; Meeusen et al., 2013). Surveys, symptom assessments, and performance markers can be used as a part of diagnosing overtraining syndrome (Carrard et al., 2021). However, to further the knowledge of the topic regarding overtraining syndrome, researchers have also performed assessments of biomarkers in adults in hopes that they can predict and prevent OTS before it reaches a point by which conventional means of rest are no longer viable (Cadegiani & Kater, 2017; Cadegiani & Kater, 2019; Carrard et al., 2021; Lee et al., 2017). The lack of research on OTS in youth creates a gap within the research considering, biochemically and hormonally, adolescents may have different levels of biomarkers and time to symptoms when experiencing an overtrained state. Therefore, understanding the long-term implications in youth athletic development, overall health and growth/development is paramount.

Statement of the Problem

There is little research investigating adverse stress indicators on performance and the health implications of overtraining syndrome in adolescents.

Purpose

To analyze the effects of over stress exercise training (overtraining) on levels of stress hormone biochemical markers, anthropometric measurements, and behavioral traits in adolescent C57BL/6 mice.

Research Question

Will overtrained adolescent mice have different levels of biochemical markers, anthropometrics, and behavioral traits compared to trained and sedentary adolescent mice?

Research Aims

Aim 1- to determine the effects of OTS on circulating biomarkers (corticosterone and IGF-1) in overtrained, moderately trained, and sedentary adolescent mice.

Aim 2- to determine the effects of OTS on anthropometric measurements (body weight, lean mass, fat mass, percent lean mass, and percent fat mass) in overtrained, moderately trained, and sedentary adolescent mice.

Aim 3- to determine the effects of OTS on behavioral traits (food consumption, open field edge time, and open field center time) in overtrained, moderately trained and sedentary adolescent mice.

Hypothesis 1

Null Hypothesis

There will be no difference in levels of blood biomarkers (corticosterone and IGF-1) among groups (sedentary, overtrained, and moderately trained)

Alternative Hypothesis

There will be a difference in levels of blood biomarkers (corticosterone and IGF-1) among groups (sedentary, overtrained, and moderately trained)

Hypothesis 2

Null Hypothesis

There will be no difference in anthropometric measurements (body weight, lean mass, fat mass, percent fat, and percent lean) among the groups (sedentary, overtrained, and moderately trained)

Alternative Hypothesis

There will be a difference in anthropometric measurements (body weight, lean mass, fat mass, percent fat, percent lean) among the groups (sedentary, overtrained, and moderately trained)

Hypothesis 3

Null Hypothesis

There will be no difference in behavioral traits (food consumption, open field edge time, and open field center time) among the three groups (sedentary, overtrained, and moderately trained)

Alternative Hypothesis

There will be a difference in behavioral traits (food consumption, open field edge time, and open field center time) among the three groups (sedentary, overtrained, and moderately trained)

Operational Definitions

Biomarkers- Any biological substance that can be used to indicate a pathological condition or can change to external or internal biological influences. (Califf, 2018)

Blood Biomarkers- Biomarkers that can be measured in the blood (plasma or serum)

Blood Samples- Samples of blood extracted from easily accessible blood vessels such as veins (submandibular vein)

Contamination- The presence of foreign entities that disrupt and or change the quality and content of a biological sample.

Overtraining Protocol- A protocol that induces overtraining syndrome through the use of downhill running (% decline) and in turn causes greater eccentric forces on musculature which leads to greater muscle tissue damage and inflammation. (Pereira et al., 2012)

Moderate Intensity Training Protocol- Training protocol to induce improvements by training mice at their maximal lactate steady state (MLSS). (Pereira et al., 2012)

Overtraining Syndrome- A physiological state where an individual is unable to adapt/recover from training loads that results in decreases in performance (Cadegiani & Kater, 2019).

Sedentary Mice- Mice that remain solely in a facility housing cage with no additional exposure to physical movement or exercise outside of the housing cage during the study period.

Wild-Type (C57BL/6 mice)- Most commonly used mouse model to represent a "wild-type" mouse and serves as the most common base strain for a wide array of research testing in mice (Mekada et al., 2009).

CHAPTER 2

LITERATURE REVIEW

Overview of Research Topic

Exercise and physical activity have been well established in their ability to improve health and increase physical capacity (Kaminsky, 2010). The positive benefits of exercise and training are key aspects in the increases in performance that athletes experience within their respective sport. Athletes' can acutely push their bodies to extreme limits when it comes to sports. Strength coaches and personal trainers alike may incorporate brief periods in time where they create an intentional overload on their athletes/clients to induce what is known as supercompensation (Hough et al., 2021; Stone et al., 1991). This supercompensation will allow a greater physiological reaction to increase physiological adaptation rate (Stone et al., 1991). However, human beings are still finite in their ability to adapt to stressors and long periods of overload with limited rest can be detrimental. When human beings surpass their physiological capabilities or stay within an overreached state for too long with minimal recovery, they can experience what is known as overtraining syndrome (OTS) (Cadegiani & Kater, 2019; Kreher & Shwartz, 2012; Saw et al., 2016; Stone et al., 1991). For athletes and recreational exercisers to experience positive adaptations for performance and health, proper recovery within their training plan must be implemented. If the ratio between training and rest is not sufficient, then athletes can experience OTS (Kreher & Schwartz, 2012). Attempting to identify overtraining syndrome before it reaches critical physiological time periods is critical for keeping athletes on their respective surfaces and for maintaining health and fitness in the recreational exerciser (Armstrong & VanHeest, 2002; Cadegiani & Kater, 2019; Cheng et al., 2020; Meeusen et al., 2013). The use of certain biological factors as possible biomarkers has shed light on predicting

and diagnosing OTS. However, many of these studies have been performed in the adult

population and there is limited such research in youth/adolescent populations. To better

understand OTS and its consequences, one must first be aware of the benefits of normal exercise

training.

<center>**The Typical Response to Exercise**</center>

Physical Activity vs Exercise

Physical activity can be defined as any activity that engages the body through the use of

skeletal muscle contraction and increases in the breakdown of substrates to produce energy

above a normal homeostatic resting state (U.S. Department of Health and Human Services,

1996). The qualities of physical activity encompass a wide range of activities that may include

walking, gardening, manual labor jobs, sitting/standing etc. Physical activity has been shown to

have positive health benefits (increased strength, endurance, stability, and metabolic regulation),

and overall ability for one to improve their ability to engage in such tasks (Kaminsky, 2010).

However, physical activity is often limited in its performance benefits in those that already have

high levels of physical fitness. Physical fitness is defined as characteristics that allow an

individual to carry out physical activities (Caspersen et al., 1985). The health-related physical

fitness characteristics include body composition, cardiorespiratory fitness, flexibility, and

muscular fitness (strength and endurance) while the skill related physical fitness characteristics

involve motor control, speed and agility, and muscular power. To gain increases in physical

performance and or health, physical activity must become structured and intentional in

frequency, intensity, time, and type (i.e., FITT Principle). Taking up "Exercise," defined as

planned or structured form of physical activity, is where such emphasis of FITT takes place (U.S.

Department of Health and Human Services,1996). Indeed, physical activity/exercise is a form of

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physical stress placed on the body, that causes a period of time where homeostasis is disrupted. However, the human body efficiently responds to this disruption in homeostasis and adapts based upon the opposed demands of exercise (Haff & Triplett, 2015).

Benefits of Appropriately Implemented Exercise

Exercise can improve not only the athletic ability but also the quality of life of individuals. It is known that exercise can improve physical fitness: cardiorespiratory endurance, muscular strength/endurance, stability, flexibility, and body composition (Kaminsky, 2010). In addition to greater increases in physical fitness, exercise has been shown to improve and or mediate chronic diseases like type 2 diabetes mellitus, multiple sclerosis (MS), heart disease, osteoporosis, chronic obstructive and restrictive lung disease, and obesity by means of physiological exercise adaptations that improve blood sugar regulation, pain sensitivity, cardiac function and blood delivery, bone building properties, respiratory muscle endurance, and metabolic rate (Kaminsky, 2010). For example, a study conducted by Schroeder et al. (2019), found that the incorporation of an eight weeklong mixed exercise program of strength training and endurance training reduced peripheral blood pressure (-4 mmHg) and increased oxygen consumption (V02 ml/kg/min) in obese sedentary and hypertensive adults with an average age of 58.

Furthermore, exercise is also known to have benefits on mental health and cognition. A study conducted by de Oliveira et al. (2019), surveyed over 200 elderly subjects on health and anxiety and found that the subjects who engaged in exercise displayed lower anxiety and better mental health with a correlation of R= 0.77 with activity engagement and better mental health scores on questionnaires and surveys. Conclusively exercise is a beneficial form of medicine and improves the quality of life, health, skill-related physical fitness, and controlling many known

diseases. The use of exercise can induce such benefits by offering the optimal levels of stress on the human body to elicit positive adaptations.

Overload Principle and The General Adaptation Syndrome

The main purpose of exercise is to stress the biological systems in such a way that they adapt according to the stress (exercise) that was placed on them. Common terminology for this load and adaptation concept is the specific adaptations to imposed demands (SAID) theory. This theory proposes that adaptations from exercise are proportional to the type and degree of physical stress provided (Haff & Triplett, 2015). An example of this theory are the adaptation and training differences seen between endurance runners and sprinters. Endurance runners train in such a way to maximize cardiorespiratory fitness, muscular endurance and running economy, while sprinters train to maximize running velocity, acceleration, and muscular power. Both athletes may use timed running training and resistance training to elicit adaptations to improve these fitness outcomes. However, the endurance athlete will focus on training styles that promote mechanical efficiency during an event and training modalities that promote adaptations in oxygen uptake and utilization (VO2), oxygen delivery (Cardiac output) and muscle fiber adaptations (increased mitochondrial density and enzyme concentrations) for aerobic fitness. The sprinter will focus on training styles that maximize take off acceleration, sustained maximal velocities, power and improvements in anaerobic energy systems (glycolysis, creatine phosphate pathways) for anaerobic high intensity fitness. However, for either of these athletes in the examples above to achieve their specific adaptations they must appropriately stress their bodies using specific exercise modalities. One common way to achieve these adaptations is to "overload" the body beyond its current physical limits (Haff & Triplett, 2015).

A widely accepted theoretical model of exercise explaining the adaptive response to stress has been eloquently described by Hans Selye's "Theory of General Adaptation Syndrome" (GAS). The GAS can be broken into four primary components as followed: alarm phase, resistance phase, supercompensation phase, and overtraining phase (Haff & Triplett, 2015). The "alarm phase" can be characterized as the stimulus or stressor from exercise where a temporary drop in performance is seen (Haff & Triplett, 2015). The "resistance phase" is the adaptation or recovery response where performance values rise above previous performance (Haff & Triplett, 2015). The "supercompensation phase" is marked as the new baseline capacity in performance (Haff & Triplett, 2015). It is at phase three (supercompensation) where the newly acquired adaptations lead to improved performance. Of course, these adaptations subsequently require a progressive stimulus (i.e., stress) from exercise for further adaptations to continue (Halson & Jeukendrup, 2004). However, if an individual does not get adequate physiological recovery between the alarm phases and resistive phases or if such phases are too high in stress, the risk of phase four (overtraining) can ensue. The "overtraining phase" can be seen when individuals do not adapt according to the first three phases, and performance may plateau or worse, decline due to catabolic responses (Haff & Triplett, 2015). However, it is normal for the initial response to a new stressor for performance to decline before adaptations occur (as seen in phase 1-2); therefore, a distinct length of time while undergoing recovery must pass before an individual can be classified as experiencing overtraining (Armstrong & VanHeest, 2002; Cadegiani & Kater, 2019; Haff & Triplett, 2015; Meeusen et al., 2013). There are performance benefits from the deliberate use of exercise loads that exceed what an individual is capable of for the purpose of enhancing supercompensation. These deliberate periods used by exercise professionals and athletes is known as "overreaching".

Overreaching vs Overtraining

Overreaching is defined as a deliberate attempt to "overstress" an athlete using training volumes and or intensities that are beyond that individual's functional capacity in performance. (Fry & Kraemer, 1997; Haff & Triplett, 2015; Halson & Jeukendrup, 2004). This method causes large decreases in performance for a brief period of time. Hence if effectively overstressed for several days to weeks, the adaptation benefits far exceed that of normal training volume adaptations (Haff & Triplett, 2015).

This form of overreaching is termed functional overreaching (FOR) and is used regularly in sports performance training blocks. Nonetheless, if the overreaching strategies are not monitored sufficiently, it can lead to detrimental overstress, which is known as non-functional overreaching (NFOR) (Fry & Kraemer, 1997; Haff & Triplett, 2015). Individuals experiencing NFOR may require weeks to recover from the training load, and in some cases, individuals may not experience any positive improvements in performance and cross over into what is known as overtraining syndrome (OTS) (Haff & Triplett, 2015). NFOR and OTS share many of the same characteristics, however, the key difference between the two is time to physiological recovery. Although NFOR may take weeks before individuals return to baseline performance levels, many individuals can still recover if given appropriate rest (Fry & Kraemer, 1997). The response differs if an individual is experiencing true OTS as it may take months up to a year before they fully recover. It is this delicate overstress scheme that exercise professionals and coaches alike must be able to discern between OTS and OR/FOR/NFOR. Although exercise is beneficial and can even be manipulated in such ways to elicit higher adaptations for a given stress, uncontrolled exercise stresses may resort to irreversible catabolic responses.

Physical Overstress with Exercise and Overtraining Syndrome

Overtraining Characteristics

Overtraining syndrome (OTS), is a complex syndrome that has led many researchers to study its impact on athletes and exercise enthusiasts alike. However, defining the syndrome is the first step in studying its consequences. The most profound characteristic of OTS is the relationship between training load and training recovery (Kreher & Schwartz, 2012). Saw et al. (2016), states "Training imposes stress on an athlete, shifting their physical and psychological well-being along a continuum that progresses from acute fatigue to overreaching, and ultimately overtraining syndrome" (pg.1). Physiological signs and symptoms of OTS involve accelerated resting and submaximal exercising heart rate, increased resting and submaximal exercising blood pressure, enhanced glycolytic metabolism leading to early glycogen depletion, altered hormone secretion, and receptor activity, and altered neuromuscular facilitation. Psychological signs and symptoms of OTS include depression, anxiety, insomnia, and mental "burnout." Hence, overtraining syndrome is defined by Brenner and American Academy of Pediatrics Council on Sports Medicine and Fitness (2007), as "a series of psychological, physiologic, and hormonal changes that result in decreased sports performance" (pg.1243).

