Title: PRO INFLAMMATORY CYTOKINE PRODUCTIO N BY POLYMORPHONUCLEAR NEUTROPHILS FOLLOWING A 12-DAY PERIOD OF INTENSIFIED TRAINING

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PRO-INFLAMMATORY CYTOKINE PRODUCTION BY POLYMORPHONUCLEAR NEUTROPHILS FOLLOWING A 12-DAY PERIOD OF INTENSIFIED TRAINING

By Josh Thorley

A thesis submitted to the University of Bedfordshire, in fulfilment of the requirements for the degree of Masters of Science.

University of Bedfordshire

Institute of Physical Activity and Research

Submitted: 15th January 2019

Authors Declaration

I, Josh Thorley declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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Abstract

This thesis investigated whether resting and/or exercise-induced interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) production by antigen-stimulated polymorphonuclear neutrophils (PMN) would alter over a 12-day intensified training period (ITP). Ten physically active males completed seventeen exercise sessions in total, including: two main trials (30-min self-paced treadmill run (RPE_{TR}), 10 km time trial), completed before (MT_{PRE}) and after (MT_{POST}) a twelve day ITP, and two $\dot{V}O_{2max}$ tests completed before (VO_{2PRE}) and after (VO_{2POST}) the ITP. Blood samples were collected via venepuncture before and after the RPE_{TR} at MT_{PRE} and MT_{POST}. PMN were isolated from whole blood and incubated for 18 h with lipopolysaccharide (LPS) antigen. IL-8 and TNF-α production by LPS-stimulated PMN was determined using enzyme-linked immunosorbent assay (ELISA) tests. TNF-α production by LPS-stimulated PMN significantly elevated in response to the RPE_{TR} at MT_{PRE} ($P = 0.004$) and MT_{POST} ($P = 0.047$). IL-8 production only significantly increased in response to the RPE_{TR} at MT_{PRE} ($P = 0.033$) but not at MT_{POST} ($P = 0.199$). The absolute RPE_{TR}-induced increase in TNF-α and IL-8 concentrations by LPS-stimulated PMN were lower at MT_{POST} compared to MT_{PRE}. Blood PMN concentration increased significantly following the completion of RPE_{TR} at MT_{PRE} ($P = 0.02$) and MT_{POST} ($P = 0.016$). Resting and RPE_{TR}-induced blood PMN concentrations did not significantly differ between MT_{PRE} and MT_{POST} ($P = 0.521$). Following the completion of the ITP, $\dot{V}O_{2max}$ ($P = 0.696$) and 10 km time to completion scores ($P = 0.457$; $d = 0.32$) did not change. The severity of upper-respiratory tract symptoms (URTS) increased in six out of ten participants following the ITP. Self-reported general ($P = 0.040$) and sport-related ($P = 0.005$) stress scores were higher at MT_{POST} compared to MT_{PRE}. The identification of increased stress states, more severe URTS, and decreased physical performance capacities in participants indicates that overreaching may have been achieved following the ITP. Reduced pro-inflammatory cytokine production in response to acute exercise following a period of intensified training may predispose athletes to impaired inflammatory responses during exercise which may contribute to the pathogenesis of reported URTS in athletes who are overtraining.

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Presentations of Thesis

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List of Abbreviations

°C	Degrees Celsius	**FOR**	Functional Overreaching
~	Approximately	**g**	Gram
µL	Microliters	***g***	Gravitational force
Mm	Micrometer	**GAS**	General Adaptation Theory
%	Percentage	**HR**	Heart rate
α	Alpha	**$HR_{average}$**	Average Heart Rate
±	Plus-minus	**HR_{max}**	Maximal Heart Rate
<	Less than	**HR_{rest}**	Resting Heart Rate
>	Greater than	**ICAM**	Intracellular Adhesion Molecule
ANOVA	Analysis of Variance	**IL**	Interleukin
ACSM	American College of Sports Medicine	**ITP**	Intensified Training Period
CD	Cluster of Differentiation	**Km**	Kilometer
CXCL	(C-X-C motif) Ligand	**mL**	Mililiter
DAMP	Damage-associated Molecular Pattern	**MT_{PRE}**	Main trial performed before the intensified training period
DNA	Deoxyribonucleic Acid	**MT_{POST}**	Main trial performed after the intensified training period
ECSS	European College of Sport Science	**sIgA**	Secretory Immunoglobin A
mRNA	Messenger Ribonucleic Acid	**TNF-α**	Tumor Necrosis Factor-Alpha
NFOR	Non-functional Overreaching	**TLR**	Toll-like Receptor
ng	Nanogram	**URTS**	Upper-respiratory Tract Symptoms

Nm	Nanometer	**$\dot{V}O_2$**	Volume of Oxygen Uptake
ES	Effect Size	**$v\dot{V}O_2$**	Velocity at Maximal Oxygen Uptake
PAMP	Pathogen-associated Molecular Pattern	**$\dot{V}O_{2max}$**	Maximal Oxygen Uptake
PBMC	Peripheral Blood Mononuclear Cell	**VO_{2PRE}**	$\dot{V}O_{2max}$ test performed before the intensified training period
PBS	Phosphate Buffer Saline	**VO_{2POST}**	$\dot{V}O_{2max}$ test performed after the intensified training period
pg	Picogram	**OTS**	Overtraining Syndrome
PMN	Polymorphonuclear		
PRR	Pattern Recognition Receptors		
ROS	Reactive Oxygen Species		
RBC	Red Blood Cell		
RPE	Rate of Perceived Exertion		
RPE_{TR}	30-min self-paced treadmill run		
SD	Standard Deviation		

Chapter 1. Introduction

The primary aim of athletic training is to improve physical performance by using effective training intensities and volumes whilst incorporating an adequate amount of recovery to promote biological adaptation (Dick, 2007). When training load is increased without a corresponding increase in recovery, then athletes may experience symptoms of fatigue and underperformance termed overtraining (Meeusen *et al*., 2013). The overtraining continuum has been categorized into three conditions, functional overreaching (FOR), non-functional overreaching (NFOR), and the overtraining syndrome (OTS) (Meeusen *et al*., 2013). FOR is characterized by a short-term reduction in physical performance capacity lasting days to weeks which may lead to a super-compensatory increase in physical performance capacity. In contrast, NFOR is characterized by the stagnation of physical performance capacity lasting weeks to months which can ultimately be restored after a sufficient recovery period (Meeusen *et al*., 2013). The OTS is thought to occur at the end of this continuum following a prolonged imbalance between training and recovery. This syndrome is defined by a long-term decrement in physical performance capacity that can last from several months to years, with some cases reporting no recovery at all (Dupuy *et al*., 2010; Meeusen *et al*., 2013).

Besides the decline in physical performance capacity, athletes who have reportedly developed NFOR or the OTS are known to experience an increased susceptibility to upper-respiratory tract symptoms (URTS). This increased susceptibility to URTS is associated with the development of immune impairments that arise following periods of intensified training (Peters and Bateman, 1983; Neiman *et al*., 1990; Verde *et al*., 1992; Gomez-Merino *et al.* 2005; Morgado *et al*., 2012). It is yet to be determined however which particular immune impairment, or the effect of multiple impairments, may lead to the pathogenesis of reported URTS in athletes.

Markers of biological responses have been proposed as potential tools to highlight the onset of overtraining, thus helping to prevent such states in the future (Meeusen *et al*., 2013). No

reliable marker however has currently been established, although many have been suggested. Decrements to physical performance capacity have been frequently used to identify states of fatigue and overtraining (Jeukendrup *et al*., 1992; Halson *et al*., 2002; Dupuy *et al*., 2010). Performance capacity can however be highly influenced by other variables such as psychosocial stressors, inadequate nutritional intake, and the development of illness (Morgan et al., 1987; Hooper and MacKinnon, 1995). Reduced heart rate (HR) responses to submaximal and maximal exercise have also been reported in athletes clinically diagnosed with NFOR or the OTS (Costill *et al*., 1988; Lehmann *et al*., 1991; Jeukendrup *et al*., 1992; Hedelin *et al*., 2000). Such responses however are influenced by individuality and external factors that limits its use as a sensitive marker.

Polymorphonuclear neutrophils (PMN) are powerful effector leukocytes that are the earliest and most abundant cell type mobilized during the inflammatory response against antigens that can cause infection or tissue damage (Abbas *et al*., 2014). PMN are enforced with microbicidal properties that are activated to support the clearance of such antigens (Abbas *et al*., 2014). The microbicidal activity of PMN is a result of 3 primary functions; (1) the uptake of antigens within vacuoles (*phagosomes*) which fuse with degrative enzymes (*lysosomes*) to destroy captured antigens (termed phagocytosis); (2) the generation of reactive oxygen species (ROS) by increasing intracellular O_2 consumption (*via respiratory burst*) which aids in the clearance of phagosome-bound and extracellular antigens; (3) the translocation of granules containing antimicrobial enzymes to the PMN membrane where they are released into the extracellular space (*via degranulation*) (Mayadas *et al*., 2012).

The microbicidal function of PMN has been reported to decrease following short-to-moderate (~ 2-5 weeks) intensified training periods (ITP) (Blannin *et al*., 1997; Robson *et al*., 1999a; Yaegaki *et al*., 2007; Robson-Ansley *et al*., 2007; Leal, 2017). The degranulation response by antigen-stimulated PMN has been reported to decrease by 20%, 26% and 30% following a two-week period of intense interval training in highly-trained runners (Robson *et al.,* 1999a), a two-week period of intense interval training in male triathletes (Robson-Ansley *et al*., 2007),

and a 5-week ITP in previously sedentary individuals (Blannin *et al*., 1997), respectively. Phagocytic activity by antigen-stimulated PMN has also been reported to decrease by 47% and 30% in males who had completed a 12-day ITP where training load was increased by 147% (Leal, 2017) and in female judoists who had completed a 20-day period of intense running, judo and weightlifting training (Yaegaki *et al.*, 2007), respectively. A decreased PMN function has therefore been regarded as a potential biological marker in highlighting states of overtraining in athletes (Gleeson, 2002; Yaegaki *et al*., 2008).

Not all PMN functions have been studied in overtrained individuals. It is well documented that PMN synthesize and release a wide range of pro- and anti-inflammatory cytokines and are therefore significant mediators of the body's inflammatory response to antigen challenge (Cassatella, 1999; Tecchio *et al.,* 2014). Cytokines are a category of signalling proteins that activate and control the mechanisms responsible for inflammation (Coico, 2015). Inflammation is a protective response to regulate antigen clearance and the remodelling process of damaged tissue (Coico, 2015). The cytokines interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) are classified as pro-inflammatory cytokines and are produced by PMN once stimulated by antigen challenge such as the endotoxin lipopolysaccharide (LPS) (Cassatella, 1999). Due to the increasing evidence that PMN microbicidal function becomes impaired following an ITP, it is possible that PMN ability to produce pro-inflammatory cytokines may also become impaired. A decreased production of pro-inflammatory cytokines by PMN following an ITP may reduce the effectiveness of the inflammatory response in successfully clearing antigen challenge from the body. Ultimately, this may be one of several immune impairments that occur after an ITP responsible for the pathogenesis of reported URTS in athletes who are overtrained.

Pro-inflammatory cytokine production by antigen-stimulated PMN may also play an important role in the acquisition of inflammatory-derived symptoms associated with states of overtraining (Fry *et al*., 1991). The cytokine hypothesis developed by Smith *et al*. (2000) suggests that muscle and connective tissue microtrauma caused by repeated exercise with limited recovery

triggers the release and accumulation of pro-inflammatory cytokines that develops a state of acute systemic inflammation. A state of acute systemic inflammation may consequently trigger neuroinflammation (Dantzer *et al.,* 2008) through blood-brain communication mechanisms that can evoke the development of sickness behaviours such as anorexigenic behaviour (Romanatto *et al*., 2007), chronic fatigue (Morris *et al*. 2015), mood decline (Miller and Raison, 2016) and an increased sensitivity to pain (Zhang and An, 2007). Because PMN play a significant role in mediating the inflammatory response, their ability to produce pro-inflammatory cytokines may contribute to the acquisition of such sickness behaviours that are associated with overtraining. It is not currently known if pro-inflammatory cytokine production by antigen-stimulated PMN is elevated by a single bout of exercise, therefore one aim of this study was to measure the production of IL-8 and TNF-α by antigen-stimulated PMN in response to a 30-minute high-intensity, self-paced treadmill run.

Chapter 2. Literature Review

2.1 Overreaching and the Overtraining Syndrome

2.1.1 The General Adaptation Syndrome

In 1936, Hans Selye developed the concept of the general adaptation syndrome (GAS). The GAS highlights how an organism can adapt once it is exposed to a novel, potentially damaging stimulus (Selye, 1936). Selye detailed two main stages of GAS which indicate the body's ability to overcome and adapt to novel stressors. The first stage is known as the 'alarm' stage. The alarm stage occurs 6-48 hours after the initial contact with the novel stress and will lead to a rapid decline to the body's physical and biological capacity. The second state is the 'resistance' stage which occurs ~48 hours after contact with the novel stress. The body begins developing a resistance against the stress by activating a series of defence mechanisms to restore homeostasis. Selye identified enlarged adrenal glands and the reduction of oedema size in his participants (Selye 1938) – we would now identify this as the actions of hormones (i.e. cortisol) and the immune system (lymphatic system) working to restore homeostasis during this stage (Desborough, 2000; Mortimer and Levick, 2004). It is also during the latter stages of resistance against the stimuli where it is believed that if the defensive mechanisms build such a resistance against the stimuli, then the individual would succumb to a full recovery by physiological adaptation. If the defensive mechanisms against the stimuli are too weak, or the potency of the stimuli is too severe, then a 3rd stage, known as the exhaustion stage, may be experienced (Selye, 1936). During this exhaustion phase, it is thought that the body would eventually lose its resistance against the stimuli and thus would begin to exhibit negative symptoms which would have been observed prior to the resistance stage (Selye, 1936). An overview of the GAS can be seen in Figure 2.1.

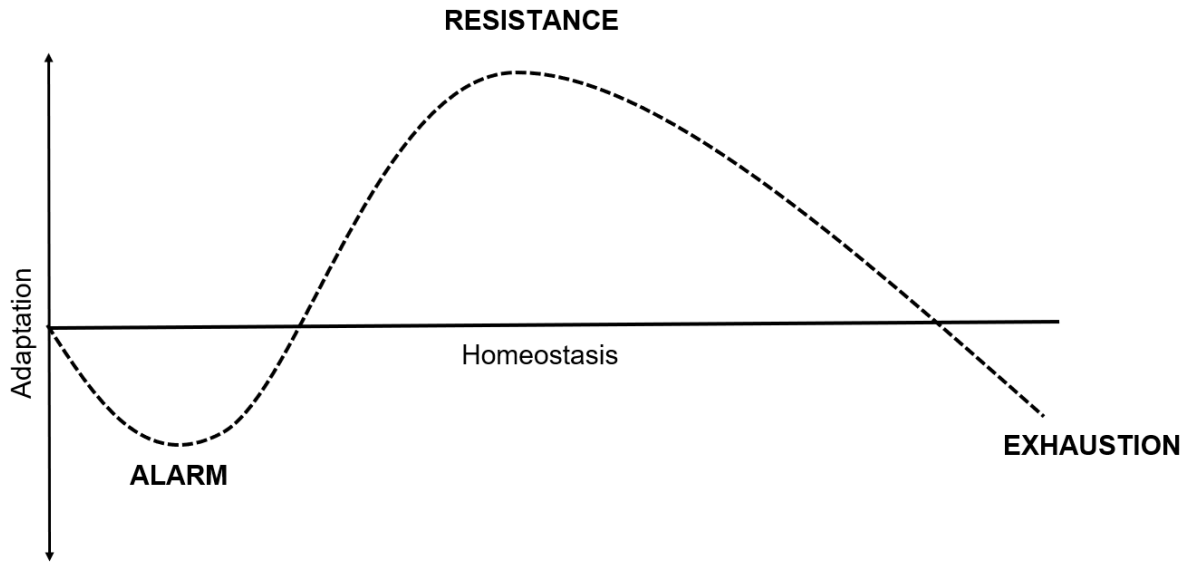

Figure 2.1: *A schematic overview of the general adaptation syndrome (GAS) (Selye, 1936).*

2.1.2 Adaptation to Exercise Stress

In order to improve physical performance capacity, the body must build a resistance and adapt to the novel stress imposed upon the body during exercise (Whyte, 2006). To achieve such adaptations, training load and recovery must be appropriately monitored to prevent an overload of stress which would cause a loss of adaptation and subsequently cause the development of maladaptation (Selye, 1950). Periodization is a method to help develop an athlete's peak performance by planning cycles of training using variations of training methods, intensities and frequencies to promote positive adaptations and to help to manage fatigue appropriately (Bompa and Haff, 2009). During preparation cycles of periodization, whereby an athlete would undergo an ITP with limited recovery (Bompa and Haff, 2009), the planned increase in training stress would lead to the initial 'alarm' stage – in line with Selye's GAS theory (Selye, 1936). Following this period, the exercise stimuli would have caused a noticeable decrease to physical performance capacity by reducing various physiological and mechanical functions of the body. This increase in training stress, the imbalance of recovery periods and a subsequent decrease to physical performance capacity is otherwise known as overtraining (Meeusen *et al*., 2013).