Consequently, overreaching and overtraining share many characteristics initially, but what sets them apart is the degree of recovery needs and severity of the aforementioned signs and symptoms. Therefore, the most widely agreed upon definition of overtraining syndrome (OTS) is defined as a physiological state where an individual is unable to adapt/recover from training loads even after two - three weeks (14-21 days) of sustained rest (Armstrong & VanHeest, 2002; Cadegiani & Kater, 2019; Meeusen et al., 2013). The relationship between training load and recovery lays the groundwork for researching OTS. However, a gap exists in

research regarding the identification of OTS presence and characteristics in adolescent population.

Etiology of Overtraining Syndrome

The complex nature of overtraining syndrome requires those who study the disease to delineate between foundational causes that support a shared definition. Cadegiani and Kater (2017) state "The imbalance between training and recovery, which can be worsened or confounded by inadequate nutrition, illness, psychosocial stressors and sleep disorders [1], among many other causes, leads to dysfunction of pathways and responses in immune, inflammatory, neurological, hormonal and metabolic systems as a maladaptation to chronic exposure to extreme metabolic and tissue environments" (pg.1). Like most diseases and conditions, overtraining syndrome shares characteristics that could be mistaken for other stress responsive states.

Although other factors can contribute to the degree of overtraining syndrome, they may not be the cause. According to Meeusen et al. (2013), the causational factors that may be responsible for overtraining syndrome should not consider diseases, dietary factors, ratio of energy supply, mineral or vitamin deficiencies to be the cause of true overtraining. Therefore, it is crucial to not mistake all other "stressors" as the direct cause of overtraining syndrome but rather that they contribute to the severity of the symptoms experienced from the imbalance of training and recovery (Cheng et al., 2020; Meeusen et al., 2013). Conforming to a sound definition and relatable cause allows researchers and physicians to understand the prevalence of physical stress overtraining in a more efficient manner.

Prevalence of Overtraining Syndrome

Due to the various signs and symptoms of OTS and many conditions that coincide with OTS, the extent and overall prevalence of overtraining syndrome is still uncertain (Kreher & Schwartz, 2012). The current prevalence rates of overtraining syndrome have been compiled from different researchers who use their own definition of overtraining syndrome as well as what sample population and variables they evaluate. A large consensus of research agrees that the length of a sport's training session, the training age of an individual and the level the athlete is stressed, also have a relationship with overtraining (Cheng et al., 2020; Carfagno & Hendrix, 2014). Meeusen et al. (2013), states "The risk of NFOR/OTS becomes compounded over the course of an athlete's career. Survey studies of elite runners report 60% of females and 64% of males indicate experiencing at least one previous episode of OTS, with a career rate of 33% in nonelite adult runners" (pg.189). Although prevalence rates of overtraining are difficult to determine accurately, physicians and researchers can assess the symptomatology for better indications of prevalence of OTS (Kreher & Schwartz, 2012; Meeusen et al., 2013). Indeed, the ability to investigate chronic effects of overtraining is yet to be elucidated, however the impact it could have on humans as they age may give insight into its prevalence and further negative impacts (Meeusen et al., 2013)

Common Symptoms of Overtraining Syndrome

Overtraining can negatively impact the central nervous system, cardiovascular system, musculoskeletal system and endocrine system (Armstrong & VanHeest, 2002; Lehmann et al., 1993). The impacts on these physiological systems create a wide array of symptoms. According to Kreher and Schwartz (2012), the symptoms of overtraining include: fatigue, depression, bradycardia, "loss of motivation," insomnia, irritability, agitation, tachycardia, hypertension,

restlessness, anorexia, weight loss, "lack of mental concentration," "heavy, sore, stiff muscles," anxiety, and "awakening unrefreshed" (pg.129, Table 2). Carrard et al. (2021), conducted a systematic review assessing the literature on symptoms of overtraining and found eight studies that identified abnormal heart rates and QRS complexes in those who were experiencing OTS as well as studies that found increases in catabolic hormones and decreases in anabolic hormones like: testosterone, GH and IGF-1 in athletes. The wide scope of overtraining yields many symptoms, however there is evidence that differently trained athletes that experience overtraining syndrome may have shared and opposing symptoms. According to Kreher and Schwartz (2012), aerobic athletes who experience OTS are more likely to have alterations in parasympathetic alterations (bradycardia, fatigue, depression), while anaerobic athletes display greater alterations in sympathetic control (insomnia, tachycardia, agitation and hypertension). The symptoms of overtraining syndrome remain vast and even may display opposite symptoms in different types of athletes (anaerobic vs aerobic), therefore, using common symptoms of overtraining as the sole diagnostic method in identifying or classifying OTS is not sufficient. A multidimensional approach must be taken if one is to understand both commonly shared qualities of OTS and unique qualities across age, sex, and athletic discipline

Diagnostic Criteria of Overtraining Syndrome

The first step in determining if an individual or athlete is experiencing overtraining syndrome is by evaluating the common symptoms associated with it. Those experiencing overtraining syndrome present drops in performance even with long periods of recovery in conjunction with changes in mood/behavior, however some may display no signs or symptoms that would cause performance decrease from other stimuli besides training (Kreher & Schwartz, 2012). However as mentioned previously by Meeusen et al. (2013), the symptoms of

overtraining must be ruled out to diagnose true overtraining. Therefore, other means of

identifying overtraining syndrome must be established. Only if an individual presents continual

decline in performance after more than 14-21 days of rest can OTS be diagnosed (Kreher &

Schwartz, 2012).

In conjunction with performance decline, the evaluation process of determining OTS

should take into consideration overall stressors and evaluate different physiological or biological

systems, hormone levels, immune function, body composition and behavior (Carrard et al., 2021;

Carfagno & Hendrix, 2014). To this effect research attempts to look further into behavior,

biomarkers, and anthropometrics in hopes of finding trends in those who are at risk or currently

experiencing OTS.

Behavioral Characteristics of Overtraining Syndrome

Behavior and Stress

Merriam Webster dictionary defines behavior as "the response of an individual, group, or

species to its environment" (Merriam Webster, 2022). Often, behavior is a response to the

external environment. However, this response in behavior may not be intentional. Both a

common external and internal influence on behavior is stress. Stress can alter the behavioral

traits of individuals including hostility and withdrawal from normal behavior (Sandi & Haller,

2015). Similarly, this type of behavior is often seen in individuals who are experiencing OTS

(Kreher & Schwartz, 2012).

Many psychologists agree socialization is a behavior influenced by one's environment.

Such environments could affect eating patterns, anxiety, exercise, social withdrawal, etc. Indeed,

abnormal eating behavior is often associated with anxiety and depression, and some propose that

stress-related hormonal response alters ones eating behavior (Konttinen et al., 2019). Exercise

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acts as a stressor on the body, and therefore, if overstressed, it can alter the behavior of individuals by way of OTS. OTS has been shown to alter eating through behavioral increased appetite or decreased appetite (Lehmann et al., 1997). Therefore, exploring behavioral traits like eating and withdrawal/motivational loss in those who may be overstressed with exercise and the risk of OTS could lend credence to OTS familiarity.

Mood and mental health surveys can be easily implemented in human studies regarding the relationship between OTS and behavior. In fact, qualitative data regarding behavior in humans can be easily obtained via oral communication and or written language. However, these methods regarding behavior analysis are not so easily done in animal studies. The use of animal models in research creates a disconnect in communication between investigator and subject, therefore only educational assumptions, quantitative measures, and observational data can be used to assess such things as feelings of anxiety in animals. To many researchers' benefit, behavioral traits like eating and anxiety can also be observed in animal research models.

Assessing Anthropometrics in Overtraining Syndrome

Measuring Body Composition

Body composition assessment is a form of anthropometry where tools are used to measure such things as the body's components and relative proportions (Kaminsky, 2010). Body composition is based on the density of particular tissues within the body (lean mass and fat mass) expressed as density= mass/volume (Kaminsky, 2010). Furthermore, relative make up of an individual can be determined from density using varying techniques (Skinfold, total body plethysmography, bioelectrical impedance, and dual-energy xray absorptiometry (DEXA)). Body composition can be expressed in absolute values (body mass, lean mass, fat mass) and relative values (% lean mass and % fat mass) (Kaminsky, 2010).

Anthropometrics and Overtraining Syndrome

Results from Cadegiani & Kater (2018), found that overtrained (OTS) subjects displayed higher % body fat and lower % muscle mass compared to healthy athletic subjects. However, when compared the OTS group displayed a higher % muscle mass and lower % body fat in comparison to sedentary control subjects. Other studies have also indicated that OTS subjects experience a lower % lean mass and a higher % fat mass when compared to healthy trained subjects (Cadegiani & Kater 2019; Carrard et al., 2022; Joro et al., 2017). These findings offer useful insight into using body composition assessments when studying the effects of OTS. Although the methods of collecting body composition can range among studies via the use of total body plethysmography, DEXA, or skinfolds, the results remain consistent across the scope of research in OTS. Behavioral characteristics like food consumption and energy expenditure have influences on body composition, however biological molecules such as hormones can also both influence and are influenced by changes in the external environment.

Biomarkers

Biomarkers Defined

Biomarkers can be any biological substance that is used as a means to explain in a larger context how the body is responding physiologically and, in most cases, in pathophysiological conditions (Califf, 2018; Capecchi et al., 2020; Mastorakos et al., 2005; Palacios et al., 2015). Furthermore, biomarkers can be used as a means to not only diagnose the current biological/physiological state, but also as a means to predict future biological/physiological states or risks (Califf, 2018). Biomarkers can range from proteins and enzymes to hormones and substrates (i.e., glucose) (Mastorakos et al., 2005).

Biomarkers for Identifying Overtraining Syndrome

Current evidence has suggested that hormones are the greatest influencer in OTS. Through the natural process of exercise, the desired goal is to increase performance by creating mechanical and physiological overload/stress on the body to signal for adaptations and repair within the cells of respective tissues (Stone et al., 1991). Conclusively exercise is considered a "stressor" on the human body, and that stress creates hormonal signaling from the brain and endocrine system (Mastorakos et al., 2005). Anabolic hormones and stress hormones are among the most commonly studied hormonal biomarkers for overtraining syndrome (Hough et al., 2021). The compounding nature of overtraining creates key shifts in the anabolic and catabolic hormones. These shifts give strong evidence to assess the time points of individuals going through overtraining (Cadegiani & Kater 2019).

The research conducted by Cadegiani & Kater (2019) evaluated testosterone, estradiol, insulin-like growth factor 1, thyroid-stimulating hormone, and many thyroid hormones. The authors performed biochemical and hormonal assays on fifty-one men that ranged from 18 to 51 years old. The men used in the study were "suspected" to be overtrained.

A comparative 25 healthy males and 12 sedentary individuals were used as controls. Conclusively the results found males appeared to have reductions in testosterone and increases in estradiol with no significant difference in IGF-1 levels or testosterone: cortisol ratio. The authors propose that the shifts in these hormones may have inhibited recovery adaptations that were present in the healthy athlete group.

IGF-1

Insulin like growth factor-1 (IGF-1) is a polypeptide hormone secreted by the liver to promote protein synthesis during growth and repair (Haff & Triplett, 2015). This hormone is stimulated and released in response to muscle damage and can act to stimulate skeletal muscle stem cells to undergo protein synthesis (Yoshida & Delafontaine, 2020). However, IGF-1 is also affected/dependent on other anabolic hormones like testosterone and growth hormone (GH). In the presence of other anabolic hormones and energy supply, IGF-1 has greater effects on protein synthesis. It is during the pubertal phase that IGF-1 and other anabolic hormones are at their highest concentrations that promote tissue growth (Pathology Tests Explained, 2022). However, IGF-1 and other anabolic hormones are inhibited by catabolic hormones like cortisol. In cases of normal hormonal regulation, brief periods of high release in catabolic hormones (i.e., acute exercise) do not have long term detrimental effects on IGF-1 but rather under chronic periods of stress (i.e., OTS) and high levels of catabolic hormones that inhibits IGF-1 activity.

IGF-1 and Normal Exercise Response

Under normal exercise conditions the response of IGF-1 increases after strenuous bouts of exercise if starting concentrations are low, but if concentrations are already elevated then IGF-1 levels remain constant and or decrease after exercise bouts (Haff & Triplett, 2015). This regulation may be influenced by energy availability, other anabolic hormones, or the presence of catabolic hormones. In both men and women normal resting concentrations of IGF-1 that are between 10-20 nmol/L are low enough to elicit further increases as a response to exercise (Haff & Triplett, 2015).

Overtraining Syndrome and IGF-1

Cadegiani & Kater (2017), found a divide in studies regarding the influence of OTS on IGF-1 concentrations. There comprehensive review found 50% of studies reported no altered IGF-1 levels due to OTS and 50 % of studies found alterations in IGF-1. This finding may further support the notion of IGF-1 activity and its dependence on its baseline levels. Although the literature shows conflicting results in the response of IGF-1 and OTS, consideration of initial hormone levels may be a key factor in understanding if OTS alters IGF-1 concentrations and activity. To this effect, studies that take into consideration pre-experimental IGF-1 concentrations may be able to better determine if alterations or lack thereof are due to OTS.

Cortisol

Cortisol is a steroid hormone in the glucocorticoid class and acts as a primary catabolic hormone in humans. Cortisol release is closely linked to the body's natural circadian rhythm and is in the highest concentrations in the morning or after an individual completes normal sleep cycles (Haff & Triplett, 2015). The catabolic effects of cortisol inhibit protein synthesis and promote the breakdown of amino acids into carbohydrates as well as combat the inflammatory response and initial steps in preparing the body for protein synthesis. Cortisol is necessary under short-duration stress conditions like exercise and is for the most part regulated well in healthy individuals and does not pose negative effects; however, when individuals are under chronic stress, cortisol's catabolic effects become unregulated and can inhibit repair processes like protein synthesis and immune cell function (Haff & Triplett, 2015).

Cortisol and Overtraining Syndrome

When individuals are under chronic states of stress such as frequent exercise bouts with high volume/intensity and low rest periods, cortisol can have detrimental effects on repair

processes and other anabolic hormones like growth hormone (GH) and IGF-1 (Haff & Triplett, 2015). According to Haff and Triplett (2015), cortisol levels must surpass 800 nmol/l to be considered a risk for OTS. When under chronic states of stress and or OTS, cortisol levels remain high and do not become inactivated via being converted to cortisone, which is a crucial regulatory mechanism to lower cortisol levels (Kraemer et al., 2020). In a study conducted by Gouarné et al. (2005), male triathletes were observed to have significant alterations in cortisol/cortisone ratios during the evening hours of high intensity training during their training season. Additionally, the two male triathletes had twice the levels of cortisol and decreases in cortisone when compared to other triathlete counterparts during this season of training. Performance assessments also indicated that the two male athletes met criteria for OTS. This study displays the importance of assessing cortisol levels across a span of time rather than at one specific time point. This would suggest that understanding cortisol's relationship to overtraining is dynamic and conclusions cannot solely be made from single time point measurements at one single hour of the day.

Cortisol and Corticosterone

Cortisol is the primary glucocorticoid in humans, but it can also be seen in rodents like mice. Cortisol can be measured in rodents, but it appears that corticosterone acts as the predominant stress hormone in rodents (Gong et al., 2015). Similarly, cortisol and corticosterone respond identically under conditions of stress (Gong et al., 2015). To this effect, corticosterone can be used synonymously with cortisol when conducting rodent studies.

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Overtraining in the Adolescent population

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Brenner and American Academy of Pediatrics Council on Sports Medicine and Fitness (2007), states "Burnout, or overtraining syndrome, has been well described in the literature for

adult athletes, but little is found regarding its applicability in youth. As previously mentioned, overtraining syndrome can be defined as a "series of psychological, physiologic, and hormonal changes that result in decreased sports performance" (pg.1243). Limited research and lack of youth assessments of overtraining has hypothesized that children experience overtraining syndrome both at similar rates and similar responses and symptomology as adults (Matos & Winsley, 2007). There has been a relationship between increased sports specialization in youth and an increased risk of burnout, injury, and depression (Myer et al., 2016). Due to the normal growth and maturation process, adolescents are at risk for experiencing negative side effects due to being in an overtrained state (Almási et al., 2021). However, based on the physiological status, children and adults are not the same regarding hormone levels during the growth and maturation processes. Hence one should not assume similar responses in hormones as a key biochemical marker within OTS of adolescents and adults. Determining these differences is crucial to expanding the research of OTS across the lifespan.

Hormonal Differences in Adults and Adolescents

It is well known that human anatomy, physiology, and biochemistry change and adapt as we age. As such, the anatomy and physiology of a child compared to an elderly adult can be observed simply by physical appearance. The dose of medicine even differs between adults and children, simply because adults and children are physiologically and metabolically different. When it comes to exercise and fitness, youth athletes respond and adapt differently than adults. Matos and Winsley (2007), state "Furthermore, with the appropriate stimulus, prepubertal and adolescent athletes can show significant increments in muscle strength (13 - 30%). Children can improve anaerobic power (3%-10% Mean Power and 4%-20% in Peak Power), although the mechanisms responsible for the improvements in children remain unclear" (pg.353). One of the

major differences between adults and children is sex characteristics. Increases in strength and aerobic performance of children primarily comes from increases in neurological activation of the heart and muscles. Testosterone is a hormone largely responsible for improvements in strength and aerobic performance. The lack of this key hormone in prepubescent children greatly limits their ability to experience large gains in strength and endurance and is most likely influenced by growth hormone: IGF-1 axis (Matos & Winsley, 2007). Although physiologic differences exist between adults and adolescents, exercise and physical activity is still important in improving health and positive performance adaptations. However, if exercise in adolescents goes beyond that of the ability to recover, hypothalamic activity and regulation of downstream hormone signaling may be altered (Bettio et al., 2020).

Insights from the Animal Research Model

Behavioral Assessments Relevant to OTS

Food consumption in mouse research models is assessed by measuring food weight per individual or group-housed mice twice weekly. By taking the difference between initial food weight and final food weight of each week, one can determine how much food an individual animal or animals consumed. Furthermore, tracking food consumption can be a direct measure of the behavioral trait that can indicate responses from their external environment like exercise training (Jung et al., 2010). The ability to observe food consumption in animal models allows researchers to make associations to behavior changes that may also be exhibited in humans.

Anxiety-related behavior can also be assessed in animal models via the use of open-field equipment and mazes. The open-field behavior test has been a commonly used experiment to assess both anxiety and cognitive disorders since it was first introduced in the 1930s (Hall, 1934; Seibenhener & Wooten, 2015). The open-field experiment can identify behavior associated with

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fear, anxiety, aggression, and or neurocognitive issues (Knight et al., 2021; Mertens et al., 2019; Seibenhener and Wooten 2015). The open-field experiment utilizes a four-walled enclosure with designated zones (inner, middle, and outer) on the floor of the enclosure. Modern renditions of the experiment use motion capture software that can track the animal's movement in the following ways: distance traveled, mean speed, time spent in each zone, and direction of movement. Often procedural protocol requires mice to be placed inside the open-field enclosure one at a time and are monitored for 10-minutes. The open-field assay analyzes mouse behavior on the premise that mice are considered prey and low on the food chain hierarchy, thus they are more likely to avoid dangerous situations. Mice will naturally explore familiar and foreign environments in search for food or other mice (Mertens et al., 2019). However, the behavioral instinct of the mice to explore their environment is dependent upon the safety of that environment. Naturally, the prey instinct hardwired within rodents causes them to display cautious behavior where they will avoid well-lit areas and hide along walls and other structures, to ensure being unseen by possible predators (Mertens et al., 2019). This behavior is mimicked in the open-field test and the level of confidence and or anxiety can be measured via how mice move within the open-field enclosure.

When mice spend a greater duration of the test time along the walls (edge zone) of the open field, it indicates anxiety related behavior, while the opposite is true, if mice spend more time in the middle (inner zone). These measures of anxiety have been observed by Mertens et al. (2019), where their study compared normal C57BL/6 mice to knockout mice (KO) that lacked the important regulatory protein- p62 which is involved in signal transduction pathways within cells in the open-field test. Their results showed that the KO mice spent more time along the edges of the open-field enclosure compared to the controls (C57BL/6). These results indicated

how the deregulation of an animal's internal environment can display different behavior in the open-field. However, animals' external environment can also alter behavior as observed in the open-field study by Zhang et al. (2019), showed that rats that underwent stress from chronic restraint displayed higher measures of anxiety behavior in the open-field compared to controls that were not restrained. The chronically restrained rats spent more time along the edge of the open-field and less time in the center of the open-field. These results show that external stressors like chronic restraint can also be seen in altering behavior in rodents.

Furthermore, the use of the open-field test can be a valuable tool in assessing anxiety-linked behavior in rodents, and if combined with other measures of stress or anxiety it can offer greater insight into how mice respond to both their external and internal environments. Both the food consumption behavior and anxiety behavior can be assessed in mice and compared to OTS. In addition, these behavioral changes can also have observable impacts on physiological systems that can affect downstream hormone levels, metabolism and body composition.

Conclusion

In summary, overtraining syndrome is a prevalent condition that is experienced by all ages and sex. However, the complex nature, etiology, and prevalence have generated extensive research to be conducted in all aspects of OTS including subjective and objective observations and analyses. We can assess overtraining syndrome with physical performance factors, psychological factors, signs and symptoms, and physiological parameters like hormone levels. Assessment of biological markers, especially hormones, are being the focus of current studies regarding overtraining syndrome in adults. Evidence do show that children also experience overtraining syndrome, however the degree and overall impact are still unknown. The hormonal and metabolic differences between adults and adolescents give insight into why researchers must

begin studying overtraining effects on adolescents. Indeed, methods conducted in human trials to

assess hormones, anthropometrics, and behavior can also be conducted in animal research

models. Therefore, this study aims to explore these markers in male adolescent C57BL/6 mice.

CHAPTER 3

METHODS

Study Design and Population Sample

This study utilized a three-group design consisting of an animal-based model for exercise testing and implementation. The animal model chosen were C57BL/6 mice. C57BL/6 mice are a widely used and viable model in animal research due to their close relationship and shared characteristics of true wild-type mice (Mekada et al., 2009; T.J.L., 2022). The research animals were obtained through Hilltop Lab Animals, Inc. (131 Hilltop Drive, Scottdale, Pennsylvania 15683). The mice received no prior alterations or external stressors outside of normal animal delivery and housing protocol prior to the start of the study. This study and all mice used were approved by the Institutional Animal Care and Use Committee (IACUC) of Marshall University prior to beginning the experimental protocol. The research completed the CITI certification training for vertebrate animal research use prior to the beginning of the study. A total of 24 male C57BL/6 mice were obtained and randomly assigned into three experimental groups, "sedentary group", "moderate-intensity training group" and "overtrained group" with an even distribution of eight (n=8) mice per group.

Inclusion and Exclusion

IACUC approval and appropriate research lab/animal handling training were completed prior to the obtainment and use of animals in the experimental protocol. *Inclusion Criteria***:** adolescent C57BL/6 male wild type mice aged four-five weeks old at the start of the study, and appearing physically healthy. *Exclusion Criteria*: mice that have any noticeable physical deformities or physical signs of disease or illness, mice that refuse to exercise or are unable to

perform experimental procedures or mice that develop diseases, health concerns or signs of distress.

Protocol & Procedures

Protocol

For the purpose of this study, the mice chosen were ordered to begin all protocols at the start of adolescent ages (4-5 weeks old). The mice were subjected to an eight-week-long intervention. Mice were assigned randomly into the three experimental groups "sedentary group," "moderate-intensity trained group," and "overtrained group". The overtrained protocol group consisted of eight mice (4-5 weeks old at start) that received the "Downhill running protocol" described by Pereira et al. (2012) (explained in detail in the procedures section). The moderately trained protocol group consisted of eight mice (4-5 weeks old at start) that received the "moderate intensity exercise training protocol" described by Pereira et al., (2012) (explained in detail in the procedures section). The sedentary group consisted of eight mice (4-5 weeks old start) that remained sedentary throughout the entirety of the study. The overtrained and moderately trained groups performed a three-day acclimation phase to the Columbus Instruments Exer-3/6 animal model treadmill (Columbus Instruments, Columbus, OH) prior to administration of their assigned group training interventions.

Instrumentation

The primary instrumentation used in this study to administer the moderate intensity and overtraining downhill running protocols was a Columbus Instruments Exer-3/6 animal model treadmill (Columbus Instruments, Columbus, OH). This treadmill can reach speeds up to 100 m/min, an incline of 20 degrees and decline of -15 degrees; has the capacity to house six mice and offers software programming to administer automated increases and changes within

acceleration, percent grade and time; and offers a shocking mechanism that encourages animals to continue running on the treadmill belt, however for this study the shocker was disabled. All the features offered by the Columbus Instruments Exer-3/6 animal model treadmill (Columbus Instruments, Columbus, OH) was suitable to perform both performance protocols within this study. According to Columbus Instruments Inc. the Exer-3/6 has been cited and used in over 480 experimental studies (Columbus Instruments, 2022).

Assessments/Data Collection

All groups underwent the same baseline and follow up experimental assessments throughout the eight-week study. The assessments included: blood collection, body weight measurements, magnetic reasoning imaging (MRI) testing for body composition, food consumption, and behavior analysis. Body weight measurements were monitored daily. Weight measurements were taken in the morning at the same time each day (8:00 am) using a standard digital scale to measure weight in grams (g). MRIs were done at baseline (pre-0 weeks), mid-point (4-weeks), and post (8-weeks) of the experimental protocol. An EchoMRI™-100H (EchoMRI, Houston, TX) was used for collection of body composition that included: lean mass, fat mass, total water, and free water.

Food consumption was monitored weekly for the entirety of the eight-week study and tracked via weight of chow (consumed and leftover) per experimental group at the beginning and end of each experimental week in the mornings (8:00 am). Food consumed was tracked by taking the difference between food at the beginning of the week and at the end of each week.

Blood collection, magnetic reasoning imaging (MRI) and behavior testing were performed at pre (baseline), mid (4-weeks), and post (8-weeks) study for all experimental groups. Blood collections were obtained using the submandibular bleeding procedure using

either a 5mm lancet or 25-gauge needle. Only 10% of circulating blood volume (CBV) or 1% of the animals' body weight was collected per mouse at baseline (0 weeks) and mid-point (4 weeks) by using the submandibular procedure. The sample volume guidelines were followed in order to prevent hypovolemic shock in mice. For the post-blood time point, blood was obtained using cardiac puncture during the endpoint procedure and sacrifice of mice. The endpoint procedure involved mice inhaling 1-2% isoflurane until anesthesia was achieved. After the mice were anesthetized, the blood was collected using a 1 ml syringe and 25-gauge needle via cardiac puncture. Blood was collected in 0.5 ml microcentrifuge tubes for pre and middle collections and a 1 ml microcentrifuge tube for post-blood collection, spun at 100 rpm for 10-minutes to separate the plasma. The plasma was separated and stored in a -80-degree Celsius freezer

Behavior data was obtained using open-field motion capture software to track animal speed (m/min), direction of movement, time spent in inner, mid, and outer zones. Each mouse was tested for 10 minutes in the open-field apparatus at pre-zero weeks, mid-point-four weeks and at post-eight weeks of the experimental protocol. The ANY- maze software and clear open-field ANY-maze container was used for the experimental protocol. At the end of the eight-week study, mice were sacrificed and all tissues were collected and stored in a -80-degree Celsius freezer.

Biochemical Assays

To measure levels of hormones or any biomarkers for the prediction of diseases, one must determine which method of collection and analysis they will perform (Brasil et al., 2010; Liu et al., 2020; Tighe et al., 2015). Perhaps the most common form of analyzing circulating levels of hormones is enzyme-linked immunosorbent assays (ELISA) (Liu et al., 2020; Tighe et al., 2015). Antibodies for specific proteins, enzymes and hormones can be used to measure and

detect levels for the corresponding biomarkers being evaluated. The sensitivity of the ELISA method is very high; however, the drawback is its high cost related to equipment and kits (Liu et al., 2020). For this study the ELISA based method was used to detect hormones found within the plasma.

Two circulating biomarkers (Corticosterone and IGF-1) were analyzed for this study. Plasma obtained after centrifugation of each mouse blood samples collected at pre, middle, and post study time points were used as samples for the 96 well plate ELISAs. Two ELISA kits were used, one for measuring mouse corticosterone levels and the other for determining mouse IGF-1 levels. The mouse corticosterone ELISA kit was obtained from Chrystal Chem, Inc., (Chrystal Chem, Elk Grove Village, IL). The mouse IGF-1 ELISA kit was obtained from LifeSpan BioSciences, Inc. (LifeSpan Biosciences, Seattle, WA). Each kit utilized a 96 well plate format which was sufficient for detecting all mice samples at all time points within the study.