2.1.3 Overreaching

Overreaching has been defined by a joint consensus statement from the European College of Sport Science (ECSS) and the American College of Sports Medicine (ACSM) (2013) by Meeusen and colleagues (Meeusen *et al*., 2013). This document defined overreaching as:

> An accumulation of training and/or non-training stress that results in short-term decrements to physical performance capacity. A decrease to performance capacity can be seen with or without any signs or symptoms of performance-related maladaptation.

There are two forms of overreaching, FOR and NFOR (Meeusen *et al*., 2013). The differentiation of these states was to provide clarity to the definition by emphasizing that overreaching could develop either positive or negative effects to an athlete's physical performance capacity depending on how well it is controlled by the athlete or coach (Meeusen *et al*., 2013). The statement by Meeusen and colleagues (2013) however did not establish the longevity of each stage and when each stage would transition along the 'overtraining continuum', i.e. when does FOR transition into NFOR.

2.1.4 Functional and Non-functional Overreaching

In line with Selye's GAS theory (Selye, 1936), intensified periods of training with insufficient rest periods over a short duration will impose a great stress upon the human body, causing a decline in the body's physical and biological capacity. This ultimately leads to a decline in physical performance capacity (Dick, 2007). With the application of appropriate recovery periods however, a 'super-compensatory' effect may occur whereby physical performance capacity can elevate to a level above that achieved after a period of regular periodized training (Dick, 2007). This adaptation to exercise training is known as FOR (Meeusen *et al.,* 2013). The benefits of FOR in relation to physical performance mean that coaches often use ITP in the form of training camps to improve their athlete's performance prior to competition (Thiel *et al.,* 2011). The recovery time for super-compensation to occur is essential. If a lack of recovery is implemented before a new overload of exercise stress is given, then a greater imbalance

between training and recovery will occur. A prolonged imbalance between training and recovery will ultimately see athlete's progress along the 'overtraining continuum' to a state of NFOR and eventually OTS. Such short-term periods of FOR are generally not accompanied by the presence of severe physiological or psychological symptoms (Birrer *et al*., 2013). It is possible for athletes to recover from FOR within a 2 week period (Halson *et al*., 2002).

If intensified training continues without the application of appropriate recovery periods, NFOR may occur (Meeusen *et al*., 2013). The only distinction between states of FOR and NFOR is the duration of time it takes for recovery to occur. Recovery from NFOR can occur and physical performance can return to baseline levels, albeit not always to the same performance capacity, however it may take athletes several weeks to months of rest for this to occur (Halson *et al*., 2002). The super-compensatory effect observed following a state of FOR will not occur following a state of NFOR (Meeusen et al., 2013). If the development of NFOR is not identified by either athlete or coach, then it may progress in severity, leading to the OTS.

2.1.5 The Overtraining Syndrome

The OTS has been classified by the ECSS and ACSM (2013) as:

> An accumulation of training and/or non-training stress that results in a long-term decrease to physical performance levels. Restoration of physical performance capacity may take from several months to years, and in severe cases, may never occur.

The only objective difference between NFOR and the OTS is the amount of time it takes for a full recovery to occur. A diagnosis of either condition can thus only be made retrospectively once the time frame of maladaptation can be observed (Sims, 2001; Meeusen *et al.,* 2013). A possible presentation of the different stages of FOR, NFOR and the OTS can be found in Table 2.1.

***Table 2.1**: The theorised progression through the different stages of FOR, NFOR and the OTS. Adapted from Bompa and Haff (2009), and Meeusen et al. (2013).*

Fatigue	Increasing state of fatigue			
Training	Ongoing exposure to exercise stress with limited recovery			
Outcome	**Acute fatigue**	**FOR**	**NFOR**	**OTS**
Recovery	Days	Days – weeks	Weeks – months	Months – years (or none at all)
Performance	Increase	Temporary decrease with potential super-compensation	Decrease with performance capacity potentially returning after sufficient recovery	Decrease

Out of 376 young athletes competing at club-international level and spanning across 19 different sports, 29% had experienced NFOR or OTS at least once in their life (Matos *et al.,* 2011). Similar prevalence percentages of NFOR and OTS have been reported in adult athletes, with 37% of elite Swedish athletes (Kenttã *et al*., 2001), ranging across 26 sports, and 35% of competitive swimmers (Raglin *et al.,* 2000) having experienced symptoms of NFOR or OTS at least once within their life.

2.1.6 Symptoms of Overtraining

For clarity, the verb 'overtraining' describes the process of over-training through the continuum of FOR, NFOR and the OTS. The action of overtraining is best characterized by a decreased physical performance capacity (Meeusen *et al*., 2013). This however appears to be the only clear measure. There is no definitive list of overtraining symptoms as they appear to be relative from person to person. Fry *et al*. (1991) compiled a list of symptoms based on their appearance in literature. Symptoms were subcategorized as physiological performance impairments, psychological/information processing impairments, immunological impairments, and biochemical impairments. Robson-Ansley and Costa (2014) compiled this list down to 6 primary symptoms which may be more commonly reported. These symptoms were:

1. Increased fatigue during exercise
2. Underperformance with an inability to increase the pace at the end of a race
3. Increased fatigue and sleepiness during the day
4. Increased URTS
5. Reduced sleep quality
6. Slow wound healing and muscle soreness

2.1.7 Markers to Detect Overtraining

Identifying a marker or markers to detect overtraining would be beneficial to athletes and coaches as it would allow for the continual monitoring of training stress which would help reduce the incidence of NFOR and the OTS. One commonality often shared between athletes who are overtraining is the decline in physical performance capacity (Jeukendrup *et al*., 1992; Halson *et al.*, 2002; Dupuy *et al.*, 2010). It has therefore been proposed that the use of physical performance tests such as time trials (TT) may be useful when attempting to detect the onset of NFOR and the OTS (Schmikli *et al*., 2011). Indeed, both Jeukendrup *et al*. (1992) and Halson *et al.* (2002) reported significantly slower time to completion scores in response to simulated TT tests when athletes had reportedly developed a state of overreaching. There are however issues with using performance markers to identify overtraining. For example, performance decrements may result from psychosocial stressors, inadequate nutritional intake, and the development of illness (Morgan *et al*., 1987; Hooper and MacKinnon, 1995) thus reducing its reliability as a marker.

For the purpose of this present study, the following markers were used in an attempt to detect whether or not participants were overreached by the end of the ITP: (1) Time to completion of a 10 km time trial (2) the development or increased severity of URTS in participants (3) Increased perception of stress levels reported by participants.

Elevated training and/or non-training stress with insufficient recovery has been reported to alter many biological functions such as the immune system (Gleeson and Williams, 2013). A

measure of immune function at rest or in response to an exercise test may therefore be a more sensitive marker to highlight a state of overtraining in athletes compared to the aforementioned performance markers.

2.1.8 Immune Markers to Detect Overtraining

The immune system appears to be sensitive to changes in exercise stress and therefore many immune parameters have been reported to alter following an ITP (Gleeson and Williams, 2013). These changes to immune function could therefore be used as potential biological markers to highlight whether an athlete is overtraining or not. Many of the previously reported immune alterations following an ITP which have been suggested as potential biological markers within the literature can be found in the forthcoming paragraph.

Secretory immunoglobin A (sIgA) is the primary antibody found in mucous membranes and can be an indicator of mucosal immunity (McGhee *et al.,* 1992). SlgA concentration at rest has been documented to decrease following both short- and long-term ITP (Mackinnon, 1996; Fahlman and Engels, 2005; Gleeson, 2006a; Bishop and Gleeson, 2009; Gleeson *et al*., 2012). The total number and function of lymphocytes – a subtype of leukocyte which comprises the highly-specific adaptive immune response against antigen challenge – has been reported to decrease (Walsh and Oliver, 2016). Circulating numbers of natural killer cells and their cytotoxic activity has been reported to decrease over a 7-month training season in elite swimmers (Gleeson *et al*., 1995) and over a 3-week intensive military training course (Gomez-Merino *et al*., 2005). CD4+ T cells and their ability to produce the cytokine interferon-gamma have been reported to decrease following 6 months of intensive cycling training (Baj *et al.,* 1994) and a 6-day period of intensified training (Lancaster *et al*., 2004). In addition, the T cell expression of the leukocyte membrane antigen CD45RO was reported to be higher in athletes who were clinically diagnosed with OTS. Reductions in B cell immunoglobin synthesis have been documented in OTS athletes (Verde *et al*., 1992). Collectively, these findings

suggest that the adaptive immune response against antigen challenge is dysregulated in overtrained athletes and may be beneficial in highlighting overtraining in athletes.

Gleeson (2002) suggested that measures of CD45RO+ T cells (Gabriel *et al*., 1998), salivary IgA concentrations (MacKinnon, 1996) and PMN function (Blannin *et a*l., 1997; Robson *et al*., 1999a) could be used to indicate an impending state of OTS in athletes. Yaegaki *et al.* (2008) suggested that PMN function could be a viable predictor of overtraining in athletes as they reported an exercise-induced reduction in ROS production by antigen-stimulated PMN following a 7-day intensive (two-fold increase in training load) judo training camp. Recent research on PMN function following a period of intensified training has examined their primary microbicidal functions including antigen-stimulated phagocytic activity, ROS production and degranulation response (Blannin *et al*., 1997; Robson *et al*., 1999a; Yaegaki *et al*., 2007; Robson-Ansley *et al*., 2007; Leal, 2017). If PMN function is to be used as a biological marker to detect the onset of NFOR and the OTS, it is important to investigate how other PMN functions are influenced following a period of intensified training. At present, no research has been undertaken to examine whether the release of pro-inflammatory cytokines by PMN is affected by a period of intensified training. This thesis will therefore examine whether a 12-day period of intensified training alters pro-inflammatory cytokine production by antigen-stimulated PMN.

2.2 The Immune System

2.2.1 Immunity

The environment is populated by an array of foreign agents such as bacteria, viruses, fungi, protozoa, prions and parasitic organisms, allergens, pollutants and other toxic substances which trigger immune responses in the human body (Alberts *et al*., 2002). Triggers of immune responses are broadly known as antigens. Foreign antigens can enter the body via inhalation, ingestion, and through physical boundaries such as the skin and mucous membranes (Janeway and Medzhitov, 2002). The human body can also be exposed to self-antigens that originate within the body, triggering autoreactive responses that can result in cell death or damage (Anders and Schaefer, 2014). To counteract these threats, the human body uses the immune system to distinguish healthy host cells from diseased, dying or dead host cells, or foreign antigens (Alberts *et al*., 2002). The immune system minimizes autoimmunity and allergy, whilst being able to eliminate malignant host cells and foreign antigens from the body. For such protective mechanisms to work efficiently, the immune system is reliant on the detection of distinct structural features upon antigens and diseased, dying or diseased cells to distinguish them apart from healthy cells (Mogensen, 2009). Recognition of such structures allows the complex workings of the immune system to attack abnormal host cells and foreign antigens without damaging healthy host cells (Janeway and Medzhitov, 2002). The immune system works through a cascade of pathways controlled by host organs, tissues, cells and proteins to detect and eliminate these abnormal cells and pathogens which may subsequently develop illness and disease within the body (Janeway, 2001).

2.2.2 The Innate Immune System

The innate immune system is comprised of cellular and chemical components which represent the immediate, non-specific immune response to antigen challenge within the body (Lydyard, 2004). The main goal of the innate immune system is to act immediately in response to antigens so that infection or prolonged tissue damage does not occur (Lydyard, 2004). The

physical boundaries of the human body such as the mucous membranes and the epithelial tissue which lines the skin, pulmonary system and gut, as well as chemical components such as sebum, saliva and gastric acid, pose as the first line of defence against foreign antigens (Clark and Kupper, 2005). Leukocytes such as dendritic cells, monocytes, macrophages, granulocytes (which includes PMN, eosinophils, basophils) and natural killer cells, as well as soluble components like complement proteins and cytokines are the second line of defence against both foreign and self-antigens (Clark and Kupper, 2005).

An array of non-specific antigen receptors located intercellularly or upon cell membranes permit these leukocytes to recognize and interact with many antigens (Turvey and Broide, 2010). Non-specific receptors called pattern-recognition receptors (PRR) target pathogen-associated molecular patterns (PAMP) such as the endotoxin LPS (Thompson *et al*., 2011). One of the most abundant groups of PRR is the Toll-like receptor (TLR) family which can interact with PAMP to initiate immune and inflammatory responses (Medzhitov, 2007). PRR also play an important role in the surveillance of tissue damage (Santoni *et al*., 2015). PRR including some TLR recognise self-antigens known as damage-associated molecular patterns (DAMP) that are released following cellular damage (Takeuchi and Akira, 2010). DAMP include intracellular heat shock proteins, high mobility group box-1 proteins, uric acid, mitochondrial DNA and serum amyloid A (Schaefer, 2014).

2.2.3 Neutrophils

PMN are large (12-15 μm in diameter) leukocytes which are characterized by the distinct 'lobed' shape of their nucleus, hence the 'polymorphonuclear' description (Abbas *et al*., 2014). PMN are also classified as granulocytes as they possess highly degradative granules known as azurophilic and specific granules within their cytoplasm (Abbas *et al*., 2014). The normal healthy range of PMN in circulation is thought to be between 2 - 7.5 x 10^9 cells.L^{-1} which constitutes to 40-80% of all circulating leukocytes (Gargani, 2012). Rises in circulating PMN

numbers is clinically referred to as neutrophilia whereas a decline is known as neutropenia (Gargani, 2012).

The development of PMN occurs in bone marrow from haematopoietic stem cells (Delves and Roitt, 1999). The differentiation process from immature stem cell to mature PMN may take up to ~13 days based on *in vivo* radiolabelling studies in humans (Patt and Maloney, 1964). The production of PMN can be triggered by various stimulants such as the cytokine granulocyte-colony stimulating factor (Abbas, *et al*., 2014).

Inactive PMN will circulate throughout the bloodstream until activated by chemoattractants that diffuse from infected or damaged tissue. Chemoattractants such as IL-8, complement protein C5a, and bacterial products such as formylated methionine-leucine-phenylalanine permit PMN to move towards the highest concentration of chemoattractant, thus mobilizing them into tissue which is infected or damaged (Borresgaard, 2010). PMN are released from bone marrow at a rate of 10^{11} per day (Furze and Rankin, 2008), this large-scale influx of PMN into the blood is equally matched by their short-life span which is thought to last for less than 24 hours (McCracken and Allen, 2014). This short life cycle is thought to be a protective mechanism to prevent the cytotoxic nature of PMN from causing harm to healthy cells (McCracken and Allen, 2014).

PMN possess microbicidal functions that aid in the clearance of antigens and cell debris. Such functions are phagocytosis, the production of ROS, degranulation, and the formation of extracellular traps (Kobayashi and DeLeo, 2009). To prime PMN, they must first recognize PAMP or DAMP using PRR such as the endocytic Dectin-1 and TREM-1 receptors, or the cell-surface TLR (Mayadas *et al*., 2014). PMN also express Fc receptors and complement receptors that recognize opsonized antigens (Mayadas *et al*., 2014).