Exercise Training Procedures

Acclimation Protocol

After pre-assessment variables were obtained (plasma, body composition, body weight, open-field edge and center time) the overtrained group and moderately trained group began a 3-day acclimation to the Exer-3/6 animal treadmill. The acclimation protocol consisted of: day one by sitting inside the treadmill apparatus for ~10 minutes; On day two the mice ran at low speeds of 3m/min for no longer than 10 minutes and on day three they ran at 3.0-5.0 m/min for 15-20 minutes. Testing procedures were done at the same time each day to maintain consistency within the study.

Incremental Load Test and Exhaustion Velocity Assessment Protocol.

The moderate-intensity training protocol followed the same procedures as previously published by Pereira et al. (2012). As explained by Pereira et al. (2012) the moderate-intensity exercise group initially underwent an incremental load test (ILT) to determine the exhaustion velocity (EV) range to conduct the moderate-intensity test. To determine each mouse's EV, they ran at 6 m/min with 0% grade on the Exer-3/6 animal treadmill. Every three minutes the speed was increased by 3 m/min until mice stopped at the end plate five consecutive times in one minute. The velocity reached during exhaustion was recorded for each mouse as the EV and served as the base velocity on which percent exercise intensities were prescribed in the moderate-intensity testing protocol.

The overtrained exercise group followed the same ILT and EV procedure as the moderate-intensity exercise group. The overtraining protocol suggested by Pereira et al., (2012) began with mice running on the Exer-3/6 animal model treadmill for one session every day for five days and 24 hours of rest between sessions. However, the overtrained group conducted two training sessions per day during week eight. The findings from Pereira et al. (2012), found their downhill running protocol was sufficient enough to induce an overtrained or non-functional overreaching (NFOR) state in 100% of the mice they tested. The results and findings from their protocol give strong reliability in the use of their model for inducing overtraining syndrome in mice. For this study, the shock feature provided by the Exer-3/6 animal model treadmill was disabled and not utilized. Manual prodding with the use of dull forceps was utilized to encourage the mice to run. Consistency in the order and placement of mice in designated lanes of the treadmill was done for the entirety of the study to create a consistent testing environment.

Moderate-Intensity Exercise Training Protocol

The moderate-intensity exercise trained group began the study protocol at the age of four weeks. The protocol began with mice running at 60% of their exhaustion velocity for one session five days a week for eight weeks at 0% grade with 24 hours of rest between each session. The first week of the protocol mice ran at the assigned intensity for a duration of 15 minutes, followed by week two at 30 minutes, week three at 45 minutes and weeks four - eight at 60 minutes. For this study, the shock feature provided by the Exer-3/6 animal model treadmill was disabled and not utilized. Manual prodding with the use of dull forceps was utilized to encourage mice to run. Consistency in the order and placement of mice in designated lanes of the treadmill was done for the entirety of the study to create a consistent testing environment.

Overtrained Exercise Training Protocol

The overtraining group began the overtraining protocol at the age of (4-5) weeks. The overtrained group ran at 60% of their EV for weeks one - five, 75% EV for week six, and 90% EV for weeks seven - eight. The duration for each session began at 15 minutes for week one, 30 minutes for week two, 45 minutes for week three, 60 minutes for weeks four - five, 75 minutes for week six and 90 minutes for weeks seven - eight.

Sedentary Group (Control)

The sedentary mice did not engage in any physical activity outside of normal behavior within the housing cage. However, to keep consistency across all groups the sedentary mice were brought to the lab that housed the Exer-3/6 animal model treadmill and was present inside their cages at the same time and duration during the moderate and overtrained exercise groups exercise sessions.

Data Analysis

Statistical analysis of this study's data was done using JMP version 15.1.0. A multivariate analysis of variance (MANOVA) with repeated measures was used as the primary statistical test in this study. The MANOVA was used to determine if interactions occurred within and between groups. The following interactions were assessed for all data in this study: time, group by time, and group. If an interaction occurred in the group by time, then a contrast was done to identify which groups were statistically different. If no interaction occurred in a group by time, then group and time were looked at independently. A One-way Analysis of variance (ANOVA) was used to assess interactions in group means for each assessment variable at baseline (pre-0-weeks). If baseline group means were statistically significant then a post hoc Tukey's method was used to assess which groups were significantly different. This was done to assess and ensure true randomization of all group variables at baseline. The significance level for all tests were set at p=0.05.

During the middle of the study, one subject within the moderate-intensity trained group became prematurely deceased. All missing data for this subject post-death were replaced via group mean computation. Seven subjects among the moderate-trained and sedentary groups displayed corticosterone concentrations below the lowest detectible range for the corticosterone-specific ELISA. Therefore, these data were excluded from the data analysis.

Two subjects' plasma samples from the overtrained group were lost and unable to be used for corticosterone and IGF-1 assays. Group mean computation was used to replace the missing data from these two subjects' samples. Additionally baseline food consumption (week-0) was unable to be determined due to misinformation between the investigator and animal facility staff in regards to the administration of food. Initial food weight at the start of week 0 was recorded but the difference in food weight (food consumed) was unable to be determined due to

food being added to each group's housing cage before the final food weight of that week could

be determined.

CHAPTER 4

RESULTS

The purpose of this study was to investigate the effects of physical over-stress/overtraining on levels of hormone biochemical markers, anthropometric measurements, and behavioral traits in adolescent male C57BL/6 mice. This study included three groups of adolescent C57BL/6 mice (4-5 weeks old at start) over an 8-week intervention. Each group was comprised of 8 subjects (n=8), with 24 total subjects in the study (N=28). Subjects (C57BL/6 mice) were randomly assigned to the respective experimental groups (moderate intensity exercise training, overtrained/high intensity exercise-trained and sedentary control). The moderately trained and overtrained groups underwent a pre-study incremental load test (ILT) to determine individual subjects' relative training intensities that were prescribed during each group's respective training protocol. As seen in Table 1, the ILT for both overtrained and moderately trained mice showed to have the same exhaustion velocity (EV) for all subjects. The sedentary group did not take part in the ILT as they did not engage in any training protocol during the study.

The mean ± Standard Deviation (SD) was used for comparing the dependent variables among each group. The mean and SD of each group for each dependent variable assessed can be further seen in Table 2 for: fat mass, lean mass, %fat mass, % lean mass, edge time, center time, corticosterone, and IGF-1. Mean and SD can be seen for body weight across eight weeks in Table 3. Results of the multiple ran One-Way Analysis of Variance (ANOVAs) for pre-study comparison of group means assessment variables are as follows: There was no significant difference in baseline (0-weeks) corticosterone concentrations (ng/ml) among groups (p=0.398567); there was no significant difference in baseline (0-weeks) body weight (g) among

groups (p=0.226062); there was no significant difference in baseline (0-weeks) lean mass (g)

among groups (p=0.360781); there was a significant in baseline (0-weeks) % lean mass among

groups (p=0.034059), and the Tukey method showed that the difference was between the

overtrained exercise group and sedentary control group; there was no significant difference in

baseline (0-weeks) fat mass (g) among groups (p=0.152295); there was no significant difference

in baseline (0-weeks) % fat mass among groups (p=0.07274); there was no significant difference

in baseline (0-weeks) edge time (sec) among groups (p=0.597201); there was no significant

difference in baseline center time (sec) among groups (p=0.056152); there was no significant

difference in baseline (0-weeks) IGF-1 concentrations among groups (p=0.61592). The results of

the pre assessment One-Way ANOVAs are further illustrated in Table 4.

Subject ID	Group	Stage 1 completed	Stage 2 completed	Stage 3 completed	Stage where exhaustion occurred	Exhaustion Velocity (m/min)
MT1	Moderate Intensity Trained	6 m/min, 0% grade	9m/min, 0% grade	12 m/min, 0% grade	Stage 3	12 m/min
MT2						12 m/min
MT3						12 m/min
MT4						12 m/min
MT5						12 m/min
MT6						12 m/min
MT7						12 m/min
MT8						12 m/min
OT1	Overtrained	6 m/min, 0% grade	9m/min, 0% grade	12 m/min, 0% grade	Stage 3	12 m/min
OT2						12 m/min
OT3						12 m/min
OT4						12 m/min
OT5						12 m/min
OT6						12 m/min
OT7						12 m/min
OT8						12 m/min

Table 1. Incremental Load Test (ILT) Determination for Moderate Intensity Trained Group and Overtrained Group

Note. All mice experienced exhaustion at stage 3. Exhaustion is defined as: when mice reach the end plate of the treadmill 5 consecutive times in 1 minute. ILT was only done for the moderate trained intensity and overtrained intensity groups (Table 1).

		Fat mass (g)		Lean mass (g)		% Fat mass		% Lean mass		Edge Time (sec)		Center Time (sec)		Corticosterone (ng/ml)		IGF-1 (ng/ml)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Sedentary	Pre (0-weeks)	0.85	0.14	14.24	1.05	5.6	1.02	92.74	1.49	480.98	34.87	67.64	23.93	130.17	45.58	0.57	0.27
	Mid (4-weeks)	0.78	0.26	20.44	1.26	3.55	1.3	91.8	1.17	357.81	96.05	49.43	26.02	144.53	71.88	0.62	0.2
	Post (8-weeks)	0.79	0.35	23.1	2.21	3.21	1.48	92.62	3.56	476.38	148.25	37.56	56.38	58.7	97.45	0.4	0.14
Moderate-trained	Pre (0-weeks)	0.72	0.09	14.8	1.11	4.58	0.45	94.43	0.96	474.45	48.27	46.11	24.05	155.47	86.86	0.68	0.35
	Mid (4-weeks)	0.73	0.3	19.49	1.17	3.41	1.52	89.83	1.45	469.89	57.98	20.41	16.43	100.48	38.79	0.66	0.33
	Post (8-weeks)	0.67	0.2	21.88	2.68	2.76	0.91	88.99	10.46	485.93	64.09	12.94	9.64	20.99	12.97	0.43	0.08
Overtrained	Pre (0-weeks)	0.76	0.14	14.94	0.62	4.82	0.86	94.5	1.44	453.63	30.18	39.19	15.61	157.1	35.99	0.57	0.19
	Mid (4-weeks)	0.7	0.19	19	1.72	3.36	1.05	89.31	1.01	435.61	79.48	44.4	22.1	53.03	24.61	0.66	0.37
	Post (8-weeks)	0.69	0.3	22.62	1.72	2.92	1.55	94	2.33	425.3	108	27.81	34.18	153.09	99.92	0.44	0.1

Table 2. Mean and Standard Deviations (SD) of Dependent variables

Note. List of mean and standard deviations of dependent variables for Sedentary group, Moderate-trained group, and Overtrained group (Table 2).

| | Body weight (g) | | | | | | | | | | | | | | | |
| | Week 1 | | Week 2 | | Week 3 | | Week 4 | | Week 5 | | Week 6 | | Week 7 | | Week 8 | |
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Sedentary	16.29	0.83	17.98	0.49	19.63	0.77	21.26	0.89	22.81	1.22	23.29	1.21	24.59	1.75	25.1	1.74
Moderate Trained	17.01	0.83	18.45	0.84	19.51	1.13	21.08	1.18	22.26	1.2	22.65	1.24	24.18	0.86	24.75	0.79
Overtrained	16.79	0.64	17.72	1.03	18.78	1.37	20.59	1.58	21.28	1.68	22.03	1.55	23.76	1.65	24.21	1.59

Table 3. Mean and Standard Deviations (SD): Body Weight (g)

Note. List of mean and standard deviations of Body weight for Sedentary group, Moderate-trained Group, and Overtrained group (Table 3).

Baseline (0-weeks) Assessment variable	F-value	P-value	Significance Not significant= (NS) Significant = *
Corticosterone (ng/ml)	0.96138	0.398567	NS
IGF-1 (ng/ml)	0.49600	0.61592	NS
Body weight (g)	1.59738	0.226062	NS
Lean Mass (g)	1.07062	0.360781	NS
Fat Mass (g)	2.06111	0.152298	NS
Percent Lean Mass (%)	3.98695	0.034059	*
Percent Fat Mass (%)	0.297696	0.07274	NS
Edge Time (sec)	0.52837	0.597201	NS
Center Time (Sec)	3.31329	0.056152	NS

Table 4. One-way ANOVA Results for Comparison of Group Baseline (0-weeks) Assessment Variables

Note. Significant difference was only observed in % Lean mass, Post Hoc Tukey's Method results found the difference to be significant between Overtrained and Sedentary groups (Table 4).

The null hypothesis being tested for the first hypothesis (H1) states that there will be no difference in levels of blood biomarkers (corticosterone and IGF-1) among groups (sedentary, overtrained, and moderately trained).

H1 results are displayed in Figure 1. Corticosterone levels among the three experimental groups show that there are significant differences in relation to group by time (p=0.0050) (figure 1). A contrast was done to identify which groups were significantly different. The contrast showed that corticosterone levels were significantly different between overtrained and sedentary groups across time (p=0.0022) but not significantly different between moderately trained and sedentary groups across time (p=0.3670). Lastly, the contrast showed a significant difference in corticosterone levels between overtrained and moderately trained groups across time (p=0.0098). As shown in Figure 1, the moderately trained groups' corticosterone levels dropped linearly across time (pre, middle, post). The overtrained group's corticosterone levels drastically dropped from pre (0-weeks) to mid (4-weeks) (157.1-53.03 ng/ml) but increased during post (8-weeks) (53.03-153.09 ng/ml). The sedentary groups' corticosterone levels increased from pre (0-weeks) to mid (4-weeks) (130.17-144.53 ng/ml) but decreased during post (8-weeks) (144.53-58.7 ng/ml).

Figure 2 demonstrates the results of IGF-1 levels among the three groups. There was no significant difference among the three groups (p=0.7354); however, there was an interaction in time but not significant (p=0.9754). Therefore, time and group were looked at independently. The results show that group differences were not found to be significant (p=0.7354). There was, however, a significance seen in time (p=0.0168). The significance in time, and lack of significance in the group indicates that despite each group changing across time there was no significant difference among or between groups at any time point. As shown in Figure 2 there

was no significant difference between groups at any of the three-time points or across time. The moderate-trained group decreased IGF-1 levels from the beginning to the end of the study (0.68-0.43 ng/ml). The overtrained and sedentary groups IGF-1 levels increased slightly from pre (0-weeks) to mid (4-weeks) (0.57-0.66 ng/ml) but decreased at post (8-weeks) (0.66-0.44 ng/ml). The significance in time shows that all three groups IGF-1 levels decreased from pre (0-weeks) to post (8-weeks). Despite minor differences between groups across time, there was no significant difference among them.