After the body is exposed to infectious or injurious stimuli, PMN are mobilized immediately into the tissue where they can recognize PAMP or DAMP using particular PRR (Prince *et al.,* 2011). Interaction with a particular molecular pattern causes the PMN cell membrane to

undergo a conformational change whereby the cell membrane protrudes and surrounds the antigen within a vacuole known as a phagosome (Alberts, 2002). The phagosome and the endocytosed antigen can be broken down by fusing with lysosomes which contain lysozyme and acid hydrolases (Alberts, 2002). This process is known as phagocytosis (Alberts, 2002). Additionally, an NADPH oxidase complex can form upon the phagosome membrane. This reaction produces ROS such as superoxide, hypochlorite, hydrogen peroxide and nitric oxide (Mayadas *et al*., 2012). The production of ROS occurs when there is a transient rise in O_2 consumption by the PMN known as the respiratory burst (Dahlgreen and Karlsson, 1999). The respiratory burst permits for the rapid release of ROS from the PMN upon the phagosome and also into the extracellular space (Dahlgreen and Karlsson, 1999).

Degranulation is another important function of PMN, whereby azurophilic and specific granules are activated within the PMN cytoplasm (Lacy, 2006). These cytotoxic granules can translocate to either the cell membrane or the phagosome membrane to be released via exocytosis into the extracellular space or into the phagosome, respectively. Extracellular traps, containing chromatin and bactericidal proteins, can also be released from PMN into the extracellular space where they can immobilize and destroy antigens (Kaplan and Radic, 2012).

PMN also contribute to the mediation of immune and inflammatory processes through the release of cytokines, due to the relevance of this function to the present study rationale, this shall be covered more in-depth in Chapter 2.2.6.

2.2.4 Inflammation

Inflammation is a fundamental protective mechanism in response to antigens that may cause infection or tissue damage (Coico, 2015). The primary aim of inflammation is to restore homeostasis by clearing the presence of antigens and mediating the remodelling process of damaged vascular and skeletal tissue (Coico, 2015). An inflammatory response is classified as either acute or chronic in nature. Acute inflammation is known as the non-specific, immediate response to antigen challenge and is mediated by the movement of primarily PMN

into tissue, followed by a secondary wave of monocytes which evolve into inflammatory macrophages (Freire and Van Dyke, 2013). Acute inflammation is first characterized by PMN adherence to blood vessel walls through intracellular adhesion molecules (ICAM) (Muller, 2013). PMN can then transmigrate across the vascular endothelial lining and into the inflamed or infected tissue in order to engulf infiltrated pathogens (Muller, 2013). The process of transmigration is primarily mediated by a chemotactic gradient. The cytokines IL-1β and IL-8 are two known chemoattractants that are released by cells at the inflammatory loci to form a chemotactic gradient in order to attract PMN to this vulnerable site. This process can also be accelerated by the production of TNF-α at the inflammatory loci which can upregulate ICAM upon endothelial cells, thus permitting more PMN to adhere to the blood vessel wall and subsequently transmigrate.

Chronic inflammation is defined by a prolonged and progressive state of inflammation that leads to a raise in circulating (systemic) levels of pro-inflammatory cytokines and a shift in leukocytes present at the site of infection or injury (Coico, 2015). A hallmark sign of chronic inflammation is the shift from non-specific neutrophils that encompass the acute inflammatory response to highly-specific lymphocytes and macrophages (Allison *et al.,* 1978). Chronic inflammation can evoke severe tissue damage and may lead to the pathogenesis of many inflammatory diseases such as stroke, chronic respiratory diseases, diabetes, cancers, and heart disorders (Coleman and Tsongalis, 2018).

The physical manifestation of inflammation is characterised by the development of swelling, redness, heat, loss of function and pain (Coico, 2015). The natural resolution of inflammation can occur through the transcriptional activation of anti-inflammatory cytokines which control the inflammatory response (Opal and DePalo, 2000). Anti-inflammatory cytokines may upregulate specific pro-inflammatory cytokine inhibitors and downregulate pro-inflammatory cytokine receptors that activate cellular inflammatory responses (Opal and DePalo, 2000).

2.2.5 Cytokines

Cytokines are low-molecular weight chemical messenger proteins that are locally produced at low concentrations by nucleated cells (Dinarello, 2007). Leukocytes are considered the primary producers of cytokines in order to regulate immune and inflammatory responses (Coico, 2015). Cytokines interact with cells through receptor signalling to regulate cellular activation, growth, proliferation, apoptosis and chemotaxis (Abbas *et al*., 2014). Cytokines are therefore considered the main intercellular signalling proteins of the immune system (Abbas *et al*., 2014). Cytokines are loosely categorized based on structure and function (Coico, 2015). Cytokines that attract leukocytes to areas of infection or injury are otherwise known as chemokines (Gargani, 2012). Broad families of cytokines exist such as the interleukins, colony-stimulating factors, interferons and TNF families (Tisoncick *et al*., 2012). Many types of cytokines have been reported to have either pro- or anti-inflammatory signalling effects. For example, the cytokines TNF-α and IL-8 are responsible for upregulating inflammatory mechanisms (Bickel, 1998; Walsh, 2013), whereas IL-10 has been reported to inhibit the inflammatory response (Couper *et al.,* 2006). Cytokines can interact in an autocrine (same cell), paracrine (close proximity) and endocrine (long distance) fashion, therefore gaining their name as the ‘hormones of the immune system’ (Dinarello, 2007).

2.2.6 Tumor Necrosis Factor-α Role in Inflammation

TNF-α is a potent mediator of inflammation, acting to mobilize leukocytes, particularly phagocytic cells, to infected or injured tissue (Walsh, 2013). In turn, the infiltration of leukocytes will result in an improved host defence against antigen challenge (Coico and Sunshine, 2015). This process is mediated by TNF-α ability to increase vascular permeability, to upregulate ICAM on endothelial cells, and to induce vasodilation (Johns and Webb, 1998; Hoffman *et al*., 2002)

In addition, TNF-α can signal the release of cytokines such as IL-1, IL-6, IL-8 and colony-stimulating factors from cells which help to potentiate inflammatory responses to clear foreign or self-antigens and to regulate other important immune responses (Maini *et al*., 1995).

Excessive production of TNF-α is implicated in the pathogenesis of many illnesses and diseases such as sepsis, cachexia, irritable bowel disease, rheumatoid arthritis and some forms of cancers (Tracey and Cerami, 1994; Blandizzi *et al*., 1991). Astrocytes found within the brain (Chang and Benveniste, 1990), as well as PMN (Dubravec *et al*., 1990; Streiter *et al*., 1990; Bazzoni *et al*., 1991), monocytes (Yao *et al*., 1997), T cells (Von Fliedner *et al*, 1992) and natural killer cells (Balboa *et al.,* 1992) can all produce TNF-α.

2.2.7 Interleukin-8 Role in Inflammation

IL-8 is a chemokine which mediates pro-inflammatory activities (Bickel, 1998) and is produced by epithelial cells, fibroblasts, endothelial cells, macrophages, lymphocytes, mast cells and PMN (Bickel, 1998). This pro-inflammatory cytokine acts as a chemoattractant to mediate the mobilization of leukocytes such as T cells, basophils and in particular, PMN (Cassatella, 1999), to sites of injury or infection (Russo *et al*., 2014). Elevated IL-8 concentrations within the blood has been shown to upregulate PMN migration into tissue by upregulating the formation of ICAM upon vascular endothelial cells to ensure more PMN are able to transmigrate across the vascular endothelium and into potentially damaged or infected tissue (Bickel, 1993). IL-8 is therefore primarily responsible for the large recruitment of PMN during the earliest phases of acute inflammation (Baggiolini *et al.,* 1994).

The production of IL-8 from leukocytes can also trigger the stimulation of histamine production from basophils which increases vascular permeability, allowing a greater passage of leukocytes to the site of infection or injury (Koch *et al.,* 1992). In addition, IL-8 production will stimulate the formation of new blood vessels via angiogenesis, thus maximising the delivery of circulating leukocytes to inflamed tissue (Koch *et al.,* 1992).

2.2.8 Cytokine Production by Neutrophils

PMN are an important source of cytokine production when activated by antigen challenge (Cassatella, 1999). It was initially thought that PMN were limited to only producing IL-8 (Altstaedt *et al*., 1996), however advances in research have demonstrated that PMN are able to express and release a wide variety of pro- and anti-inflammatory cytokines (Tecchio *et al*., 2014). The fact that PMN are the immediate and most abundant responders to antigen challenge, and will outnumber other cytokine-producing leukocytes within tissue during the earliest phases of acute inflammation (McCracken and Allen, 2014) highlights the importance pro-inflammatory cytokine production by PMN has in mediating the immediate inflammatory response.

Cytokine production by PMN will occur following ligand-receptor signalling with antigens such as PAMP or DAMP (Cassatella, 1999; Tecchio *et al*., 2014). A range of PMN receptors have been reported to stimulate the production of cytokines following ligand-receptor signalling such as colony stimulating factor receptors, TLR, Fc receptors and complement receptors (Cassatella, 1999). An endotoxin known as LPS which can be located on the cell membrane of gram-negative bacteria is frequently used *in vitro* to stimulate the production of cytokines by PMN as it can bind to TLR4 which is abundant upon the PMN cell membrane (Prince *et al*., 2011). The binding of LPS-TLR4 upon the cell membrane initiates the movement of the intracellular NF-kB transcription factor to the PMN nucleus where it can bind to specific DNA sequences. This subsequently leads to the transcription and translation of DNA which will conclude with the protein expression of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α (Djeu et al., 1990; Dubravec *et al*., 1990; Streiter *et al*., 1990; Bazzoni *et al*., 1991; Means *et al*., 2000).

PMN can also store ‘preformed’ cytokines such as IL-8 within their cytoplasm (Pellmé *et al.,* 2006). The exact location of storage for these preformed cytokines is uncertain however it is likely to be in specialised intracellular vesicles, granules or lysosomes.

2.3 Exercise and Neutrophils

2.3.1 Acute Exercise and Neutrophils

Acute exercise has been reported to alter the concentration and function of circulating PMN (Gleeson, 2006b). The general consensus is that neutrophilia occurs immediately and transiently during acute exercise (Pyne, 1994). It appears PMN concentration may exhibit a biphasic response to exercise whereby a transient increase during exercise is followed by a decline to resting values 30-60 minutes post-exercise. A delayed neutrophilia response may also occur 2-4 hours post-exercise (Mills *et al*., 2000). Neutrophilia appears to occur in an intensity-dependant fashion (Neiman *et al*., 1994) as acute bouts of low-intensity exercise evoke small to no changes (Saito *et al*., 2003; Neves *et al*., 2015) in PMN mobilization whereas high-intensity to exhaustive exercise can elicit significant increments (Field *et al*., 1991; Neiman *et al*., 1994; Quindry *et al*., 2003). Previous studies have reported that high-intensity to exhaustive exercise can provoke a functional change in PMN such as a modest decrease to *in vitro* antigen-stimulated degranulation response and oxidative burst capacity (Robson *et al*., 1999b).

Exercise-induced neutrophilia may result from a corresponding rise in immunoregulatory glucocorticoids such as cortisol which are produced through the activation of the hypothalamic-pituitary-adrenocortical axis (McMurray and Hackney, 2000) in response to high-intensity exercise (Verde *et al*., 1992; Gray *et al*., 1993; VanBruggen *et al*., 2011; Hough *et al*., 2011; Hough *et a*l., 2013). Elevated concentrations of circulating cortisol can directly bind to cytosolic glucocorticoid receptors and decrease the number of ICAM upon PMN, thus inhibiting PMN mobilization into tissue (Tessier *et al*., 1996; Cavalcanti *et al*., 2007). Likewise, cortisol increases the production of granulocyte-colony stimulating factor from cells which upregulates the production of PMN from bone marrow reserves (Cavalcanti *et al*., 2006). PMN apoptosis can also be inhibited following cortisol infusion, resulting in a longer life span in circulation (Liles, *et al*., 1995).

In contrast, exercise-induced neutrophilia may be modulated by the rise of cytokines such as granulocyte-macrophage colony-stimulating factor, TNF-α and leukotriene B_4 (Shephard, 2003) which have been reported to similarly downregulate the expression of ICAM on endothelial cells (Griffin *et al*., 1990).

2.3.2 Chronic Exercise and Neutrophils

An ITP lasting 2 – 5 weeks can significantly decrease the phagocytic function of PMN (Yaegaki *et al*., 2007; Leal, 2017), as well as PMN degranulation response (Blannin *et al*., 1997; Robson, 1999a; Robson-Ansley *et al*., 2007) and ROS production (Yaegaki *et al.,* 2008). It has been proposed that these functional changes may increase the incidence of URTS in athletes by inhibiting the efficiency of antigen clearance by PMN (Smith and Pyne, 1997). Very few theories have been proposed as to why PMN function is reduced following a period of intensified training, however Robson-Ansley *et al*. (2007) proposed that it may be due to an influx of immature PMN with less efficient functions compared to mature PMN. In addition, they proposed that the stimulation of PMN throughout an ITP by antigen challenge could dampen PMN ability to function in response to a secondary stimulation.

The completion of high-intensity exercise has been reported to elevate cortisol concentration (Verde *et al*., 1992; Gray *et al*., 1993; VanBruggen *et al*., 2011; Hough *et al*., 2011; Hough *et a*l., 2013) which will effectively regulate the release of immature PMN from the bone marrow into circulation (Cavalcanti *et al*., 2006). Following successive exercise sessions, the pool of mature PMN may deplete due to apoptosis or necrosis, thus leaving immature PMN as the predominant subtype in circulation. It has been reported that immature PMN have a lower phagocytic activity and less granular enzymes compared to mature PMN (McCarthy and Dale, 1988; Yang and Hill, 1991), therefore this may be responsible for the lower PMN function reported after periods of intensified training. Furthermore, following stimulation by antigen challenge, it appears PMN respond less effectively to a secondary stimulation with recovery of its functionality occurring over time (Prasad *et al*., 1991). During the repeated exercise

performed over an ITP, circulating PMN would be particularly exposed to an increased number of DAMP derived from skeletal tissue microtrauma. The continual exposure of DAMP to the circulating PMN may therefore weaken a response to future antigen challenge.

Resting and exercise-induced neutropenia has been reported in athletes who completed an 8-day period of intensified training (Svendsen *et al*., 2016) and in swimmers over a 6-month competitive season (Hooper *et al*., 1995). In contrast, Robson-Ansley *et al*. (2007) reported an acute rise in resting PMN concentration following a 12-day period of intensified training which corresponded with an acute elevation to plasma cortisol levels which may have restricted the PMN from migrating into inflamed tissue, thus elevating the number of PMN in circulation.

2.4 Cytokines and the Susceptibility to Upper-Respiratory Tract Symptoms

Intensified periods of training which may lead to a state of NFOR or the OTS have previously been associated with the depression of several immune functions (Gleeson and Williams, 2013). This impaired immune function following an ITP has also been associated with an increased susceptibility to URTS (Peters and Bateman, 1983; Neiman *et al*., 1990; Verde *et al*., 1992; Gomez-Merino *et al.* 2005; Morgado *et al*., 2012). There is little clarity however on which particular immune impairment is causing the increased susceptibility to URTS.

The development of URTS appears to be the most common reason for athletes to visit sport medicine clinics (Neiman, 1994) and is the primary medical condition reported by elite athletes at both the winter and summer Olympic games (Robinson and Milne, 2000; Reeser *et al*., 2002; Nabhan *et al*., 2016; Yoon *et al*., 2018). The development of URTS in athletes during such a critical period of training is likely to have repercussions on an athlete's physical performance capacity and even participation of events. Therefore, it is of importance to determine the aetiology of URTS present in athletes following a period of intensified training.

The aetiology of URTS is often thought to derive from the infiltration of infectious antigens, such as the Epstein-Barr virus (Gleeson *et al.,* 2002; Cox *et al*., 2004). Research completed by Spence *et al*. (2007) however reported that out of 37 URTS episodes reported by elite athletes during a 5-month training and competition period, infectious antigens were only clinically present in 11 episodes. It is therefore encouraged that physicians should consider both infectious and non-infectious mechanisms, such as allergens and pollutants, when athletes report of URTS (Cox *et al*., 2007; Spence *et al.,* 2007). Consequently, this present study will refer to episodes of upper-respiratory discomfort as URTS rather than upper-respiratory tract infection, otherwise referred to as URTI in the literature, as this investigation could not objectively define the aetiology of upper-respiratory discomfort without a clinical diagnosis.

Numerous studies have reported an inverse relationship between a decreased sIgA concentration and an increased susceptibility to URTS in overtrained athletes (Neiman *et al*., 1990; Heath *et al*., 1992; Mackinnon and Hooper, 1996; Gleeson *et al*., 1999; Neiman, 2002; Fahlman *et al*., 2005; Neville *et al*., 2008; Gleeson and Bishop, 2013). Very few studies however have examined whether an association exists between blood immune parameters and the susceptibility to URTS in overtrained athletes. It has been reported that following a 6-week elevation of training load in elite swimmers, a reduced production of pro-inflammatory cytokines (IL-1, IL-6, IL-12, TNF-α, MIP-1) by antigen-stimulated monocytes and dendritic cells was associated with an increased number of URTS episodes (Morgado *et al*., 2012). In contrast, Gleeson and Bishop (2013) reported no changes in pro-inflammatory cytokine production by antigen-stimulated whole blood culture in athletes who were reportedly more susceptible to illness compared to healthy controls. The difference in findings may be due to the fact that Gleeson and Bishop (2013) used whole blood culture which would include total pro-inflammatory cytokine production from a milieu of nucleated cells present rather than a particular, purified cell type such as monocytes or dendritic cells.

Nonetheless, pro-inflammatory cytokine production from leukocytes such as PMN may reflect an individual's capacity to mediate an inflammatory response against antigen challenge (Gleeson and Bishop, 2013). PMN is the primary leukocyte mobilized during the acute inflammatory response to antigen challenge (Freire and Van Dyke, 2013), therefore their ability to produce pro-inflammatory cytokines may significantly contribute to the inflammatory response against antigen challenge. Decreased pro-inflammatory cytokine production by antigen-stimulated PMN may therefore reduce antigen clearance which could leave the body susceptible to foreign antigens. Reduced antigen clearance may ultimately lead to the increase in reported URTS in overtrained athletes. This thesis will therefore examine whether a 12-day period of intensified training alters the production of TNF-α and IL-8 by antigen-stimulated PMN. It will also examine whether participants have a greater incidence of self-reported episodes of URTS during the ITP.

2.5 Cytokines and Symptoms of Overtraining

Many hypotheses have been proposed to explain the signs and symptoms associated with overtraining such as the '*glycogen depletion'* theory (Costill *et al*., 1988), the '*glutamine theory'* (Newaholme *et al.,* 1991) and the '*monotony theory of overtraining*' (Foster and Lehmann, 1997). None of these hypotheses however account for all of the observed symptoms associated with overtraining. The '*cytokine hypothesis of overtraining'* by Smith (2000) implies that many reported symptoms associated with overtraining could derive from a prolonged state of systemic (blood-bound) inflammation induced by an elevation in pro-inflammatory cytokines. Exercise which requires the eccentric contraction of skeletal tissue is associated with the development of muscle and connective tissue microtrauma (Peake *et al*., 2005). The onset of microtrauma leads to the release of DAMP that initiates an inflammatory response through the release of pro-inflammatory cytokines from circulating leukocytes (Perry, 1992). Often with sufficient recovery, this inflammatory response would be alleviated by therapeutic mechanisms such as anti-inflammatory cytokine release (Opal and DePalo, 2000). When excessive exercise is performed with limited recovery however, it is theorised that the mechanisms to mediate the inflammatory response are insufficient. A dysregulated inflammatory response will thus evoke a chronic inflammatory response which results in systemic inflammation (Smith, 2000). Smith (2000) postulates that the development of systemic inflammation can result in the development of the signs and symptoms associated with overtraining.

Many of the symptoms associated with overtraining may occur from central nervous system dysfunction, such as loss of appetite, increased fatigue, increased sensitivity to pain, and a loss of motivation (Fry *et al*., 1991). Evidence suggests that the development of systemic inflammation can mediate an immune system-to-brain communication by activating the vagus nerve and acting upon TLR found upon macrophage-like cells in circumventricular organs and choroid plexus (Dantzer *et al*., 2008). Engagement of immune system-to-brain communication

leads to the activation of microglia and astrocytes cells (Chesnokova *et al.,* 2016). Microglia and astrocytes are glial cells that support and protect neurons (Hartenstein and Giangrane, 2018). When activated, microglia and astrocytes can release pro-inflammatory molecules such as cytokines, chemokines, ROS, and nitric oxide, thus developing a state of neuroinflammation (Chesnokova *et al.,* 2016). Neuroinflammation is implicated in the development of non-specific psychological and behavioural changes that are collectively known as sickness behaviours (Kent *et al*., 1992). These sickness behaviours include anorexigenic behaviour (Romanatto *et al*., 2007), chronic fatigue (Morris *et al*. 2015), mood decline (Miller and Raison, 2016) and an increased sensitivity to pain (Zhang and An, 2007). Sickness behaviours are often reported by athletes who have developed NFOR or the OTS (Fry *et al*., 1991; Meeusen *et al*., 2013). It is possible that the persistent production of pro-inflammatory cytokines from excessive exercise may lead to a state of acute systemic inflammation that is responsible for the development of sickness behaviours that are reported by NFOR/OTS athletes.

In her description of the *'cytokine hypothesis for overtraining'*, Smith (2000) failed to identify which particular cell-type may be leading to the over-production of pro-inflammatory cytokines within the blood. As PMN are the primary leukocyte mobilized during a state of acute inflammation (Freire and Van Dyke, 2013), their ability to produce pro-inflammatory cytokines may significantly contribute to initiating the inflammatory response against antigen challenge. Persistent exercise-induced increases in pro-inflammatory cytokines by antigen-stimulated PMN may therefore significantly contribute to a state of acute systemic inflammation that evokes the development of sickness behaviours reported by NFOR/OTS athletes.

A level of acute systemic inflammation will be countered by a delayed release of anti-inflammatory cytokines in an attempt to mitigate the inflammatory response and prevent it from becoming chronic (Opal and DePalo, 2000). Exposure to anti-inflammatory cytokines, such as IL-10, may lead to the downregulation of pro-inflammatory cytokine receptors (Zhang and An, 2007). IL-10 has been reported to downregulate the expression of pro-inflammatory cytokines

from macrophages and PMN (Fiorentino *et al*., 1991; Wang *et al*., 1994) which may explain the reduction of pro-inflammatory cytokine production by antigen-stimulated dendritic cells and monocytes (Morgado *et al.,* 2012) as detailed in Chapter 2.4, and could imply that pro-inflammatory cytokine production by PMN may also be affected after an ITP. A reduced pro-inflammatory cytokine activity by PMN may ultimately mediate a less effective inflammatory response that impairs the body's ability to successfully clear antigen challenge. Consequently, this could be one of several immune impairments responsible for the pathogenesis of reported URTS in athletes who have NFOR or the OTS, as highlighted in Chapter 2.4.

2.6 Thesis Rationale, Aim and Hypothesis

Rationale

PMN are the primary leukocyte mobilized during the earliest phases of acute inflammation (Freire and Van Dyke, 2013), therefore their ability to produce pro-inflammatory cytokines will contribute to the initiation and propagation of the inflammatory response against antigen challenge.

PMN microbicidal function decreases following an ITP (Robson *et al.*, 1999a; Robson-Ansley *et al*., 2007; Yaegaki *et al.*, 2007; Yaegaki *et al.,* 2008; Leal., 2017). It is however not known whether the ability of PMN to mediate an inflammatory response via the production of pro-inflammatory cytokines is affected by an ITP. An altered pro-inflammatory cytokine response by antigen-stimulated PMN may lead to a less effective inflammatory response which impairs antigen clearance from the body. Ultimately, this could be one of several immune impairments responsible for the pathogenesis of reported URTS in athletes who have developed NFOR or the OTS.

The reported decrease in PMN microbicidal function following a period of intensified training has been suggested as a potential biological marker in highlighting stress states such as NFOR and the OTS (Gleeson, 2002; Yaegaki *et al*., 2008). If a reduced pro-inflammatory cytokine response by antigen-stimulated PMN is evident following a period of intensified training then this would further suggest that a comprehensive overview of PMN function could be used as a sensitive biological marker to highlight the onset of overtraining in athletes.

Aim

The aim of this study therefore was to investigate whether a 12-day ITP would alter resting and/or exercise-induced IL-8 and TNF-α production by antigen-stimulated PMN.

Hypothesis

Resting and exercise-induced IL-8 and TNF-α production by antigen-stimulated PMN will decrease following the ITP.

Chapter 3. Methods

3.1 Ethical Approval

This study was granted ethical approval by the University of Bedfordshire Research Ethics Committee. Ethical approval (Appendix 8.1) was completed in agreement with the principles of medical research involving human subjects established by the World Medical Association Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013). All potential participants were thoroughly briefed with the study structure, aims and potential risks, as highlighted in the participant information sheet (Appendix 8.2). Prior to participation, participants provided informed consent (Appendix 8.3) and completed a health screen and physiological testing questionnaire (Appendix 8.4).

3.2 Statement of Collaborative Work

This Masters by Research thesis document forms part of a wider group project including work completed by Dr. Diogo Luìs Campos Vas Leal during his PhD at the University of Bedfordshire, titled '*The Use of Endocrine and Immune Biomarkers to Highlight Overreaching'* (Leal, 2017). This current MSc thesis presents data for ten participants, two of which were recruited during the 12 month MSc project. The other eight participants were recruited as part of Dr. Leal's work. However, the author of this present thesis played an integral role in the recruitment process and data collection for the eight participants presented in Dr. Leal's PhD thesis. Thus, Mr. Thorley was involved in conducting the trials and exercise protocols for all participants presented here, two of which he completed independently to Dr. Leal. These participants both completed the protocol over the timeframe necessary for the completion of the Masters by Research project. Other participants were recruited during the MSc project but they did not complete the entire protocol and thus are not included. Data collected and analysed by Mr. Thorley included anthropometric measures, physiological responses to acute exercise and to the ITP, TRIMP scores, and blood PMN concentration. The hypothesis tested within this MSc thesis is novel and is independent of Dr. Leal's work. IL-8 and TNF-α

concentrations by antigen-stimulated PMN reported in this present thesis were not investigated by Dr. Leal. Thus, all cytokine assays, data analysis and interpretation presented here is novel and is solely Mr. Thorley's work.

3.3 Participants

Ten male participants were recruited who were aged between 18-40 years (Table 3.1). Participants were required to live a healthy lifestyle in order to participate in this study. In accordance to the Physical Activity and Public Health joint stance by the American College of Sport Medicine and the American Heart Foundation (2007) (Haskell *et al.,* 2007), participants verbally confirmed with the researcher that they had been involved in either five days of moderate-aerobic exercise per week for a minimum of thirty minutes or alternatively three days of vigorous-intensity aerobic exercise per week for a minimum of twenty minutes. Confirmation of each participants weekly exercise regime would be determined when initially quantifying participants' normal training load prior to the study, as seen in Chapter 3.7.

Participants were recruited on the basis that they did not possess any traits highlighted in the exclusion criteria which was detailed within the participant information sheet. Participants verbally confirmed that they did not meet any of the exclusion criteria. The exclusion criteria for this study was the following:

- **Night shift workers:** Night shift workers were unable to participate in this study due to potentially irregular hormonal and immune secretory patterns influenced by a distorted circadian rhythm (Arendt, 2010). Irregular hormonal patterns can distort immune function (Taneja, 2018). A lack of sleep often highlighted by night shift workers could also impact psychological, performance and general health parameters during the study (Watson, 2017).
- **Smokers:** Smokers were prevented from participating as this habit can negatively influence pulmonary variables such as $\dot{V}O_{2max}$, forced expiratory function, respiratory muscle strength and forced vital capacity (Tantisuwat and Thaveeratitham, 2014).

Pulmonary dysfunction would impair physical performance levels during the $\dot{V}O_{2max}$ test, the main trials and the ITP.

- **Females:** The menstrual cycle has been reported to impact leukocyte numbers and modulate their activity (Oertelt-Prigione, 2012), therefore to prevent this influence on the present study, females were excluded.
- **History of URTS:** Previous experience with URTS prior to the completion of the study may influence the interpretation of URTS results. Therefore, participants verbally confirmed with the researcher that they had not experiences any URTS within the last 2 weeks prior to the study and that they were in a healthy state when beginning the study.

The participants anthropometric and physiological measurements can be found in Table 3.1.

Table 3.1: *Anthropometric and physiological measurements of participants. Values expressed as mean ± SD.*

	Mean + SD
Age (years)	21.1 ± 4.4
Height (cm)	177.4 ± 6.9
Body mass (kg)	73.0 ± 5.9
$\dot{V}O_{2max}$ ($mL.kg^{-1}.min^{-1}$)	58.1 ± 5.2
HR_{max} ($beats.min^{-1}$)	194 ± 9

N =10

Participants were expected to attend seventeen trials over the course of the study (Figure 3.1). These trials included:

- **1** Familiarisation trial
- **2** Experimental main trials, completed before (MT_{PRE}) and after (MT_{POST}) the ITP.
- **2** $\dot{V}O_{2max}$ tests, completed before (VO_{2PRE}) and after (VO_{2POST}) the ITP.
- **12** Intensified exercise sessions

All trials took place at the University of Bedfordshire Sport Science laboratories with the exception of one participant who completed their weekend training at a local leisure facility as they were unable to visit the laboratory at weekends. To ensure this participant completed their training over the weekend when unsupervised, participants were requested to provide photographic evidence of HR data (from the allocated HR monitor) and treadmill velocity data (from the treadmill interface) so that it could be reported back to the researcher immediately following the completion of exercise. Likewise, the participant was also required to video himself completing the training sessions to ensure the accuracy of the HR and treadmill interface data. An in-depth review of each trial completed during the study can be found in Chapter 3.6.