These results indicate there was a significant difference among groups in corticosterone levels but not in IGF-1 levels. Because significant differences were found among groups the null hypothesis for the first hypothesis (H1) is rejected.
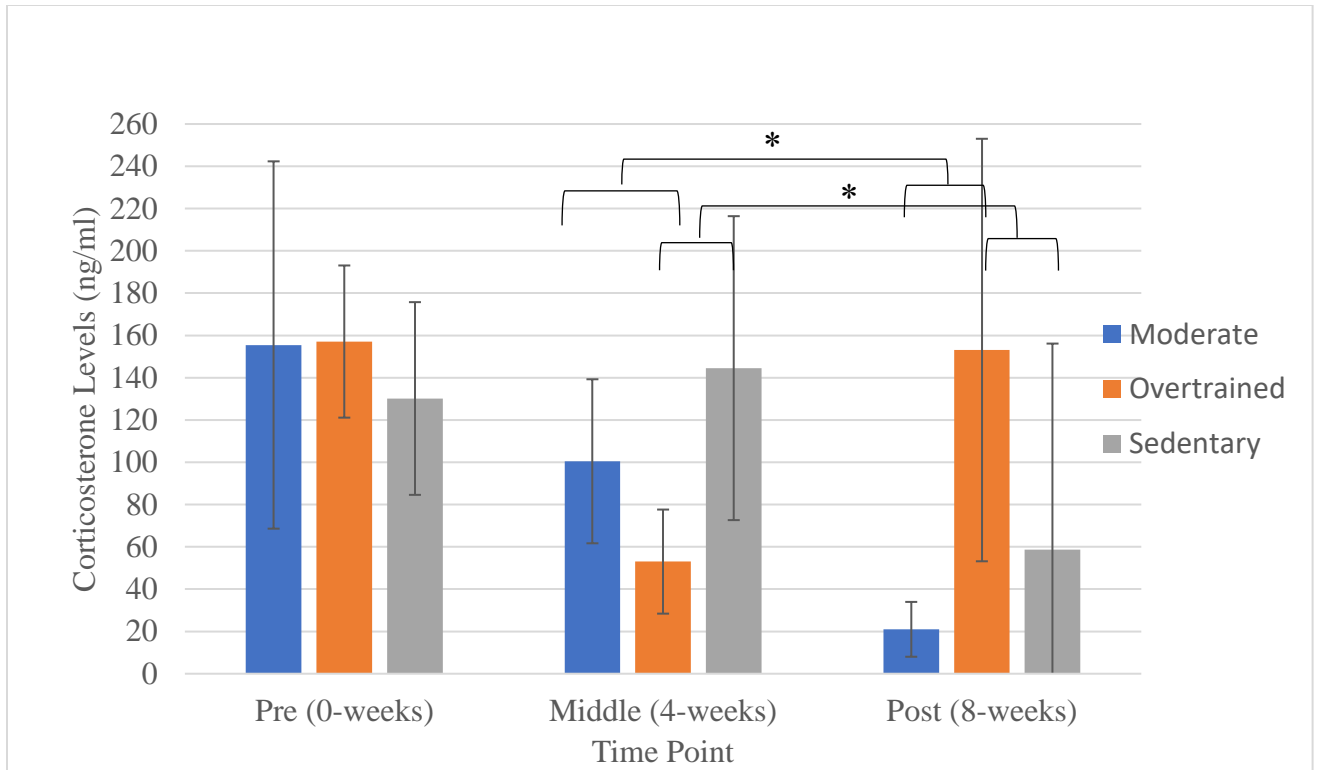
Figure 1. Corticosterone Levels Among Groups

Note. Figure 1 data includes mean ± S D. Interaction between Group and time (p=0.0050).

Overtrained vs sedentary significance (p=0.0022). Moderate vs sedentary No significance

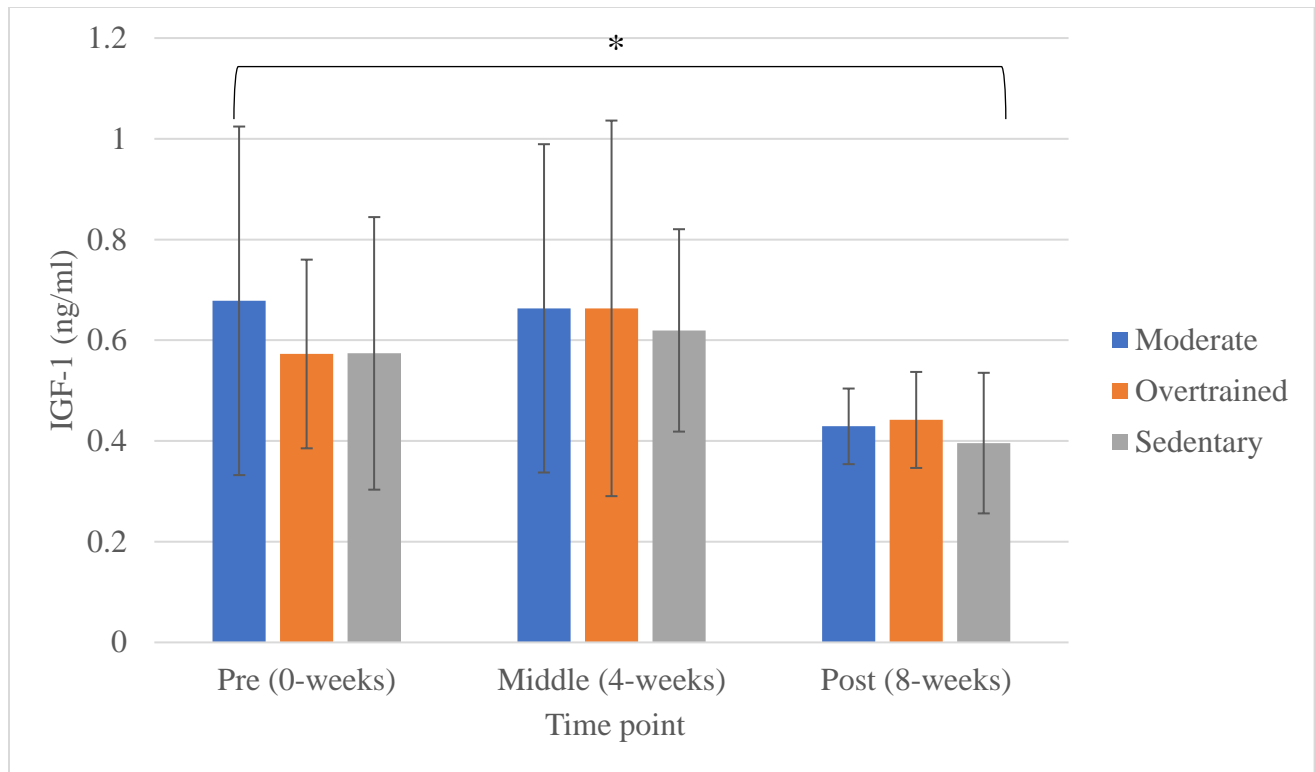(p=0.3670). Overtrained vs moderate trained significance (p=0.0098).

Figure 2. IGF-1 Levels Among Groups

Note. Figure 2 data includes mean ± SD. No interaction in group by time (p=0.9754). No significant difference among groups (p=0.7354). Time was significant (p=0.0168), and IGF-1 dropped for all groups across time.

The Null Hypothesis for the second hypothesis (H2) in this study states there will be no difference in anthropometric measurements (body weight, lean mass, fat mass, percent fat, and percent lean) among the groups (sedentary, overtrained, and moderate trained).

Results of body weight showed no significant difference among groups throughout the intervention (Figure 3). There was no interaction in group by time for body weight (p=0.1541); therefore, group and time were observed independently. The results show that group differences were not significant (p=0.389). There was, however, significance in time (p<0.0001). All three

groups increased in body weight linearly across time from weeks one to eight. The overtrained group, on average, maintained the lowest body weight but was not significantly different from the other two groups.

Results for lean mass showed no significant difference in the group by time comparisons (p=0.1326). There was no significant difference in the group (p=0.6573). However, there was an interaction in time. All groups increased lean mass from baseline to follow-up (p<0.001). Results for lean mass can be seen in Figure 4.

Results for fat mass showed no significant difference in group by time interactions (p=0.9806). There was no significant difference in group (p=0.4938). Lastly, there was no significant difference in time (p=0.5075). Results for fat mass can be seen in Figure 5.

Results for percent lean mass show there was a significant difference in the group by time (p=0.0005). Therefore, the results of the contrast show where the significance lies. There was a significant difference across time between overtrained and sedentary (p=0.0003). There was a significant difference across time between moderate and sedentary (p=0.0028). There was no significant difference across time between overtrained and moderate (p=0.1895). Results for percent lean mass can be seen in Figure 6.

Results show no significant difference in the group by time for percent fat mass (p=0.7369). There was no significant difference in the group (p= 0.5121). There was, however, a significant difference in time; all groups decreased percent fat mass over time (p<0.0001). Results for percent fat mass can be seen in Figure 7.

These results show there was a significant difference among groups across time for percent lean mass. However, there was no significant difference in the group by time for lean

mass, fat mass, or percent fat mass among groups. Based on these results, the null hypothesis
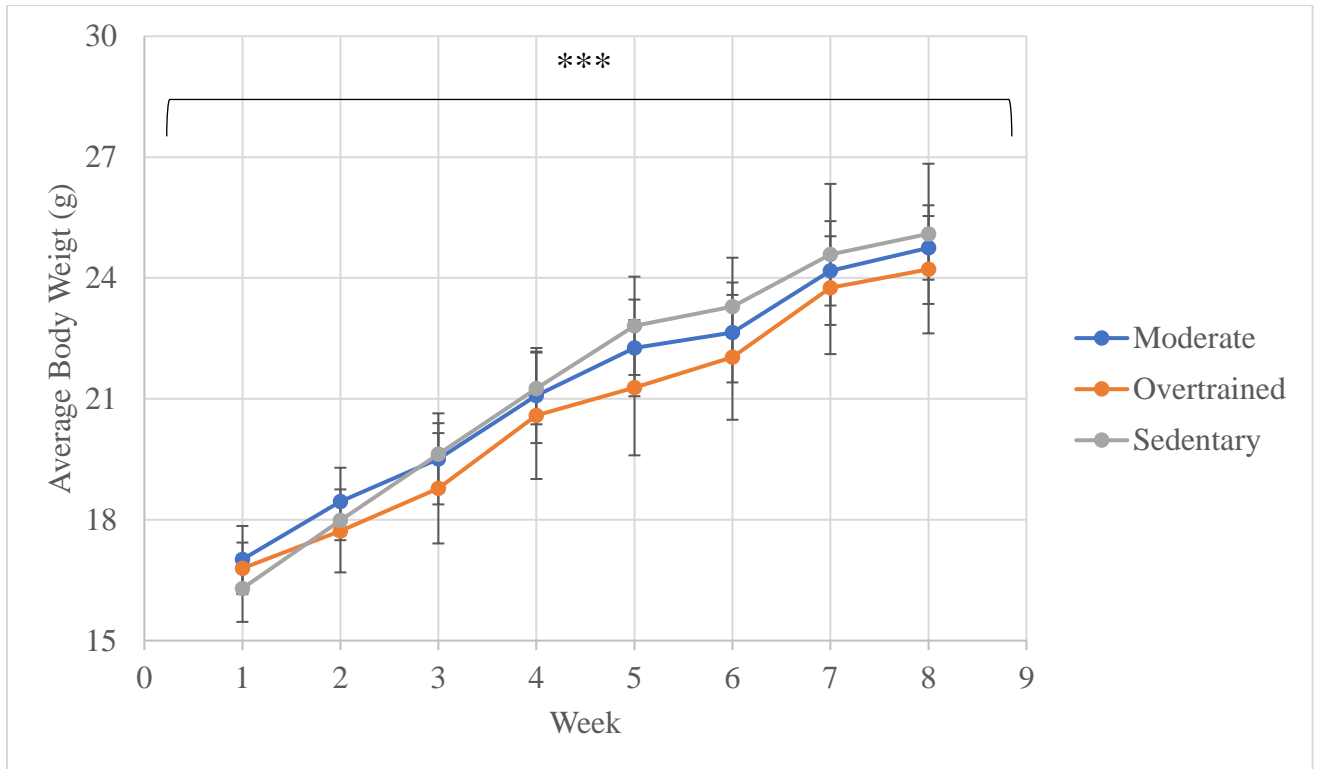
was rejected for the second hypothesis (H2).

Figure 3. Body Weight Among Groups

Note. Figure 3 data includes mean ± SD. There was no interaction in group by time (p=0.1541).