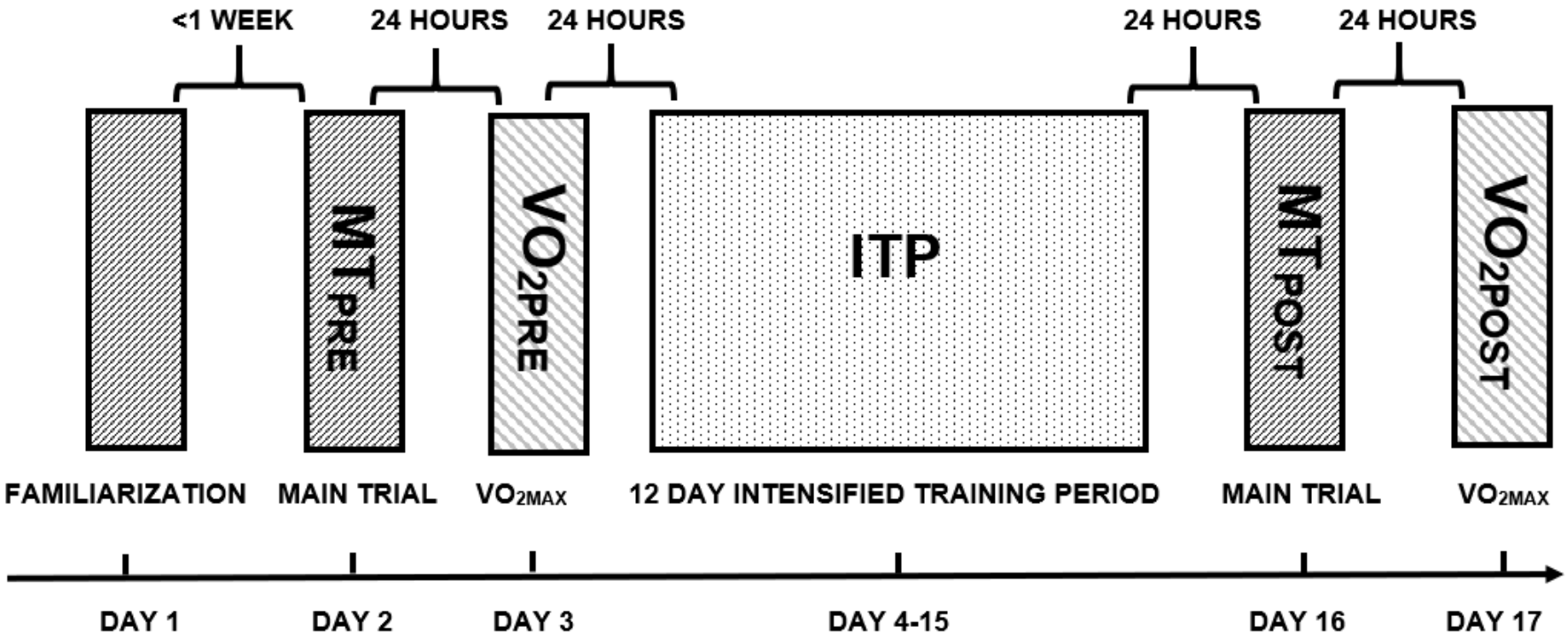

***Figure 3.1**: Study design schematic.*

3.4 Questionnaires

3.4.1 Jackson Score Upper-respiratory Tract Symptom Questionnaire

All participants were required to complete a Jackson score URTS questionnaire during MT_{PRE} and MT_{POST} (Appendix 8.5). This questionnaire recorded the severity of the following URTS:

- Sneezing
- Headache
- Malaise
- Nasal Discharge
- Sore Throat
- Cough
- Ear Ache
- Hoarseness
- Joint Aches and Pains
- Fever
- Chilliness

Each symptom was marked upon its degree of discomfort using the Jackson score (Jackson *et al*., 1958). Discomfort was measured using a Likert scale which ranged from 0 (none at all), 1 (mild), 3 (moderate), and 4 (severe). The total sum of all URTS scores was calculated so that MT_{PRE} and MT_{POST} scores could be compared to see whether URTS had worsened.

3.4.2 Recovery-Stress Questionnaire for Athletes

The RESTQ-76 Sport questionnaire assesses the recovery – stress state in athletes which indicates the degree of physical/mental fatigue accumulated from both sport-specific and external stressors over the past three days and nights (Kellmann and Kallus, 2001) (Appendix 8.6). This questionnaire was completed by participants during MT_{PRE} and MT_{POST}.

Participants answered seventy-seven questions relating to their perception of stress and recovery. These questions were divided into four main scales named general stress, general recovery, sport stress and sport recovery. Within the ‘general’ scales of stress and recovery, there were forty-eight non-specific questions and within the ‘sport’ scales are twenty-eight sport-specific questions. Within each main scale, a number of different subscales are represented that reflect specific changes to general and sport-related stress and recovery. Table 3.2 depicts each main scale and its given subscale.

Table 3.2: *Main scales and subscales used within the RESTQ-76 Sport Questionnaire.*

Scale	Subscale
General Stress	General Stress
	Emotional Stress
	Social Stress
	Conflicts
	Fatigue
	Lack of Energy
	Somatic Complaints
General Recovery	Success
	Social Relaxation
	Somatic Relaxation
	General Wellbeing
	Sleep Quality
Sport Stress	Disturbed Breaks
	Burnout
	Fitness/Injury
Sport Recovery	Being in Shape
	Personal Accomplishments
	Self-Efficiency
	Self-Regulation

Each question depicts a Likert scale that ranged from 0 (being never) to 6 (always), participants were required to mark their proposed answer. High scores received from the stress-related questions reflected a high level of subjective stress whilst high scores from the recovery-related questions indicated a good recovery from exercise and an increased sense of well-being.

3.5 Diaries

3.5.1 Training Diary

If participants were performing exercise that was not associated with the set study design, they were required to complete a training diary so that these sessions could be incorporated into the calculation to determine individual training load. (Appendix 8.7). The training diary required participants to detail the following information:

- The date of the session
- Sport completed
- Exercise completed
- Resting HR (HR_{rest})
- Average HR ($HR_{average}$)
- Maximal HR (HR_{max})
- RPE of session

Participants were given a HR monitor (Polar FS1, Polar, Kempele, Finland) and instructed to record their HR variables for each external training session. This data would be used to quantify training load, as presented in Chapter 3.7.

3.5.2 Food Diary

Participants were required to complete a food diary 24 hours prior to MT_{PRE} (Appendix 8.8). The completion of the food diary allowed for the standardization and replication of caloric and macronutrient intake prior to MT_{POST}. Standardization of caloric intake prevented dietary habits

from influencing energy expenditure which have altered the participants work capacity and RPE between MT_{PRE} and MT_{POST}. A digital weighing scale (Salter 1036BKSSDR, HoMedics Group Ltd, Kent, UK) was given to participants so that consumed food could be weighed and thus replicated to prevent miscalculation of caloric intake. Participants were encouraged to standardize their weighing habits when completing the food diary, for example weighing food prior to being cooked. Participants were reminded the day before MT_{POST} to replicate the exact food diary that they had reported on the MT_{PRE} food diary.

3.6 Trials

Below is a list of all trials [*in chronological order*] that were completed by participants during the study.

3.6.1 Visit 1: Familiarisation trial

A familiarisation trial was performed at least ~1 week before the commencement of the study to familiarize each participant with the logistics and equipment used throughout each main trial. Participants were requested to arrive at the University of Bedfordshire Sport Science laboratories at 11:40 am to begin the familiarisation process. Familiarisation and main trials were completed at the same time of day to ensure immune function was not influenced by circadian alterations to endocrine or immune function which may alter cytokine production by PMN. No blood samples were collected during the familiarisation. The exercise completed during this trial was an exact replica of what participants would be completing during the main trials. No blood samples were taken however participants did complete all the required questionnaires and forms (consent form, health screening questionnaire, URTS questionnaire, and RESTQ-76 Sport questionnaire) that would be expected to be completed during each main trial.

3.6.2 Visit 2: Main trial (MT_{PRE})

The first main experimental trial (MT_{PRE}) was completed within a week of the familiarisation session so information regarding the proposed logistics of MT_{PRE} would not be forgotten. Participants were requested to attend the University of Bedfordshire Sport Science Laboratory in a fasted state (> 4 hours fasted) and sufficiently hydrated to prevent hypohydration and changes to electrolyte balance which could compromise performance (Sawka *et al*., 2007). In accordance with the ACSM Position Stand on Exercise and Fluid Replacement (2007), participants were required to consume ~500 mL of fluid at least 2 hours before exercise to promote adequate hydration (Sawka *et al*., 2007). Upon arrival (~11:40 am), participants were immediately escorted to the bathroom where they provided a urine sample for analysis. Urine osmolality was determined using a urine refractometer (Pocket PAL-OSMO, Atago Vitech Scientific, Horsham, UK). A value of < 700 mOsmol.kg^{-1} indicated that the participant was in a euhydrated state (Sawka *et al*., 2007) and therefore was eligible to complete the main trial. If participants exceeded 700 mOsmol.kg^{-1} then the trial was rearranged for a different day as it was essential that participants began the main trial at 12:00.

On return to the laboratories, participants read and completed the following documents; Health Screen and Physiological Testing Questionnaire, REST-Q 76 Sport Questionnaire and the Jackson URTS questionnaire. Participants were fitted with a HR monitor and HR_{rest} was measured using a HR monitor (Polar FS1, Polar, Kempele, Finland) whilst participants completed the documents. Body mass and height were measured using a weighing scale (Tanita, BWB0800, Tanita, Amsterdam, Netherlands) and stadiometer (HAR-92.602, Holtain, Crymych, Wales), respectively. At ~11:55 am, blood was collected from participants' antecubital fossa via venepuncture.

Participants initially completed a warm-up which consisted of a 3 minute jog at 6 km.h^{-1} prior to the completion of exercise. At 12:00 pm, participants began the experimental exercise protocol (abbreviated as RPE_{TR}) which was completed on a motorised treadmill (D-79576,

Weil am Rhein, Germany). The RPE_{TR} was a 30 minute, self-paced treadmill run which alternated between 1 and 4 minute stages. During each stage, participants were required to alter the treadmill velocity to correspond to a rating of perceived exertion (RPE) score which was determined using a Borg scale (Borg, 1970). For all 1 minute stages, participants were required to run at an RPE of 11, which equated to light exertion. For all 4 minute stages, participants ran at a speed corresponding to an RPE of 15, or a hard exertion. Participants were not allowed to see their respective treadmill velocity as this may have influenced their relative RPE to each stage. A 1% incline gradient was set to mimic the environmental cost of outdoor running (Jones and Doust, 1996) and HR, RPE and treadmill velocity were collected in the final 15 seconds of each stage (Appendix 8.9 for data sheet).

Immediately following the completion of RPE_{TR}, a second blood sample was collected. A 1 hour rest period commenced where participants were instructed to remain seated. Following the 1 hour rest period, a 10 km time trial (TT) performance test was completed on a motorised treadmill at a 1% incline. Verbal support was given to encourage participants to finish the time trial as fast as possible. Participants were blinded to the time or distance completed during the 10 km TT as this may have influenced their time to completion. As a result, the treadmill display interface was covered. An overview of the main trial can be found in Figure 3.2.

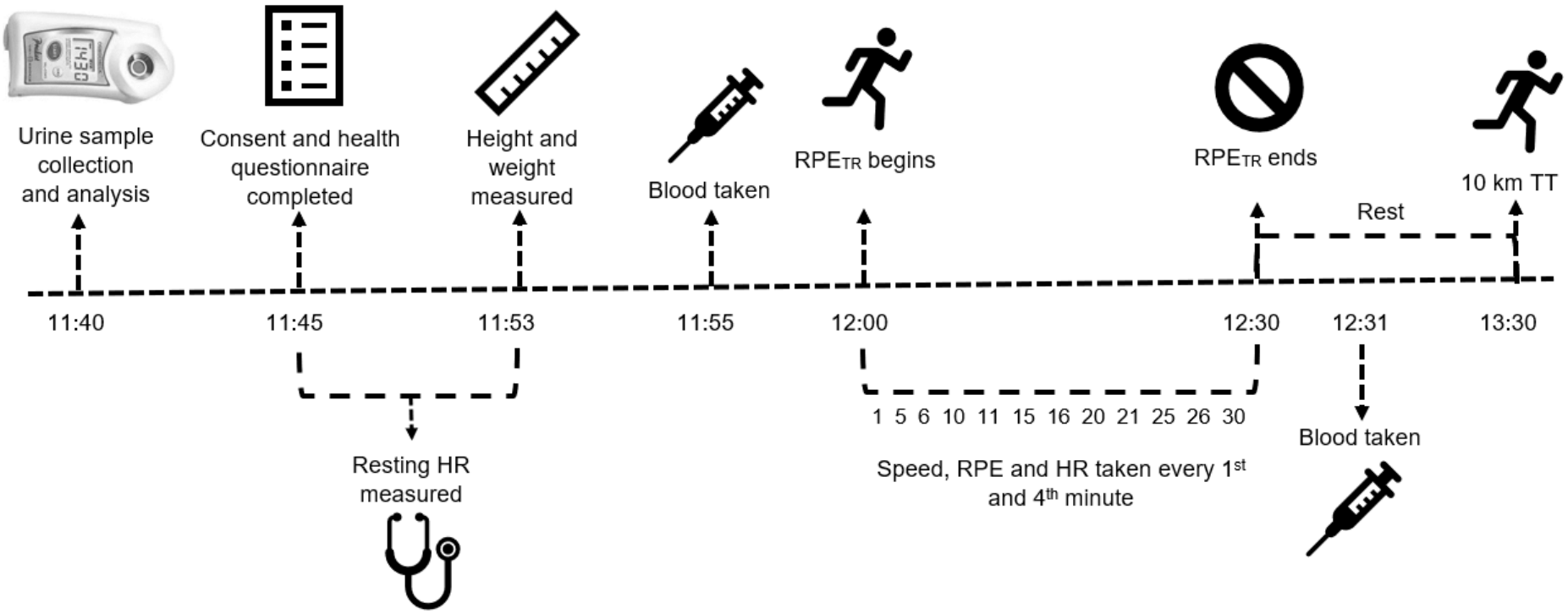

Figure 3.2: *Visual timescale of MT_{PRE} and MT_{POST}*

3.6.3 Visit 3: $\dot{V}O_{2max}$ Test (VO_{2PRE})

Participants were required to complete a $\dot{V}O_{2max}$ test before the completion of the ITP, this baseline test has been abbreviated as 'VO_{2PRE}' throughout the thesis.

A $\dot{V}O_{2max}$ test is a gold-standard method of measuring the maximum amount of O_2 a person can utilize during intense aerobic exercise (Tipton, 2006) and can therefore reflect an individual's health status and relative work capacity (Tipton, 2006). VO_{2PRE} was performed 24 hours following MT_{PRE} and 24 hours prior to the 1st session of the ITP to determine the participants' baseline aerobic capacity. The ACSM defined that the average aerobic capacities of normally active 20-29 year old males is 45-51 mL.kg.min^{-1} (Bushman, 2017). Participants were therefore required to exceed a $\dot{V}O_{2max}$ score of 45 mL.kg.min^{-1} to ensure that they were aerobically fit enough to complete the entire ITP. VO_{2PRE} was completed 24 hours after MT_{PRE} so that the high-intensity nature of the $\dot{V}O_{2max}$ test would not influence resting immune function on the morning of MT_{PRE}.

Upon arrival to the laboratories, the participants were re-familiarised with the equipment and completed a warm-up prior to the test, this was completed for 3 minutes at 6 km.h^{-1}, with an incline gradient set at 1%. A submaximal treadmill running test at a 1% incline gradient was then performed for 16 minutes, which was divided into 4 x 4 minute stages. Participants were encouraged to choose a speed between 7 km.h^{-1} to 12 km.h^{-1} to begin the test. Prior to the test, participants were given the opportunity to test each speed intensity to see which speed best suited their relative aerobic fitness capacity. After the completion of each 4 minute stage, the treadmill speed was increased by 1 km.h^{-1}. This test was used to measure participants' oxygen uptake ($\dot{V}O_2$) at submaximal velocities.

Participants were given a 20 minute rest period where they remained seated for the entirety. To determine $\dot{V}O_{2max}$, an incremental treadmill running test was run to volitional exhaustion.

The test was completed in 1 minute stages, with the first stage beginning at a 1% increment gradient and increasing by 1% after each stage until $\dot{V}O_{2max}$ criteria was achieved.

$\dot{V}O_{2max}$ was established in accordance with The British Association of Sport and Exercise Science Position Statement on the Physiological Assessment of the Elite Competitor (1992) (Bird and Davison, 1997) which states that 3 of the following 5 criteria must be achieved by the participant:

- An observed plateau in $\dot{V}O_2$ in correspondence with increased exercise intensity. This plateau can be defined as less than a 3% increase (< 2 $mL.kg^{-1}.min^{-1}$) in $\dot{V}O_2$ after an increase in exercise intensity.
- An achieved HR which is 10 $beats.min^{-1}$ away from the participants' age-predicted HR_{max}. This can be calculated by 220 – participants age (years).
- An achieved respiratory exchange ratio of > 1.15.
- An RPE of > 18.
- Volitional exhaustion achieved.

The treadmill speed was consistent throughout the entirety of the test and was set at the speed that was achieved during the submaximal test when participants' HR reached 150 $beats.min^{1}$. HR and RPE were collected in the last 15 seconds of each stage. An overview of VO_{2PRE} can be seen in Figure 3.3.

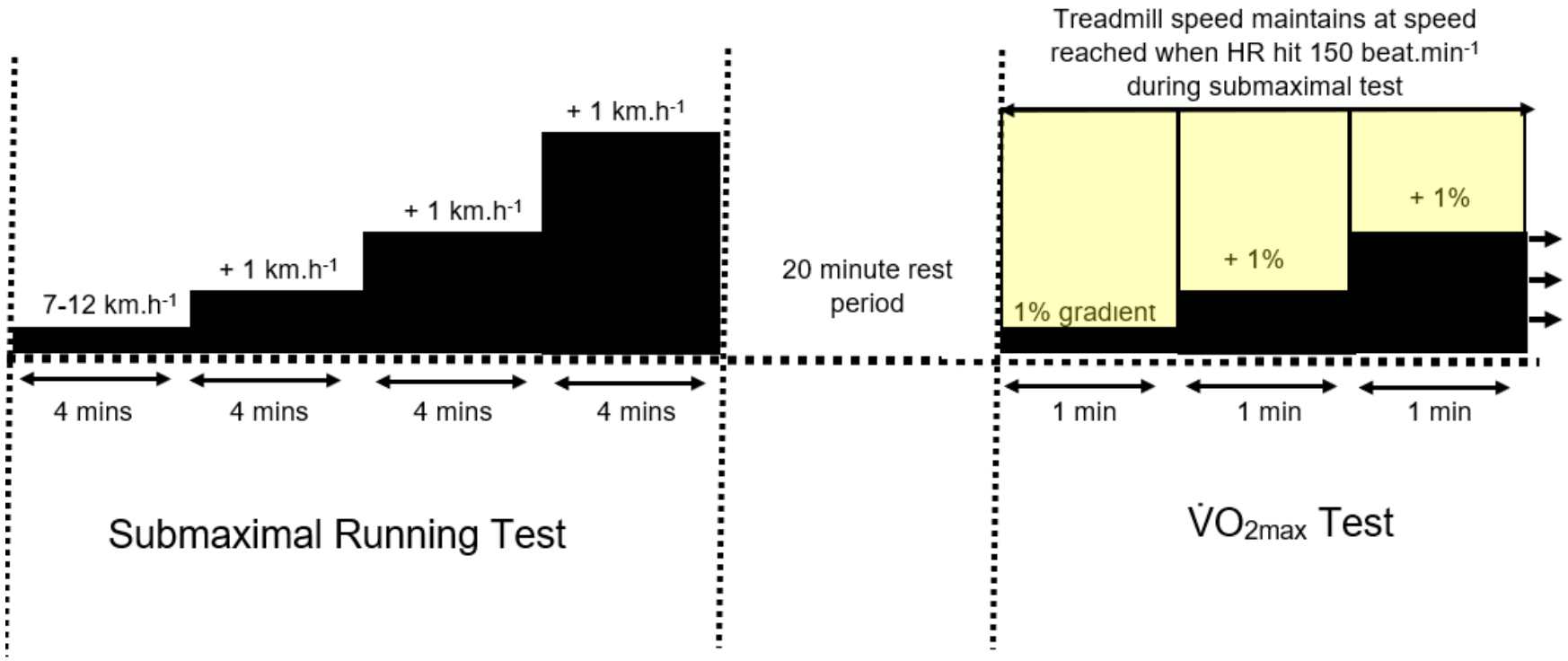

Figure 3.3: *Visual timescale of VO_{2PRE} and VO_{2POST}*

3.6.4 Calculation of Velocity at $\dot{V}O_{2max}$

During the 12 day ITP, each participant was given individualised exercise intensities based off their given velocity at $\dot{V}O_{2max}$ ($v\dot{V}O_{2max}$). Specifically, participants were asked to run for 70 minutes at 55% of $v\dot{V}O_{2max}$ and 20 minutes at 75% $v\dot{V}O_{2max}$ during a 90-minute continuous treadmill run which was employed throughout the 12 day ITP. To calculate the individualised percentages of $v\dot{V}O_{2max}$, $\dot{V}O_2$ was regressed upon the increases of speed from the submaximal test completed during VO_{2PRE} and a linear equation was obtained. The speed-$\dot{V}O_2$ relationship from the submaximal test was then extrapolated to obtain 55% and 75% of $v\dot{V}O_{2max}$.

3.6.5 Visit 4 – 15: Intensified Training Period (ITP)

24 hours following VO_{2PRE}, participants began the first exercise protocol of the 12 day ITP. Participants completed 3 different exercise protocols which repeated 4 times over the 12 days. The variety of such protocols was aimed to reduce the monotony of the training protocols which could have led to participants losing interest. The protocols used within the study design were as follows:

1. A 90-min continuous treadmill run, divided into one stage of 70 minutes at 55% of the individuals' $v\dot{V}O_{2max}$ and one 20 minutes stage at 75% $v\dot{V}O_{2max}$.
2. 5 km time trial
3. A 70 minute, self-paced treadmill run at a speed corresponding to an RPE of 12 (light) on the Borg scale for the first 30 minutes, at 13 (somewhat hard) for the following 30 minutes, and at 15 (hard) for the final 10 minutes

Treadmill incline remained at 1% throughout the entirety of each exercise protocol. Participants were allowed to drink water *ad libitum* during each protocol. Treadmill velocities,

distances, HR_{rest} and $HR_{average}$ were collected over each exercise protocol to quantify the total training load of the ITP, as highlighted in Chapter 3.7.

3.6.6 Visit 16 – 17: Main trial and $\dot{V}O_{2max}$ Test (MT_{POST} and VO_{2POST})

A second experimental main trial (abbreviated as MT_{POST}) was completed 24 hours following the completion of the ITP. MT_{POST} would measure whether the ITP had altered participants' physiological, immune, psychological, and performance variables..

Likewise, a second $\dot{V}O_{2max}$ test (abbreviated as VO_{2POST}) was completed 24 hours following the completion of the MT_{POST} main trial. VO_{2POST} was performed 24 hours after the ITP to determine whether changes to physiological stress or fitness level influenced changes to immune function. MT_{POST} and VO_{2POST} were performed by replicating the same protocol that was used during MT_{PRE} and VO_{2PRE}, respectively.

3.7 Training Load

The premise of the ITP was designed to increase (or 'intensify') participants' training load compared to their normal training load. A training impulse (TRIMP) equation was used to quantify whether participants' training load had increased during the ITP compared to their baseline training load. Twelve days prior to the beginning of the study protocol, participants were asked to complete a training diary of their daily physical exercise activities. During each exercise session, participants were required to measure their HR_{rest}, $HR_{average}$ and HR_{max}, plus the duration of each session. Measurement of these HR variables was completed using a HR monitor (Polar FS1, Polar, Kempele, Finland) that was provided to the participant. This 12 day period was known as the 'baseline' period and was used to determine participant's normal training load prior to participation. The TRIMP for each session was calculated using the following formula developed by Banister (1991):

$$time\ of\ session\ (mins) \times\ \Delta HR\ \times y$$

$$\Delta HR\ = \frac{(HRaverage - HRrest)}{(HRmax - HRrest)}$$

$$y = weighting\ factor\ (0.64e^{1.92x})$$

$$e\ = base\ of\ the\ Napierian\ logarithm$$

$$x = \Delta HR$$

Banister (1991)

The sum of TRIMP scores measured after each exercise session during the baseline period were compared to the sum of TRIMP scores of each exercise session measured during the ITP (from the set exercise sessions and from external training sessions). This would determine whether participants were truly training with an elevated training load during the ITP.

3.8 Whole Blood Collection and Isolation Techniques

3.8.1 Venepuncture

Whole blood was collected from an antecubital vein via venepuncture using a 21G needle with quickshield (Vacuette, Greiner Bio-One, Stonehouse, UK) immediately pre RPE_{TR} and immediately post RPE_{TR}. Blood was collected by a trained phlebotomist with an up-to-date hepatitis B vaccination. Three 5 mL Vacuette containers, treated with K_3 Ethylenediaminetetraacetic acid (K_3EDTA) (Vacuette, Greiner Bio-One, Stonehouse, UK) were collected and immediately used for analysis.

3.8.2 Neutrophil Isolation

To isolate PMN from whole blood, 5 mL of Lympholyte-poly™ (Cederlane Laboratories, Burlington, Canada) was gently dispensed into a 15 mL falcon tube (BD Biosciences, Oxford, UK). An equal amount of heparinised whole blood was carefully layered on top of the Lympholyte-poly™ medium. Lympholyte-poly™ is a density gradient medium (density of 1.133 g/mL^{-1}) that provokes a separation of granulocytes from other blood cells based on their particular buoyant density. The falcon tube was placed into a temperature-controlled centrifuge (Heraeus, Heraeus Multifuge X3R, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and spun for 35 minutes at 500 x *g*, with the temperature set at 20 °C. Centrifugal acceleration and deceleration levels were set to 2 and 1 respectively.

Following the centrifugal process, a density gradient between blood cells had formed (Figure 3.5). Peripheral blood mononuclear cells (PBMC) have a lower buoyant density (< 1.133 $g.mL^{-1}$) than PMN and red blood cells (RBC) (> 1.133 $g.mL^{-1}$). Granulocytes and RBC can thus sediment through the Lympholyte-poly™ medium whilst PBMC maintain at the medium interface. Granulocytes segregate within a separate band located below the PBMC whilst the RBC further sediment through the medium due to their significantly higher buoyant density. The RBC will subsequently aggregate at the bottom of the tube. The PMN are located in the

granulocyte band which also included basophils and eosinophils, therefore the PMN harvested are not 100% PMN and may contain a very small fraction of contamination.

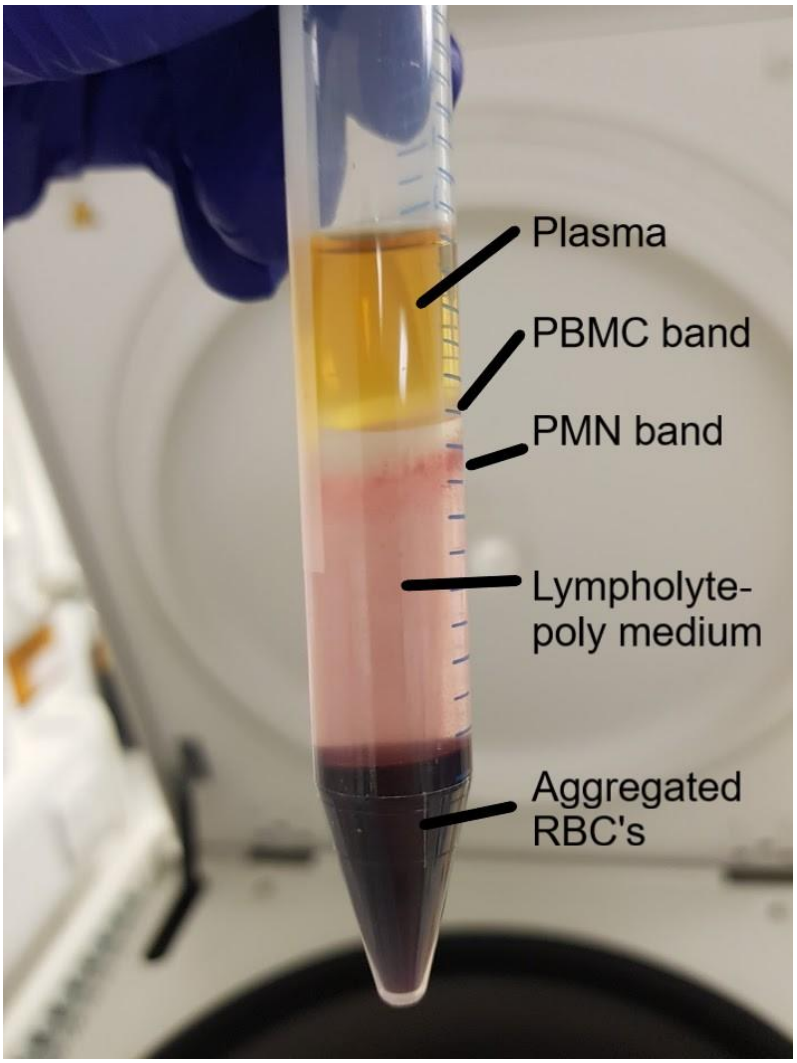

Figure 3.4: *Example result of 5 mL heparinised whole blood / 5 mL Lympholyte-poly™ post-centrifuge. Cell types of banded based on their buoyant density.*

If the granulocyte band was clearly observable, a Pasteur pipette was used to discard the plasma and PBMC layer so that PMN could be reached and harvested. A previously made solution consisting of 25 mL sterile-filtered water (BioReagent, Sigma-Aldrich, Gillingham, Dorset, UK) and 25 mL Dulbecco's Modified Eagles Cell Medium (Sigma-Aldrich, Gillingham, Dorset, UK) supplemented with glucose (4500 $mg.L^{-1}$), sodium pyruvate (110 $mg.L^{-1}$) and L-glutamine was added to the harvested PMN and centrifuged for 10 minutes at 400 x *g* to suspend, wash and culture the PMN. After 10 minutes, the supernatant was discarded and 1

mL of AIM-V Serum Free Cell Medium (AlbuMAX^{TM}, Gibco, Waltham, Massachusetts, USA) supplemented with L-glutamine, gentamicin sulfate (10 µg.mL^{-1}) and streptomycin sulphate (50 µg.mL^{-1}) was suspended upon the PMN pellet. The PMN pellet was unsuspended into the cell medium and the supernatant was used for the following cell counts and cytokine assay.

3.9 Determination of Blood Neutrophil Concentration

The PMN supernatant, as described in Chapter 3.8, was used to determine participants' blood PMN concentration. PMN supernatant (20 µL) was suspended in 100 µL of 10X, pH 7.4 phosphate buffer saline (PBS). Cells were counted using 2 different methods. Determination of blood PMN concentration was completed using a handheld cell counter (Scepter, Merck Millipore, Germany). One participant's blood PMN concentration were counted using hemocytometry due to a fault with the handheld cell counter.

Cell counting via the handheld cell counter was completed by attaching a 60 µm disposable tip (PHCC60050, Scepter, Merck Millipore, Billerica, Massachusetts, USA) onto the device and then following the instructions onscreen. PMN were detected using Ohm's Law:

$$V = IR$$

$$V = Voltage;\ I = Current;\ R = Resistance$$

As cells pass through the counters aperture, resistance increases causing a subsequent increase in voltage. Voltage changes are recorded as spikes and spikes of the same magnitude are allocated into a histogram and interpreted as a cell concentration.

Hemocytometry is the cell counting technique using a hemocytometer grid and a microscope. A cover slip was first fixed upon the hemocytometer by moistening the surface using an exhaled breath. The observation of 'Newton's rings' would determine whether the cover slip was appropriately affixed upon the hemocytometer. 20 µL of 0.2% trypan blue dye was mixed with 20 µL of PMN suspended in AIM-V. 10 µL of the trypan blue stain/PMN mix was then pipetted at the edge of the cover slip which would travel onto the hemocytometer grid. The

hemocytometer grid was placed under the microscope to be counted. Live cells were counted from 5 squares of the grid (4 corners squares, 1 centre square). Live PMN would appear colourless and bright whereas dead cells would stain blue and become non-refractile. This reaction is based on the principle that live cells possess intact cell membranes which do not allow the dye to enter whereas necrotic or PMN which underwent apoptosis allow the dye to accumulate within the cell (Strober, 2001). To calculate the particular cell concentration per mL, the following equation was used:

$$Number\ of\ PMN\ in\ 5\ squares\ \times number\ of\ counted\ squares\ (5)\ \times dilution\ factor \times 10^4$$

3.10 Neutrophil Stimulation Assay

This assay was performed to elicit IL-8 and TNF-α production by the harvested PMN. PMN were stimulated with a commercially-available LPS extracted from *Escherichia-coli* (Sigma Aldrich, Gillingham, Dorset, UK). Commercially-available LPS was prepared by reconstituting 1 mg LPS in 1 mL cell medium (1 $mg.mL^{-1}$).

A 24-well plate was acquired and wells were divided into 'stimulated' and 'unstimulated' for each time point (pre and post RPE_{TR}). For all wells, 250 µL of PMN cultured with AIM-V was added, followed by a further 750 µL of AIM-V Serum Free Cell Medium to prevent any serum component contamination. For all 'stimulated' wells, 10 µL of reconstituted LPS was added. Samples were placed into an incubator (HERA CELL 240i, Thermo Scientific, Loughborough, UK) for 18 hours at 37 °C, 5% CO_2. It was expected that the incubatory period would allow the PMN to release an optimal concentration of both IL-8 and TNF-α in response to LPS stimulation into the culture (Bazzoni *et al*., 1991; Djeu *et al*., 1990).