There was an interaction in time (weeks), (p<0.0001), and all groups increased body weight (g)

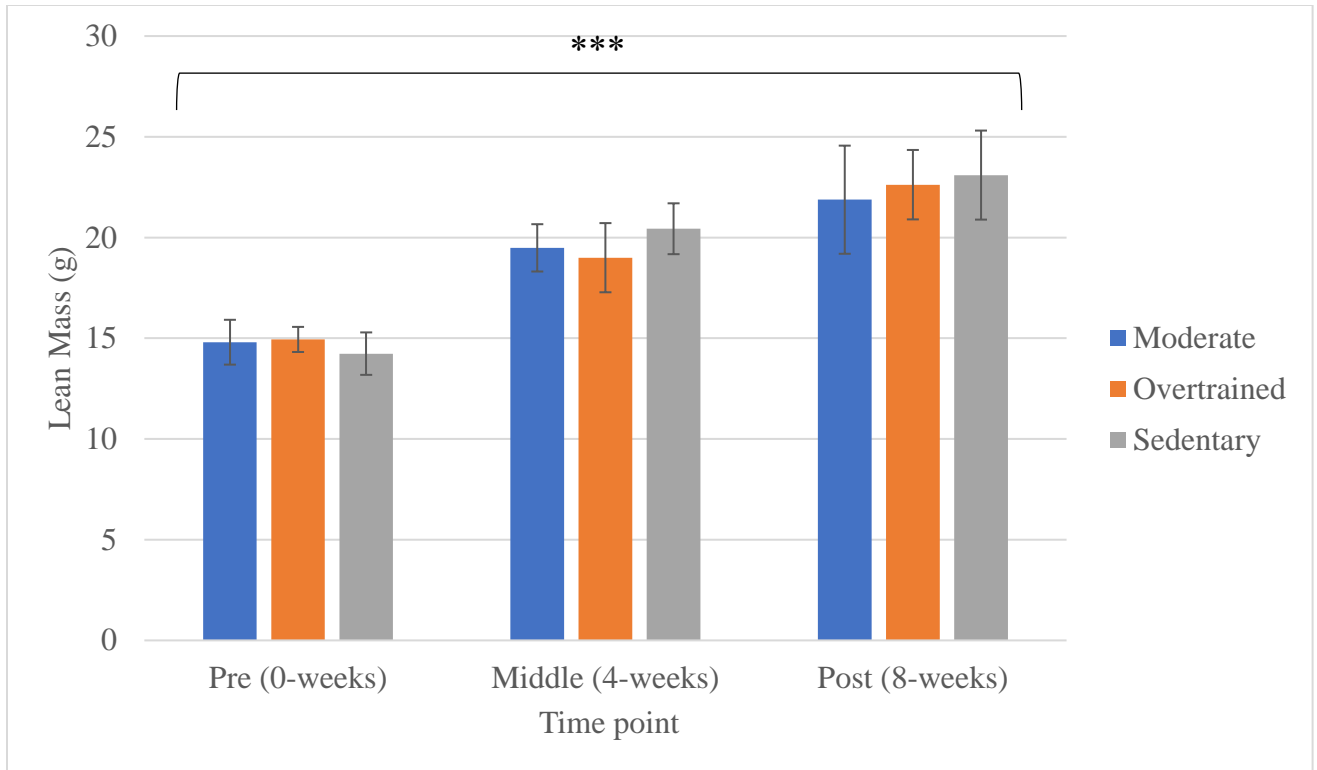over time. There was no significant difference among groups (p=0.389)

Figure 4. Lean Mass Among groups

Note. Figure 4 data includes mean ± SD. There was no interaction in group by time (p=0.1326).

There was no significant difference in groups (p=0.6573). Time was significant (p<0.0001), and

all groups increased lean mass.
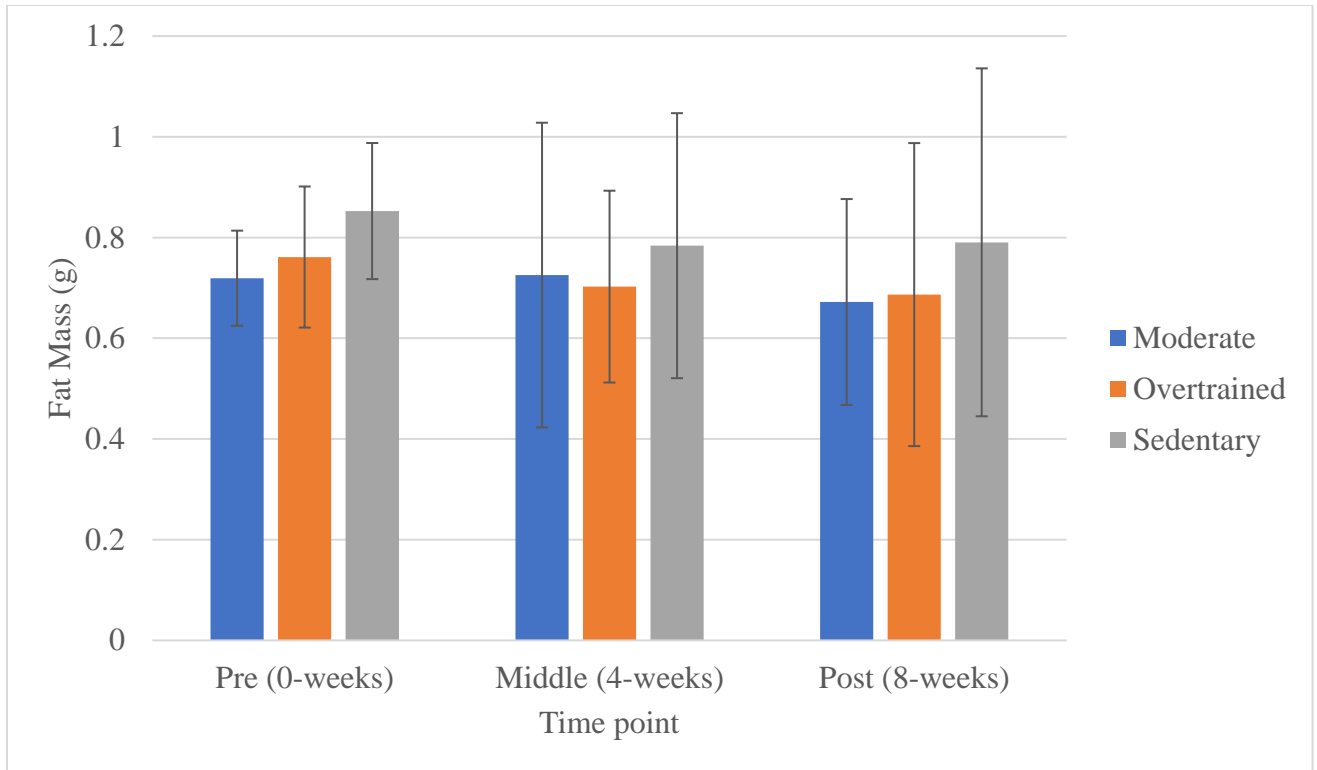
Figure 5. Fat Mass Among Groups

Note. Figure 5 data includes mean ± SD. There was no interaction in group by time (p=0.9806).

There was no significant difference in group (p=0.4938). There was no significant difference in

time (p=0.5075).
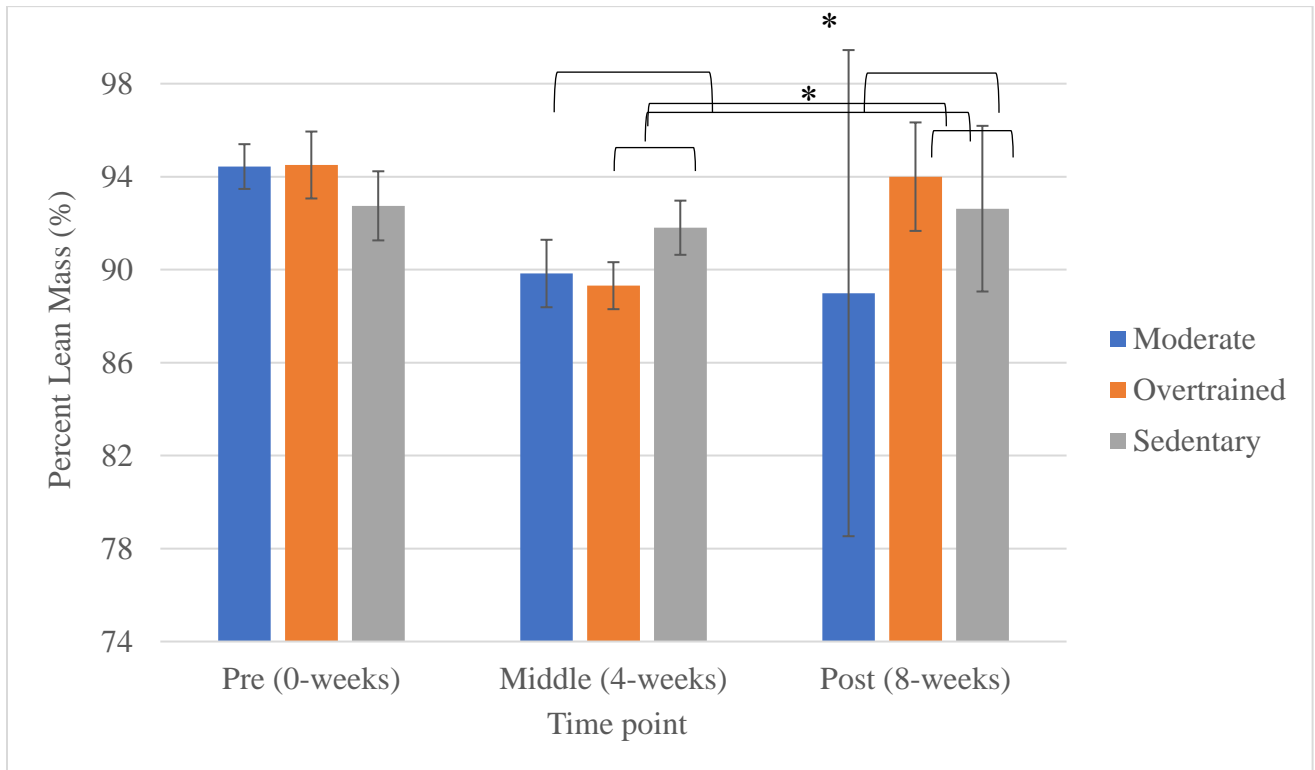
Figure 6. Precent Lean Among Groups

Note. Figure 6 data includes mean ± SD. There was a group-by-time interaction (p=0.0005).

Overtrained vs sedentary significance (p=0.0003). Moderate vs sedentary significance

(p=0.0028). Overtrained vs moderate trained no significance (p=0.1895).
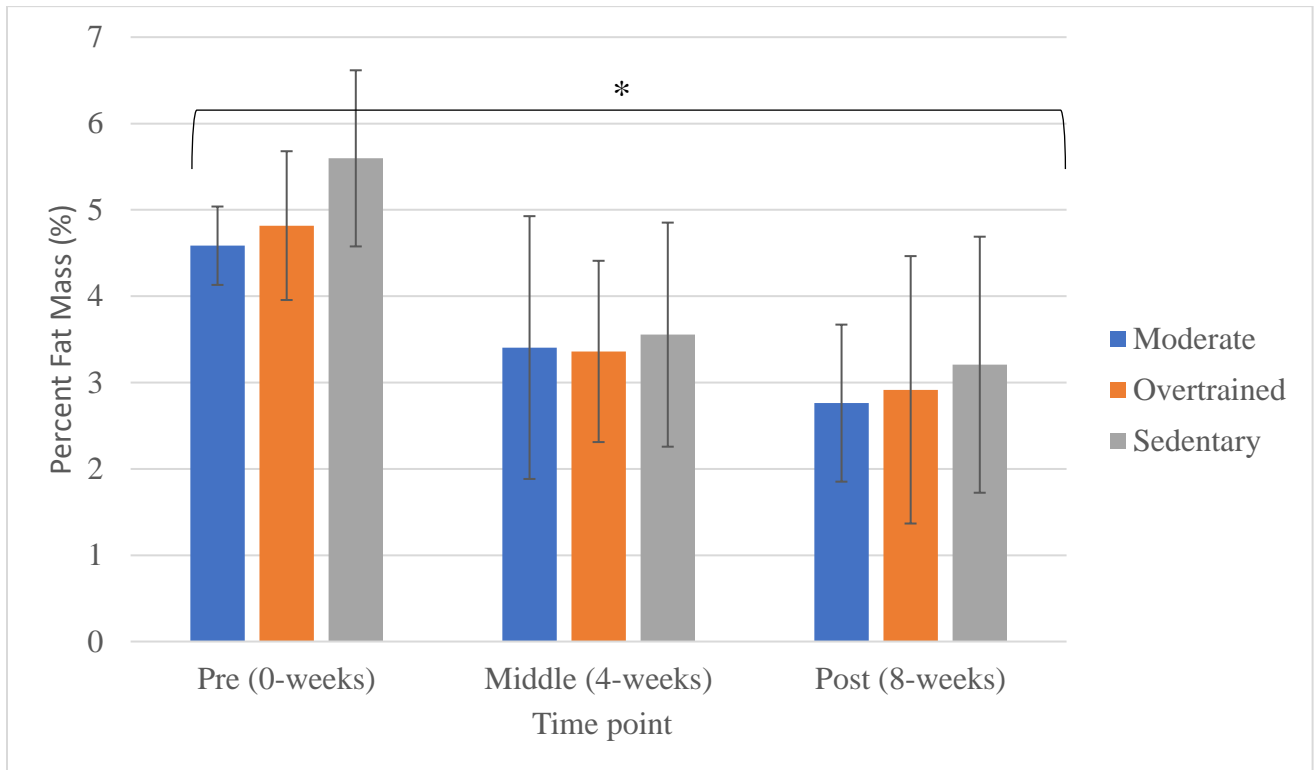
Figure 7. Percent Fat Among Groups

Note. Figure 7 data includes mean ± SD. There was no group-by-time interaction (p=0.7369). There was no significant difference in the group (p=0.5121). Time was significant (p<0.0001), and all groups decreased in % fat mass.

The Null hypothesis for the last hypothesis (H3) in this study states there will be no difference in behavioral traits (food consumption, open field edge time, and open field center time) among the three groups (sedentary, overtrained, and moderately trained). Results can be found in Figures 8-10.

Results shown in Figure 8 display results for food consumption and there was an interaction in the group by time (p<0.0001). There was a significant difference in both time and group (p<0.0001). Because there was a significant difference in the group by time the contrast

shows where the differences are indicated. There was a significant difference in food consumption between overtrained and sedentary groups across time (p<0.0001). There was a significant difference in food consumption between overtrained and moderately trained groups over time (p<0.0001). Lastly, there was also a significant difference in food consumption between moderately trained and sedentary groups across time (p<0.0001).

All three groups increased food consumption in the first three weeks. During week-four all three groups dropped in food consumption, with the sedentary group having the most drastic drop and the overtrained group having the least significant drop. Following week 4 all groups showed an increase in food consumption during week five. However, the main difference in food consumption between groups is seen from weeks five-eight. The overtrained group increased food consumption from weeks five-eight; however, there was little increase in food consumption from the sedentary group from weeks five-eight. Lastly, the moderate-trained group had the most significant drop in food consumption from weeks five-eight. Results for food consumption can be further seen in Figure 8.

Results for edge time show that there was an interaction in the group by time (p=0.0280). The contrast shows which groups had significantly different edge times across time. There was a significant difference in edge time between overtrained and sedentary groups across time (p= 0.0223). There was no significant difference in edge time between overtrained and moderately trained groups across time (p=0.7607). Lastly, there was a significant difference in edge time between moderately trained and sedentary groups across time. Results for edge time can be seen in Figure 9.

Results for center time (figure 10) show that there was no interaction in the group by time. Therefore, the main effects of group and time were looked at independently. There was no

significant difference by the group for center time (p=0.0651). There was, however, a significant difference in time for all groups (p=0.0383). All three groups had a decrease in center time across time. Results for center time can further be seen in Figure 10.

These behavioral results show there was a significant difference in food consumption among the groups across time. There was a significant difference in edge time across time between overtrained and sedentary, and moderate trained and sedentary. However, there was no significant difference in edge time between overtrained and moderate-trained groups across time. There was no significant difference among groups for center time across time. There was also no difference among groups for edge time. There was, however, an interaction in time for center time, where all groups decreased in center time across the length of the study. Based on these results null hypothesis was rejected for the third hypothesis statement (H3).
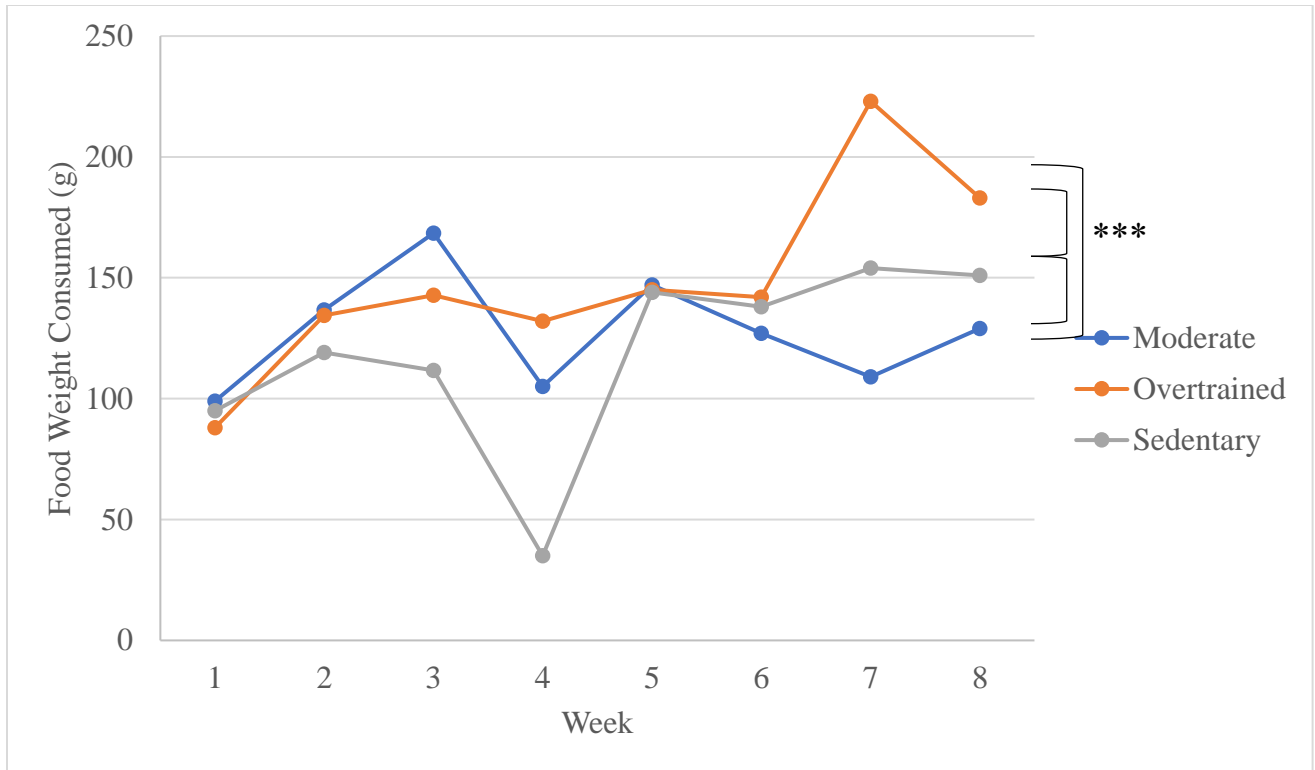
Figure 8. Food Consumption Among Groups

Note. Figure 8 data includes a single measurement of food weight per group and does not include any mean or Standard Deviations. There was an interaction in group by time (p<0.0001). Overtrained vs sedentary was significantly different across time (p<0.0001). Moderate trained vs sedentary was significantly different across time (p<0.0001). Overtrained vs moderate trained was significantly different across time (p<0.0001).
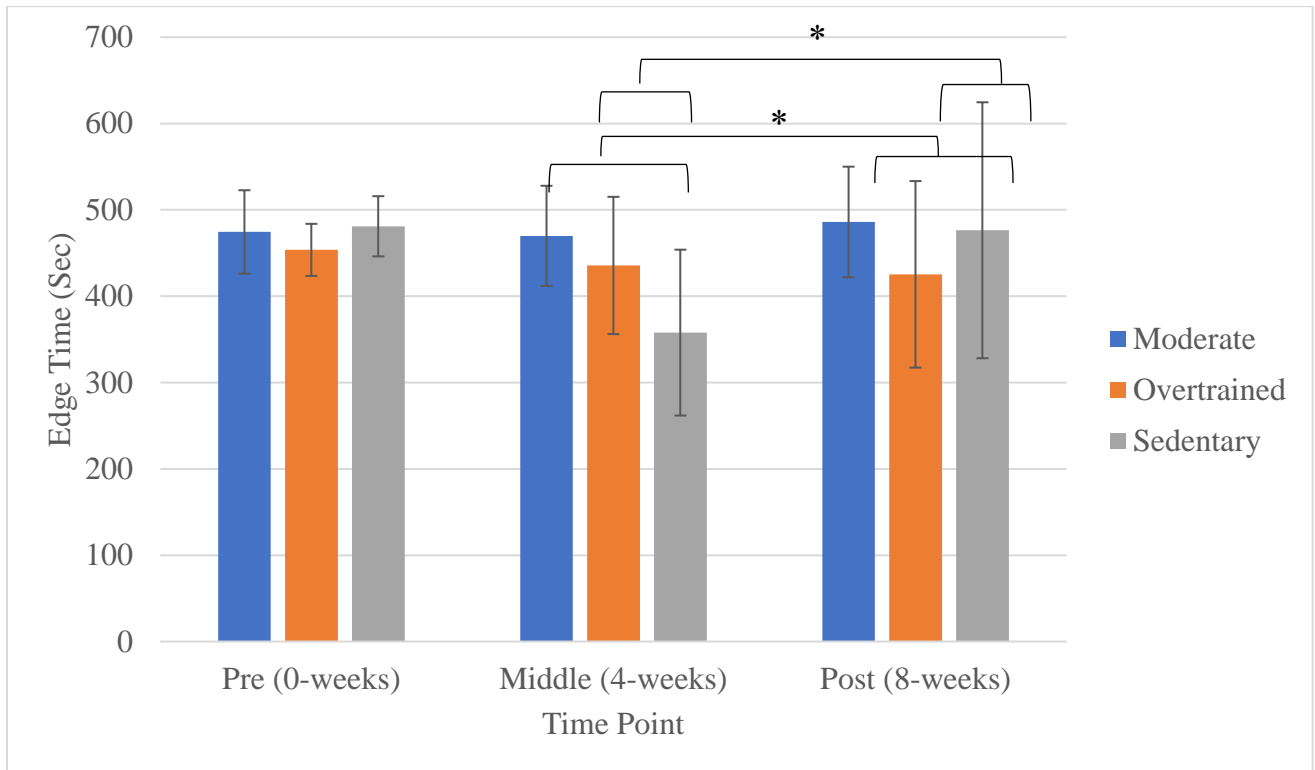
Figure 9. Edge Time Among Groups

Note. Figure 9 data includes mean ± SD. There was a group-by-time interaction (p=0.0280). Overtrained vs sedentary was significantly different (p=0.0223). Overtrained vs moderate trained was not significantly different (p=0.7607). Moderate trained vs sedentary was significantly different (p=0.0137).

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Figure 10. **Center Time Among Group**

Note. Figure 10 data includes mean ± SD. There was no interaction in group by time (p=0.2610).
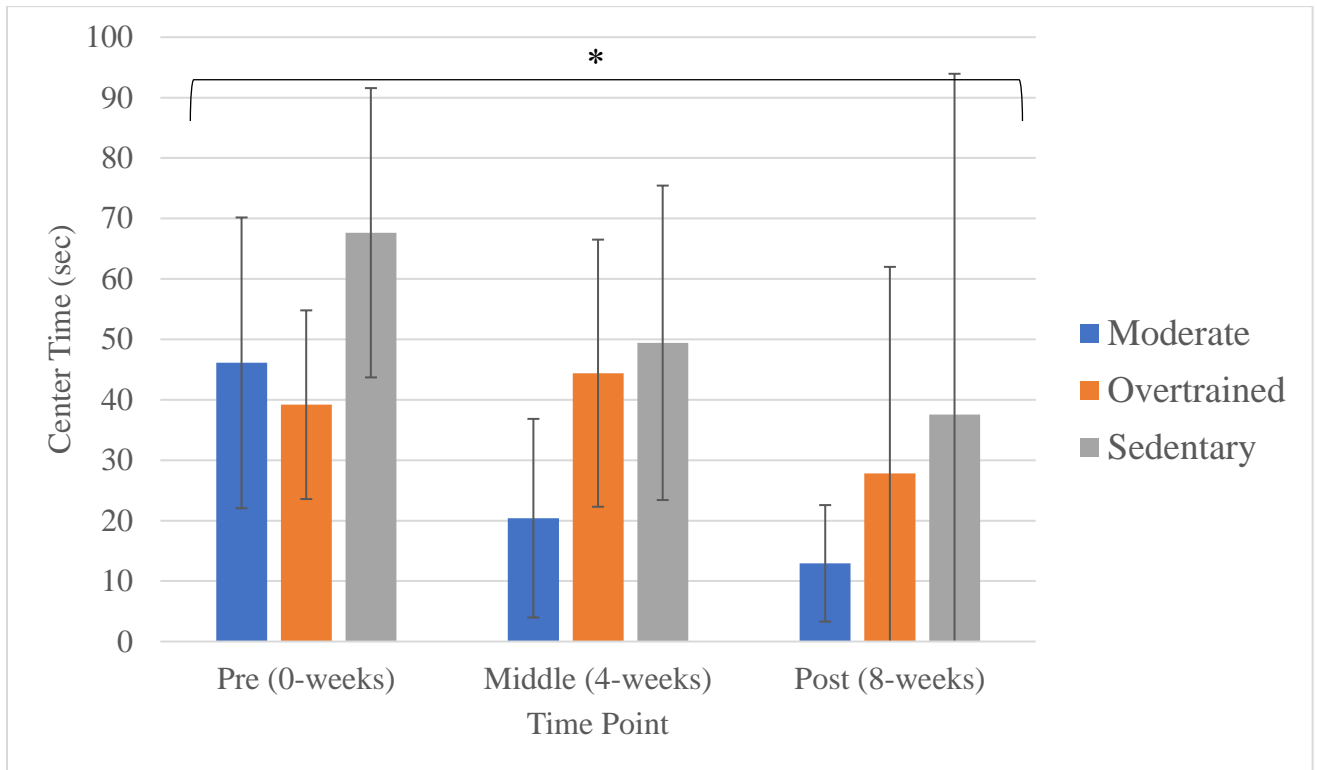
There was no difference by group (p=0.0651). There was an interaction by time (p=0.0383).

Center time dropped for all groups.

CHAPTER 5

DISCUSSION

Currently, there is little research investigating the use of biomarkers and behavioral symptoms in assessing overtraining syndrome in adolescents. Therefore, the purpose of this study was to investigate the effects of physical over-stress/overtraining on levels of hormone biochemical markers, anthropometric measurements, and behavioral traits in adolescent age C57BL/6 mice.

The primary research question of this study was: Will overtrained adolescent mice have different levels of biochemical markers, anthropometrics, and behavioral traits compared to moderately trained and/or sedentary adolescent mice?

Subjects in this study included adolescent male C57Bl/6 mice. The subjects were four-weeks old at the start of the experimental protocol and included a total of 24 subjects. The subjects were divided into three groups (sedentary, moderately-trained, and overtrained), with 8 subjects per group. Each group underwent a separate unique protocol for the length of the 8-week study. Similar assessment methods were used to determine changes in hormone biochemical markers, anthropometric measurements, and behavioral assessments due to the specific interventions.

Results of the hormone biochemical markers indicated that IGF-1 concentrations (Figure 2) among the three experimental groups were not significantly different across time or significantly different among groups without the factor of time (Figure 2). However, there was an interaction in time for IGF-1 concentration for all groups, showing that all three experimental groups decreased in IGF-1 concentrations from pre-time point (0-weeks) to post-time point (8 weeks) (Figure 2). These findings for IGF-1 concentrations within this study indicate that there

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was no significantly different impact on IGF-1 levels among sedentary, moderately trained and overtrained adolescent mice. Previous research in human adult subjects displayed similar findings.

According to Cadegiani & Kater (2019), there appears to be no significant difference in IGF-1 levels among adult male athletes who either were overtrained, trained healthy, or sedentary. This would support the argument that overtraining may have similar effects on IGF-1 in the adolescent population as it does in adults, being that it had little to no influence. IGF-1 levels decreasing for all experimental groups may be a result of normal age-related effects. It is known that IGF-1 levels gradually decline throughout the aging process in most mammals (Junnila et al., 2013). These findings suggest that IGF-1 may not be a significant marker when determining overtraining syndrome in youth. However, it is important to note that specific training interventions may or may not elicit different findings in IGF-1 from that seen in this study. The complexity of using IGF-1 as a marker of overtraining may need to be further explored in follow-up studies.

Results for corticosterone concentrations (Figure 1) showed that there was an interaction in group by time (Figure 1). There was a significant difference in corticosterone concentrations across time between: overtrained vs sedentary and overtrained vs moderate trained groups. There was no significant difference in concentrations between moderate trained and sedentary groups across time (Figure 1). All three groups had similar concentrations at the pre-time point of the study (0-weeks). The moderate trained group had a continual decline in corticosterone concentrations from pre- time point (0-weeks) to post time point (8-weeks). Interestingly non-linear changes were seen in both the overtrained and sedentary groups. The sedentary group displayed an increase in corticosterone concentrations from pre time point (0-weeks) to mid time

point (4-weeks) with a decrease in concentrations at post time point (8-weeks) lower than the pre time point concentrations. The opposite was seen in the overtrained group. The overtrained group displayed a decrease in corticosterone concentrations during the mid-time point (4-weeks) with an increase in concentrations at post-time point (8-weeks) similar to that of their pre-time point concentrations.