The following day, each well was transferred into individual Eppendorf's. All samples were placed into a microcentrifuge (Pico 17, Heraeus, Thermo Scientific, Loughborough, UK) and spun for 400 x *g* for 5 minutes to suspend the PMN. The PMN culture were then transferred into new Eppendorf's and frozen at -80 °C until cytokine analysis was performed. The non-stimulated cytokine concentrations reflect the basal level of cytokine production by PMN to

any stimulation which may have occurred pre-harvest and therefore act as control measurements. The cytokine concentration by antigen-stimulated PMN reflects the level of IL-8 and TNF-α produced in response to an *in vitro* sterile infection.

3.11 Cytokine Analysis

In this present study, TNF-a and IL-8 concentrations by antigen-stimulated and non-stimulated control PMN were determined using direct sandwich Enzyme-Linked Immunosorbent Assay (ELISA) tests (Bender MedSystems GmbH, Vienna, Austria). All ELISA tests were performed by the same researcher to avoid inter-experimenter variability. Each assay was completed by following the manufacturers guidelines. All completed ELISA tests for the present study can be found in Table 3.3.

Table 3.3: *Information of ELISA tests used within the present study.*

ELISA	Range ($pg.mL^{-1}$)	Sensitivity ($pg.mL^{-1}$)
Uncoated Human TNF-α	4 – 500	4
Uncoated Human IL-8	2 – 250	2

Uncoated 96-well microplates (Costar 9018, Corning™, New York, USA) were used to plate all samples collected throughout the study. Prior to its use, each microplate underwent a coating process which involved adding pre-titrated, purified anti-human IL-8 and TNF-α capture antibodies to each well followed by a 24 hour incubation which provoked the hydrophobic binding of the capture antibody to the plastic well. All samples used on the IL-8 ELISA were diluted 1:25 with 1X PBS (pH 7.4) to fit onto the ELISA standard curve as previous attempts of the IL-8 ELISA with undiluted samples significantly exceeded the maximal range. Samples were aliquoted into duplicates to identify any pipetting error or well contamination

that could cause skewed concentrations. The intra-assay coefficient of variance for each assay are displayed in Table 3.4. To reduce interassay variation, samples from each individual participant were run on the same ELISA.

***Table 3.4**: Intra-assay coefficient of variance (%) of all completed ELISA tests.*

ELISA	Number of Samples	Intra-assay Coefficient of Variance (%)
Uncoated Human TNF-α	32	10.3
Uncoated Human TNF-α	40	14.5
Uncoated Human TNF- α	8	6.6
Uncoated Human IL-8	40	4.5
Uncoated Human IL-8	40	10

A plate washer (Asys Atlantis, Biochrom Ltd, Cambridge, UK) was used to rinse and aspirate wash buffer (1X PBS, 0.05% Tween -20) when required. A 450 nm plate reader (Tecan, Infinite F200 Pro, Männedorf, Switzerland) was used to calculate the optical densities of the ELISA plate. The software Magellan™ version 7.1 (Tecan GmbH, Männedorf, Switzerland) was used to automatically determine the standard curve from the optical densities which provided the raw concentrations of each sample. 25 samples violated the standard curve, these were the following samples:

- **Participant 1: (1)** MT_{PRE}, post RPE_{TR}, unstimulated (IL-8) **(2)** MT_{PRE}, post RPE_{TR}, stimulated (IL-8).
- **Participant 2: (1)** MT_{PRE}, pre RPE_{TR}, unstimulated **(2)** MT_{POST}, post RPE_{TR}, unstimulated (IL-8).

- **Participant 3: (1)** MT_{PRE}, pre RPE_{TR}, stimulated (IL-8) **(2)** $MT_{PRE,}$ post RPE_{TR}, unstimulated (IL-8) **(3)** MT_{PRE}, post RPE_{TR}, stimulated (IL-8) **(4)** MT_{PRE}, pre RPE_{TR}, unstimulated (IL-8); **(5)** MT_{PRE}, pre RPE_{TR}, stimulated (IL-8) **(6)** MT_{PRE}, post RPE_{TR}, stimulated (IL-8).
- **Participant 5: (1)** MT_{PRE}, pre $RPE_{TR,}$ unstimulated (IL-8) **(2)** MT_{PRE}, pre RPE_{TR}, stimulated (IL-8) **(3)** $MT_{PRE,}$ post RPE_{TR}, stimulated (IL-8) (**4)** MT_{PRE}, pre RPE_{TR}, unstimulated (IL-8) **(5)** MT_{POST}, pre RPE_{TR}, stimulated (IL-8) **(6)** MT_{POST}, post RPE_{TR}, unstimulated (IL-8) **(7)** MT_{POST}, post $RPE_{TR,}$ stimulated (IL-8).
- **Participant 6: (1)** MT_{PRE}, post RPE_{TR}, unstimulated (IL-8) **(2)** MT_{PRE}, post RPE_{TR}, stimulated (IL-8) **(3)** MT_{POST}, post RPE_{TR}, unstimulated (IL-8) **(4)** MT_{POST}, post- RPE_{TR}, stimulated (IL-8).
- **Participant 7: (1)** MT_{PRE}, post RPE_{TR}, stimulated (IL-8) **(2)** MT_{POST}, pre RPE_{TR}, unstimulated (IL-8**) (3)** MT_{POST}, post RPE_{TR}, unstimulated (IL-8).
- **Participant 8: (1)** MT_{PRE}, post RPE_{TR}, stimulated (IL-8).

A linear equation was produced to extrapolate the standard curve to calculate the raw concentration of these violated samples from their mean optical density. An example of this process is demonstrated in Figure 3.6.

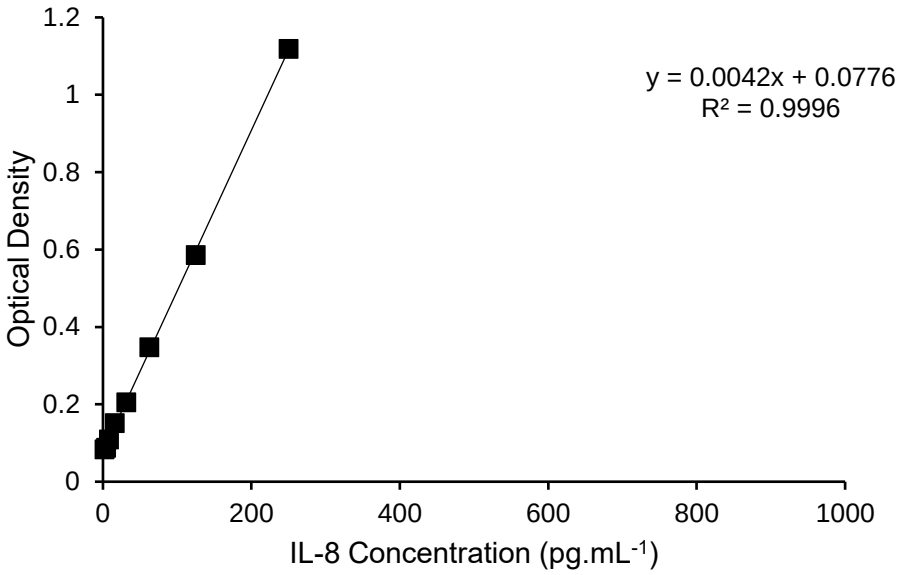

Figure 3.6: *Linear equation* $(y = mx + c)$ *produced from the standard curve of a completed IL-8 ELISA. The linear equation was rearranged to:*

$$x = (y - c)/m$$

y = mean optical density of sample (in duplicates)

x = IL-8 concentration (pg.mL^{-1})

23 samples were undetectable (< 2 pg.mL^{-1} for IL-8 ELISA; < 4 pg.mL^{-1} for TNF-α ELISA) during analysis, these were the following:

Participant 1: (1) MT_{PRE}, pre RPE_{TR}, unstimulated (IL-8 + TNF-α) (2) MT_{PRE}, post RPE_{TR}, stimulated (TNF-α) (3) MT_{PRE}, pre $RPE_{TR,}$ unstimulated (TNF-α) (4) MT_{POST}, post RPE_{TR}, unstimulated (TNF-α).

Participant 2: (1) MT_{PRE}, pre RPE_{TR}, unstimulated (TNF-α) (2) MT_{PRE}, post RPE_{TR}, unstimulated (TNF-α) (3) MT_{POST}, pre RPE_{TR}, unstimulated (TNF-α); MT_{POST}, post RPE_{TR}, unstimulated (TNF-α).

Participant 3: (1) MT_{PRE}, pre RPE_{TR}, unstimulated (IL-8)

Participant 4: (1) MT_{POST}, pre RPE_{TR}, unstimulated (TNF-α).

Participant 7: (1) MT_{POST}, pre RPE_{TR}, unstimulated (TNF-α); (2) MT_{POST}, post RPE_{TR}, unstimulated (TNF-α).

Participant 8: (1) MT_{POST}, pre RPE_{TR}, unstimulated (TNF-α); (2) MT_{POST}, pre RPE_{TR}, unstimulated; (3) MT_{POST}, post RPE_{TR}, unstimulated (TNF-α).

Participant 9: (1) MT_{PRE}, pre RPE_{TR}, stimulated (IL-8)

Participant 10: (1) MT_{PRE}, pre RPE_{TR}, unstimulated (TNF-α) (2) MT_{PRE}, pre RPE_{TR}, stimulated (TNF-α) (3) MT_{PRE}, post RPE_{TR}, unstimulated (TNF-α) (4) MT_{PRE}, post RPE_{TR}, stimulated (TNF-α) (5) MT_{POST}, pre RPE_{TR}, unstimulated (IL-8 + TNF-α) (6) MT_{POST}, post RPE_{TR}, stimulated (IL-8 + TNF-α).

3.12 Statistical Analysis

SPSS version 22 (SPSS inc., Chicago, IL.) was used to complete all statistical analyses. Prior to analysis, data was checked for normality using Q-Q plots and the Shapiro-Wilk Test. A paired samples t-test was used to compare training load variables, hydration status, $\dot{V}O_{2max}$ scores, 10 km time to completion, URTS scores and the RESTQ-76 Sport Questionnaire before and after the ITP. A two-way repeated measured analysis of variance (ANOVA) (trial (MT_{PRE} *x* MT_{POST}) x time (pre RPE_{TR} *x post* RPE_{TR})) was completed on HR, treadmill velocity, RPE, blood PMN concentration and IL-8 and TNF-α production by antigen-stimulated PMN. Where significant main effects were found, Bonferroni post-hoc corrections were used to examine the difference between individual trial and time conditions.

A calculation of Cohen's *d* effect size (ES) was used when statistical significance was not accepted. This was completed on 10 km time to completion results, URTS scores and the absolute exercise-induced change of TNF-α and IL-8 concentrations by antigen-stimulated PMN to measure the size of difference between the means (Cohen, 1988). Effect sizes of 0.2, 0.5 and 0.8 were considered small, medium and large, respectively (Durlack, 2009). The following equation was used to calculate ES:

$$d = \frac{Mean1 - Mean2}{SDpooled}$$

$$SDpooled = \sqrt{(SDgroup1^2 + SDgroup2^2)}/2$$

Cohen (1988)

All data was presented as mean ± standard deviation (SD). Statistical significance was accepted at $P < 0.05$. To clarify which work was completed during the duration of the Masters by Research, the results section is presented in two section.

3.13 Data Presentation

The data presented within Results will be divided into two sections. Chapter 4 will present data that was collected from the two participants (participant 9 and 10) who were recruited and analysed by Mr. Thorley during this Masters by Research project (~12 months). Chapter 5 will present a retrospective analysis of all 10 participants who completed the entire protocol (Figure 3.1), that is: eight participants recruited by Dr. Leal and Mr. Thorley plus two participants recruited solely by Mr. Thorley. This clarifies exactly which aspects of recruitment and data collection was completed by Mr. Thorley during the period required for the award of Masters by Research.

Chapter 4. Results (Two Participants)

4.1 Changes to Training Load

4.1.1 Total Exercise Duration

Total exercise duration was longer (885 ± 187 min, P = 0.171) during the ITP in both participants compared to the total exercise duration measured during the baseline period (350 ± 21 min) (Figure 4.1).

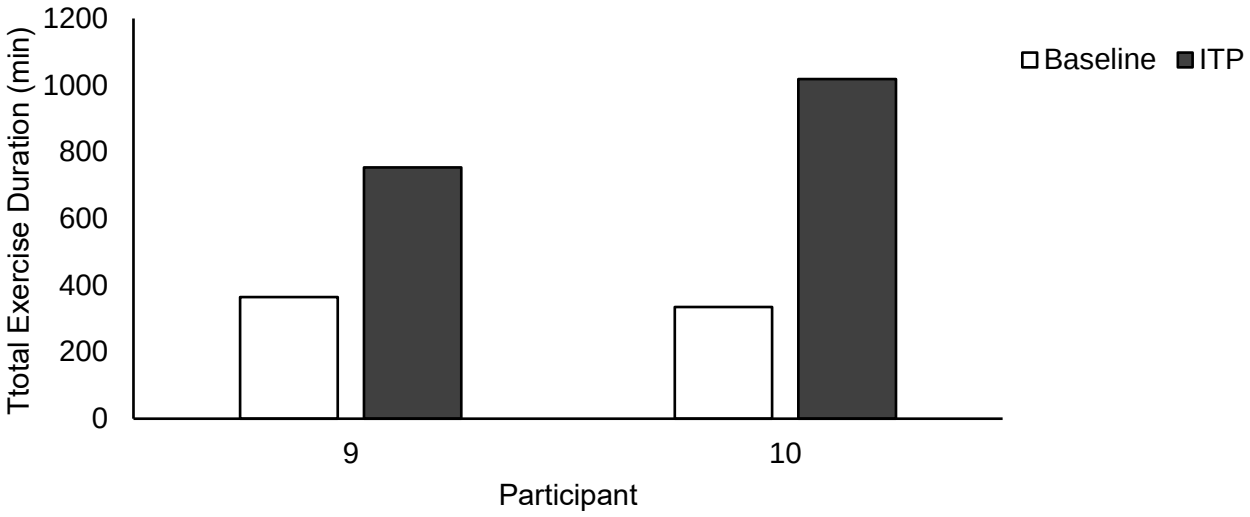

Figure 4.1: *Total exercise duration (min) measured during the ITP compared to the total exercise duration measured during the baseline period.*

4.1.2 Total Sum of TRIMP Scores

The total sum of TRIMP scores measured during the ITP was higher (1509 ± 426, P = 0.234) during the ITP in both participants compared to the sum of TRIMP scores measured during the baseline period (568 ± 88) (Figure 4.2)

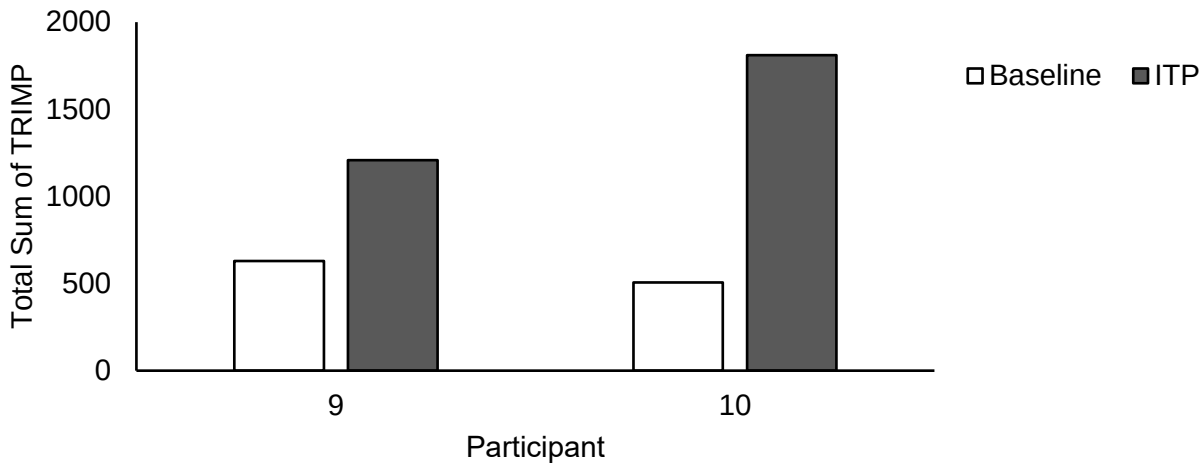

***Figure 4.2**: Sum of TRIMP scores measured during the ITP compared to the sum of TRIMP scores measured during the baseline period.*

4.2 Hydration Status

Urine osmolality was 105 ± 37 mOsmol.kg^{-1} higher at MT_{POST} (350 ± 198 mOsmol.kg^{-1}) when compared to the urine osmolality measured at MT_{PRE} (245 ± 162 mOsmol.kg^{-1}) (P = 0.751).