These results may indicate that the moderate training group acclimated to the training protocol and had a positive effect on corticosterone levels. The overtrained group also followed this decrease similarly to the moderate trained group at the mid-time point (4-weeks) which would be expected as the two groups training protocols were identical from weeks one - four. Although the overtrained group had an increase in corticosterone concentrations at the post-time point (8-weeks) and was significantly different from the moderately trained and sedentary groups this is not a conclusive indication that the overtrained group was experiencing OTS. Also, the large SD of the overtrained group may indicate that not all mice in that group had such increases in corticosterone, thus limiting further implications that corticosterone was a reliable marker in this study.

These results rather suggest that the overtrained group displayed a greater stress response to the overtraining protocol simply due to the increase in protocol intensity rather than true overtraining. There is no way to conclude based on this study's results that the overtrained group was experiencing OTS. This is due to the lack of a post EV test to determine if performance values decreased. It is also important to note the actual change in the overtrained groups' corticosterone concentrations from the mid-point to the post-point of the study. Average corticosterone levels of the overtrained group were 53.03 ± 24.61 ng/ml at the mid-time point and 153.09 ± 99.92 ng/ml at post-time point. On average, an increase of 100 ng/ml from mid-

time point to post-time point was observed for the overtrained group however the large SD would not suggest congruency among subjects within the overtrained group.

It is important to note the catabolic effects that cortisol or corticosterone have on anabolic hormones like IGF-1. It is crucial to remember that corticosterone is the primary stress hormone expressed in rodents, while cortisol is the primary stress hormone expressed in humans. Cortisol and corticosterone respond similarly under normal conditions within the stress response (Gong et al., 2015). Therefore, observations and implications made using corticosterone can be synonymous with that of cortisol. Cortisol levels are seen to be elevated after exercise bouts, with levels normalizing after sufficient recovery from stress; however, under chronic stress, these levels can remain elevated and or have negative effects on anabolic hormones like IGF-1 (Kraemer et al., 2020). Based on these results, the response to elevated corticosterone levels in the overtrained align with the literature on elevated levels of cortisol or corticosterone as a response to chronic stress, as in this study, the primary stressor was exercise. However, the results of this study cannot discern whether the increase in corticosterone levels of the overtrained group had a negative impact on the overtrained group's IGF-1 levels, as the two control groups (moderate trained and sedentary) also displayed decreases in IGF-1 levels over time. This would suggest that the significant higher levels of corticosterone observed in the overtrained group had no impact on IGF-1 levels, suggesting that the overtrained group did not align with literature stating that increases of corticosterone causes decreases in IGF-1. These results further support that the overtrained group was not experiencing a true overtrained state.

Considering the literature has stated that IGF-1 activity can be inhibited as a result of large increase in cortisol and or corticosterone (Haff & Triplett, 2015). Consideration of

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variability among subjects within groups could shed light on individual responses in hormone concentrations because of training status (overtraining, moderately training, and sedentary).

Results of body anthropometric measurements from this study show that there was no interaction in the group by time for body weight (g) (Figure 3) of the three groups (sedentary, moderately trained, overtrained). However, time was significant, and all groups increased in body weight linearly from week zero - one to week eight. Minor differences were seen among the groups' body weights, but these were not significantly different. On average, the sedentary group had the highest body weight (g) throughout the study, and the overtrained group had the lowest body weight (g) throughout the study. This could suggest that the overtraining protocol had little to no effect on body weight in adolescent C57BL/6 mice. However, one of the common symptoms reported in overtraining is a reduction in body mass (Kreher & Schwartz 2012; Stone et al., 1991). These results may support findings in the literature where body weight (g) reduction may be a confounding variable to overtraining syndrome.

Lean mass and fat mass measurements from this study further showed little difference in absolute measurements of body composition among groups (Figure 4). Lean mass (g) results showed no interaction in the group by time. Thus, no significant differences were seen in lean mass (g) among groups across time. Time was significant, being that all groups increased lean mass (g) across time. This would suggest that the overtrained protocol had little to no effect on changes in absolute lean mass (g). Consideration of all groups, increasing lean mass over time would suggest normal growth and development of all groups. Fat mass results showed no interaction in the group by time (Figure 5). Thus, no significant difference was seen among groups' fat mass (g) across time. There was also no difference among groups without regard to time, nor was there a significance in time for all groups. Despite minor decreases in fat mass

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across time for all groups, it was not significant, and all groups maintained relatively stable fat mass (g). These data would suggest that the training protocols had little effect on changes in fat mass.

Previous literature suggests that weight reduction due to overtraining would be a result of decreased lean mass. A common symptom observed in overtraining is a decrease in body mass due to decreases in lean mass accompanied by increases in fat mass (Carrard et al., 2021; Kreher & Schwartz 2012; Stone et al., 1991). The findings of this study contradict that which is often seen with lean mass and fat mass. This would suggest that lean mass and fat mass may not be reliable indicators of overtraining syndrome for this intervention or duration of the study. These results further elucidate the complexity of using symptomology as an indicator of overtraining syndrome. This study did, however, take into consideration relative lean mass and fat mass via % lean mass and % fat mass.

The percent lean mass from this study (Figure 6) showed that there was an interaction in group by time. Thus, there was a significant difference in % lean mass among the groups across time. Significant differences were seen between overtrained and sedentary, and moderate trained vs sedentary. There was no significant difference in % lean mass across time between overtrained and moderate trained groups. Although significant differences were seen among groups, the lack of significant difference between overtrained and moderate trained groups would suggest that the overtrained group had similar % lean mass changes to that of the moderate trained group. This would suggest that the changes in % lean mass of the overtrained group was not negatively impacted. In fact, the overtrained group ended up having the highest % lean mass among all groups at the post-time point (8-weeks). The moderate trained group ended

up having the lowest % lean mass among groups. These results may demonstrate an ineffective moderate and overtrained protocol design.

Percent fat mass from this study (Figure 7) showed that there was no interaction in group by time. Thus, there were no significant differences in % fat mass among groups across time. However, time was significant, all groups decreased % fat mass over time. This would suggest that the overtrained group displayed a similar linear trend in decline of % fat mass to that of the sedentary group and moderate trained group. This relative assessment of fat mass in % fat mass aligns with the results of this study for fat mass and would suggest that overtraining may not have increased fat mass.

Results of behavioral characteristics showed there was an interaction in group by time for food consumption (Figure 8). Thus, there was a significant difference among groups food consumption across time. The results show significant differences between overtrained and moderate trained, moderate trained and sedentary, and lastly overtrained and sedentary. The results show similar increases in food consumption between overtrained and moderate trained from weeks one-six, but food consumption drastically increased in the overtrained group from week six – eight, whereas food consumption decreased in the moderate trained group week six - eight. A study conducted by Fry et al. (1994), determined that overtraining syndrome caused decreases in appetite among males who were acutely overtrained.

Additionally, recent literature has also reported that overtraining causes suppressed or decreased appetite in individuals who are experiencing overtraining syndrome (Armstrong & Vanheest, 2002; Carrard et al., 2021; Kreher & Schwartz, 2012). The findings of this study go against the literature regarding food consumption and overtraining. However, the findings of this study only observe an eight-week period using an overtraining protocol for the overtrained

group. Longitudinally if the mice within this study continued with the protocol, food consumption could eventually decline, however this study did not make any observations beyond the eight-week protocol.

Edge time results (Figure 9) showed that there was an interaction in group by time. Thus, there was a significant difference among group edge time across the length of the study. There were significant differences in edge time across time between overtrained vs Sedentary groups, and moderate trained vs sedentary groups. There was no significant difference in edge time across time between overtrained vs moderate trained groups. All three groups had relatively similar edge times during the pre-time point (0-weeks). Very little difference can be seen between the overtrained group and moderate trained group for edge time. The overtrained group maintained lower mean edge time compared to the moderate-trained group across the entire study. However, these differences were not significantly different. The sedentary group displayed the highest amount of variability across time with a decrease in edge time at the mid-time point (4-weeks) and an increase from mid time point to post-time point (8-weeks). Although significant differences were seen, these do not inherently mean that the overtrained group displayed the highest markers of stress-related or anxiety-induced behavior. This can be seen when comparing the overtrained group results to the moderate trained. No significant differences were seen between these two groups. This would suggest that the overtrained group and moderate trained group displayed similar stress-related behavior. Again, these results may be due to insufficient protocols.

Results for center time showed no interaction in group by time (Figure 10). Thus, no significant differences were seen among the groups. There was however an interaction in time; all groups decreased center time across time. These results would suggest that all groups

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decreased in confidence displaying behavior across the length of the study. Although no significant differences were seen among groups, the sedentary groups maintained on average, the highest center time across time, while the moderate trained group displayed the lowest center time across time. This would suggest that the sedentary group displayed more confident type behavior and the moderate trained group displayed the least amount of confidence type behavior.

Behavior testing via open field motion capture is used to identify mouse behavior in the following areas; agitation, fear, aggression, or neurocognitive issues (Mertens et al., 2019). The open field test determined mouse behavior based on the prey mentality (Seibenhener & Wooten, 2015). When mice spend more time along the edge of the open field walls, it indicates levels of anxiety, while the opposite is true for time spent in the middle, as this displays levels of confidence or security (Knight et al., 2021; Mertens et al., 2019; Seibenhener & Wooten, 2015). It has been established that one common symptom of overtraining is disturbances in mood and feelings of depression and anxiety (Armstrong & Vanheest, 2002; Cadegiani & Kater, 2017; Cadegiani & Kater, 2019; Califf, 2018; Lee et al., 2017; Lehmann et al.,1993). When considering the literature, the results of this study would suggest opposite findings of anxiety from overtraining. However, the multifaceted nature of overtraining and the results of this study may indicate that open field testing might require secondary behavioral tests or a battery of anxiety-related behavioral tests.

This study assumed that all procedures and protocols (blood collection, MRI, open-field testing, and administration of exercise protocols) were carried out appropriately and with minimal error. It was also assumed that all mice were relatively healthy and had no underlying health conditions or diseases that would impact the results. Lastly it was assumed that the overtraining exercise protocol including the ILT would be sufficient enough to induce an

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overtrained state in the overtrained exercise group. However, there are several limitations within this study. Perhaps one of the greatest limitations within this study was the absence of determining if the overtrained group experienced true overtraining via a post-test determination in performance decline and recovery with no subsequent improvement in performance. The lack of determining a performance indicator of overtraining in the "overtrained" group of this study could impact the inferences made from the results. A high level of confidence was placed in the use of the overtraining protocol of this study and in the investigator's ability to accurately follow the protocol. A second limitation of this study was only measuring two hormones among the three groups. Only observing two hormones greatly limits this study's further application and connection to the current body of knowledge.

Thirdly, the large number of variables being assessed in this study is a limitation as it creates a higher risk for human error when carrying out multiple assessments and procedures in a short period of time. Fourthly, the large amount of variability observed within groups may have limited the reliability of group mean comparisons. This high amount of variability can be seen within the standard deviations (SD) (Tables 2-3) and may suggest that the ILT and subsequent training protocols were not adequate in providing the appropriate relative intensities for the moderately trained and overtrained mice.

Additionally, examining and identifying the mice's perception of fatigue, anxiety, and loss of motivation is a limiting factor. This study also did not utilize female C67BL/6 mice. This lack of cross-sex comparison limits this study's findings and inability to make connections to female adolescents and conclusions can only be made regarding its application to male adolescents. And lastly the length of the study may serve as a limitation as a longer duration

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study may give better insight to long term growth and development outcomes and possible variables outcomes among groups.

Future research should establish reliable tests to determine if exercise-induced overtraining syndrome is established post protocol. Future research should also consider longer duration longitudinal interventions, for instance, observing overtraining syndrome from adolescents into adulthood. Lastly, future research should make cross comparisons between overtrained adolescents and adult counterparts. Furthermore, considerations to cortisol or corticosterone may yet need to be further elucidated in adolescent OTS. And lastly future research should compare male and female adolescents and use secondary sex hormone markers (Testosterone, Estradiol) during a post pubertal OTS induced protocol.

In conclusion, this study examined three main hypotheses. The null hypothesis being tested for hypothesis 1 (H1) stated that there would be no difference in levels of blood biomarkers (corticosterone and IGF-1) among groups (sedentary, overtrained, and moderate trained). This study did show differences among groups for blood biomarkers (corticosterone and IGF-1); thus, the null was rejected.

The null hypothesis being tested for hypothesis 2 (H2) stated there would be no difference in anthropometric measurements (body weight, lean mass, fat mass, percent fat, and percent lean) among the groups (sedentary, overtrained, and moderate trained). This study did show that there were differences among groups in anthropometric measurements (body weight, lean mass, fat mass, percent fat, and percent lean); thus, the null was rejected.

The null hypothesis being tested for the last hypothesis (H3) stated there would be no difference in behavioral traits (food consumption, open field edge time, and open field center time) among the three groups (sedentary, overtrained and moderate trained). This study did show

that there were differences among groups in behavioral traits (food consumption, open field edge time, and open field center time); thus, the null was rejected.

Results of this study found unique and similar characteristics of overtraining syndrome in adolescents to that of adults as well as significant differences among adolescent C57BL/6 mice under different training conditions. However, the etiology, physiology, and prevalence of overtraining syndrome are still incomplete, especially in the youth/adolescent population. Exercise professionals and researchers should continue to better understand its implications, risks, etiology, and influence on athletes and the general population. Until a better understanding of overtraining syndrome is developed, it remains challenging to make implications on how adolescents experience overtraining syndrome. Furthermore, greater extent of research is needed and moving forward reliable OTS induced protocols need to be established for relevant data to emerge in assessing OTS in the adolescent population.

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APPENDIX A: APPROVAL LETTER

MARSHALL UNIVERSITY.
www.marshall.edu

Office of Research Integrity

October 17, 2022

Scotty Davis
1111 86th Avenue, Unit C305
Greeley, CO 80634

Dear Scotty:

This letter is in response to the submitted thesis abstract entitled *"Examining the Effects of Exercise Induced, Physical Stress Overtraining on Stress Biomarkers in Adolescent, C57BL/6 Mice."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #514. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract, you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

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