4.3 Physiological Responses to RPE_{TR}

4.3.1 Heart Rate Responses to RPE_{TR}

No trial effect (MT_{PRE} x MT_{POST}) was present in HR responses across the RPE_{TR} ($F_{1,\ 1}$ = 4.298, P = 0.286) (Figure 4.3). $HR_{average}$ of the RPE_{TR} was 152 ± 18 beats.min^{-1} during MT_{PRE} and 153 ± 24 beats.min^{-1} during MT_{POST}.

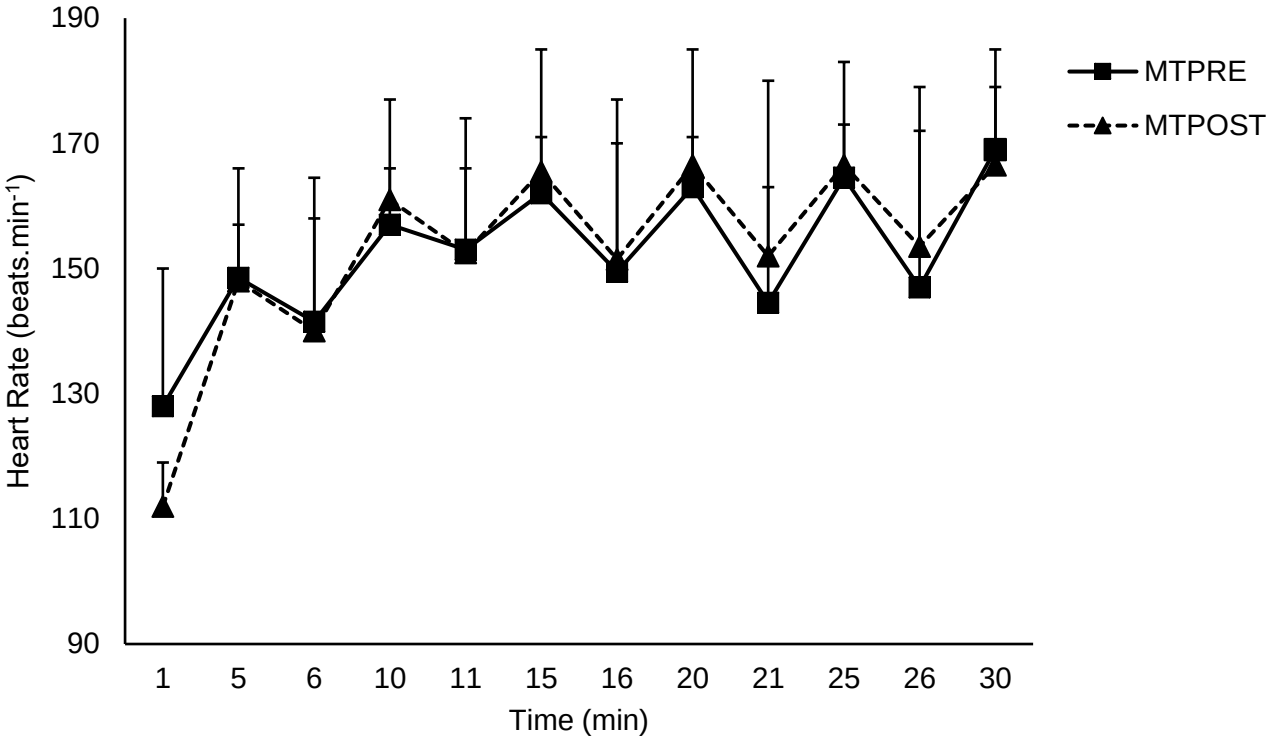

Figure 4.3: *HR responses (beats.min^{-1}) across the RPE_{TR} measured at MT_{PRE} and MT_{POST}.*

Values expressed as mean ± SD of 2 participants.

4.3.2 Treadmill Velocity Responses to RPE_{TR}

No trial effect (MT_{PRE} x MT_{POST}) was present in treadmill velocity responses across the RPE_{TR} ($F_{1,\ 1}$ = 1.993, P = 0.392) (Figure 4.4) Average treadmill velocity of the RPE_{TR} was 9.8 ± 2.2 km.h^{-1} at MT_{PRE} and 10.8 ± 2.8 km.h^{-1} at MT_{POST}

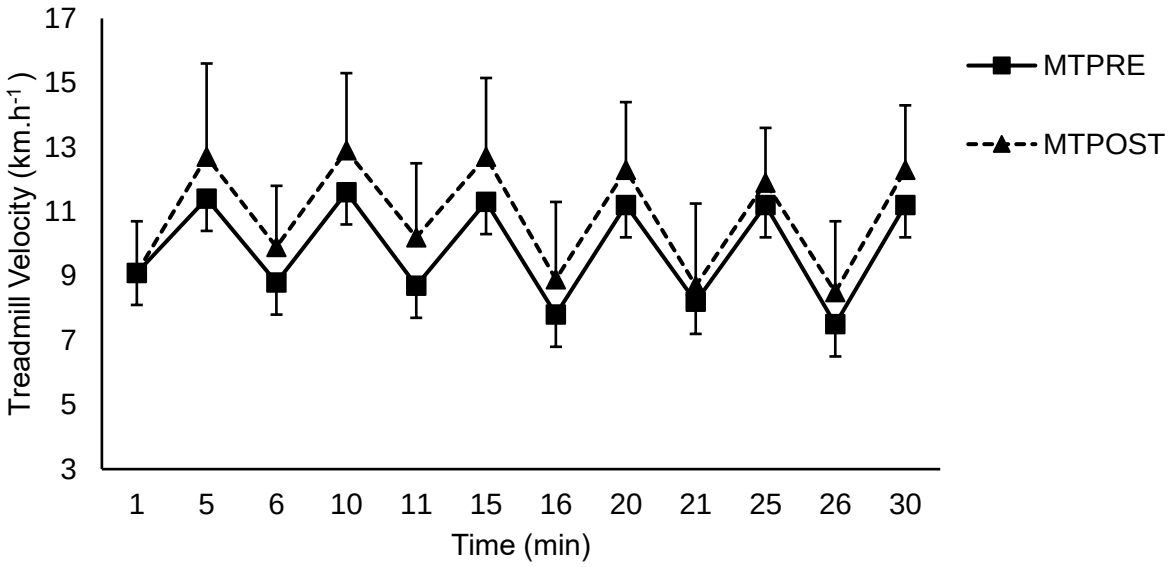

Figure 4.4: *Treadmill velocity (km.h^{-1}) measured across the RPE_{TR} at MT_{PRE} and $MT_{POST.}$*

Values expressed as mean ± SD of 2 participants.

4.4 Maximal Volume of Oxygen Uptake ($\dot{V}O_{2max}$)

Mean $\dot{V}O_{2max}$ values decreased by 1.5 ± 2.1 mL.kg^{-1}.min^{-1} at VO_{2POST} (54.5 ± 3.5 mL.kg^{-1}.min^{-1}) compared to VO_{2PRE} values (56 ± 1.4 mL.kg^{-1}.min^{-1}) (P = 0.5) (Figure 4.5).

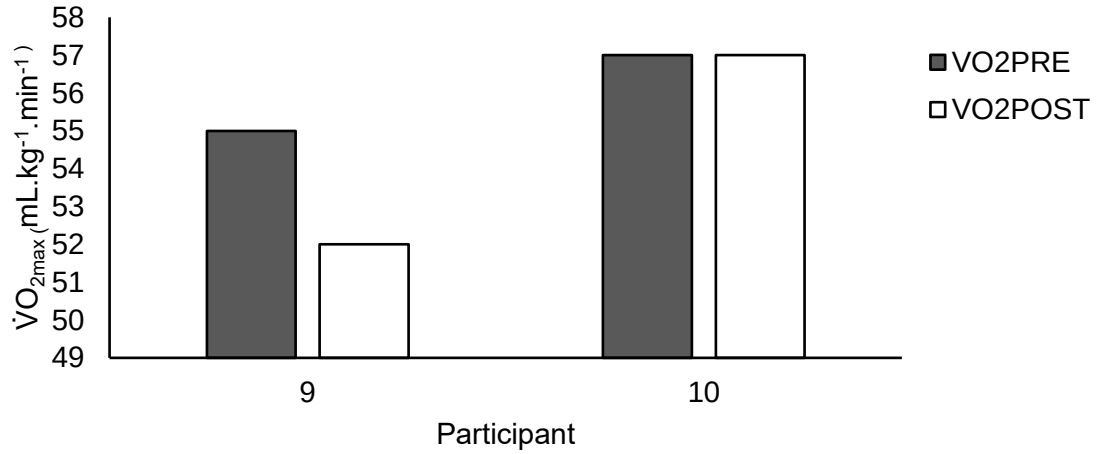

Figure 4.5: *Comparison of Participant 9 and 10's individual $\dot{V}O_{2max}$ values at VO_{2PRE} and VO_{2POST}*

4.5 Markers of Overtraining

4.5.1 Changes to 10 km Time Trial Time to Completion

The time to completion scores of the 10 km TT completed by participant 2 was 01:20 (mm:ss) longer at MT_{POST} (54:16) compared to MT_{PRE} (52:56) (Figure 4.6). Participant 9 did not complete this test.

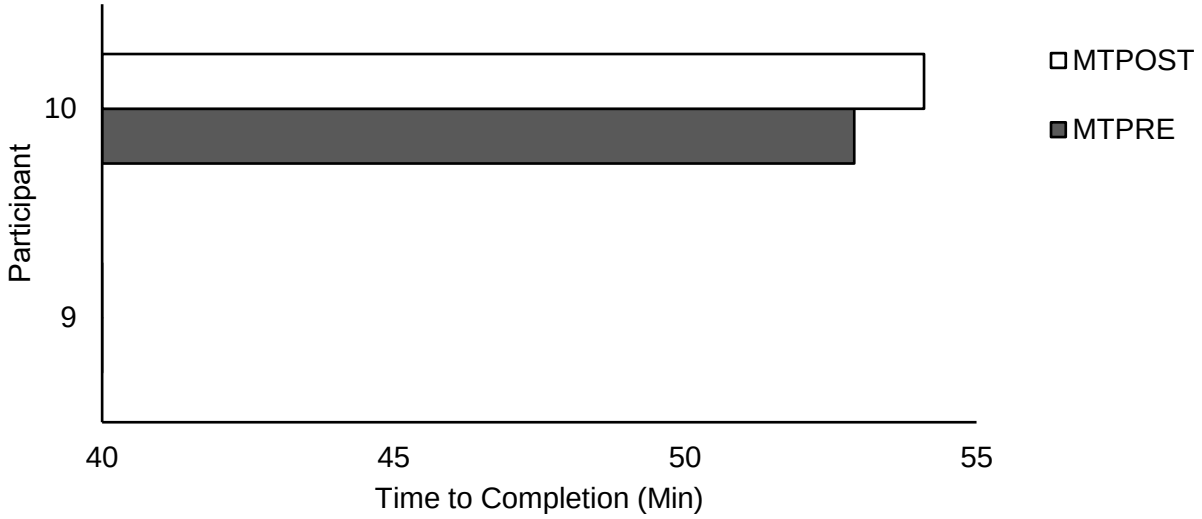

***Figure 4.6**: Time to completion (min) scores at MT_{PRE} and MT_{POST}.*

4.5.2 Changes in the Severity of Upper-respiratory Tract Symptoms

Statistical analysis could not be performed on this variable as participant 1 and 2 did not report any URTS at MT_{PRE} or MT_{POST}.

4.5.3 Changes to Perceived Stress and Recovery

The '*general stress'* scores increased by 0.1 ± 0.9 from MT_{PRE} (3.9 ± 0.9) to MT_{POST} (4 ± 0) (P = 0.951) (Figure 4.7). The 'sport stress' scores increased by 3.7 ± 0.5 from MT_{PRE} (3.2 ± 0.7) to MT_{POST} (6.9 ± 1.2) (P = 0.061). The '*general recovery'* scores increased by 0.1 ± 0.4 from MT_{PRE} (13.8 ± 3.1) to MT_{POST} (13.9 ± 3.5) (P = 0.795). The '*sport recovery'* scores decreased by 2.4 ± 1.5 from MT_{PRE} (15 ± 0.4) to MT_{POST} (11.7 ± 1.6) (P = 0.260).

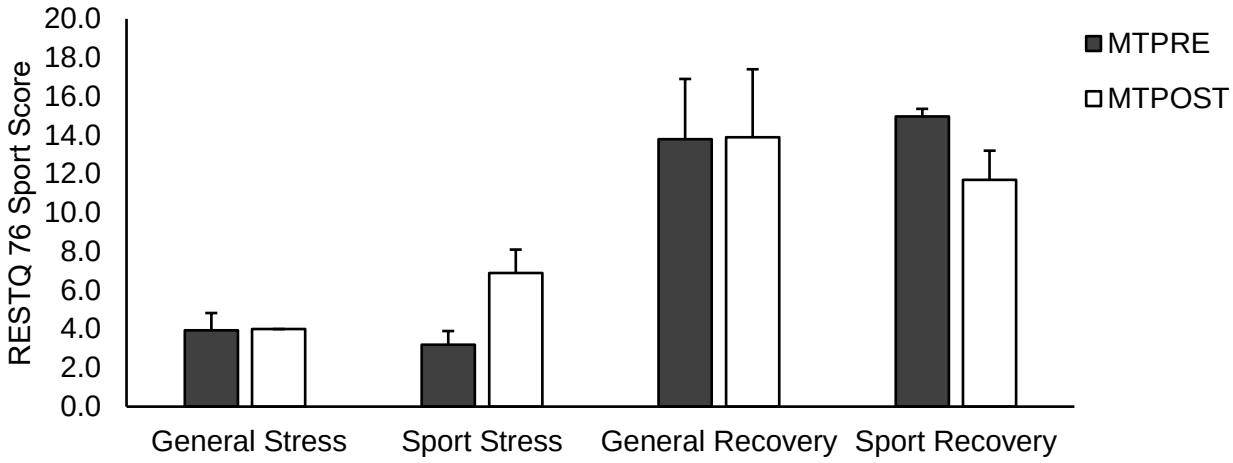

Figure 4.7*: Comparison of RESTQ 76 Sport scores measured at MT_{PRE} and MT_{POST}.*

Values expressed as mean ± SD of 2 participants.

4.6 Changes to Blood Neutrophil Concentration

No main effect for trial (MT_{PRE} x MT_{POST}) was present in the concentration of blood PMN ($F_{1,1}$ = 1.059, P = 0.491). Mean blood PMN concentration was 0.3 ± 0.4 cells $x10^6.mL^{-1}$ higher at MT_{POST} (2.0 ± 0.7 cells $x10^6.mL^{-1}$) than at MT_{PRE} (1.7 ± 0.3 cells $x10^6.mL^{-1}$). There was also no main effect for time (Pre RPE_{TR} x Post RPE_{TR}) ($F_{1,\ 1}$ = 6.547, p = 0.237). Mean blood PMN concentration was 0.3 ± 0.1 cells $x10^6.mL^{-1}$ higher at post RPE_{TR} (2 ± 0.6 MT_{PRE} cells $x10^6.mL^{-1}$) than at pre RPE_{TR} (resting) (1.7 ± 0.5 cells $x10^6.mL^{-1}$). No interaction effect of time over trial was present ($F_{1,1}$ = 0.648, P = 0.722) (Figure 4.8).

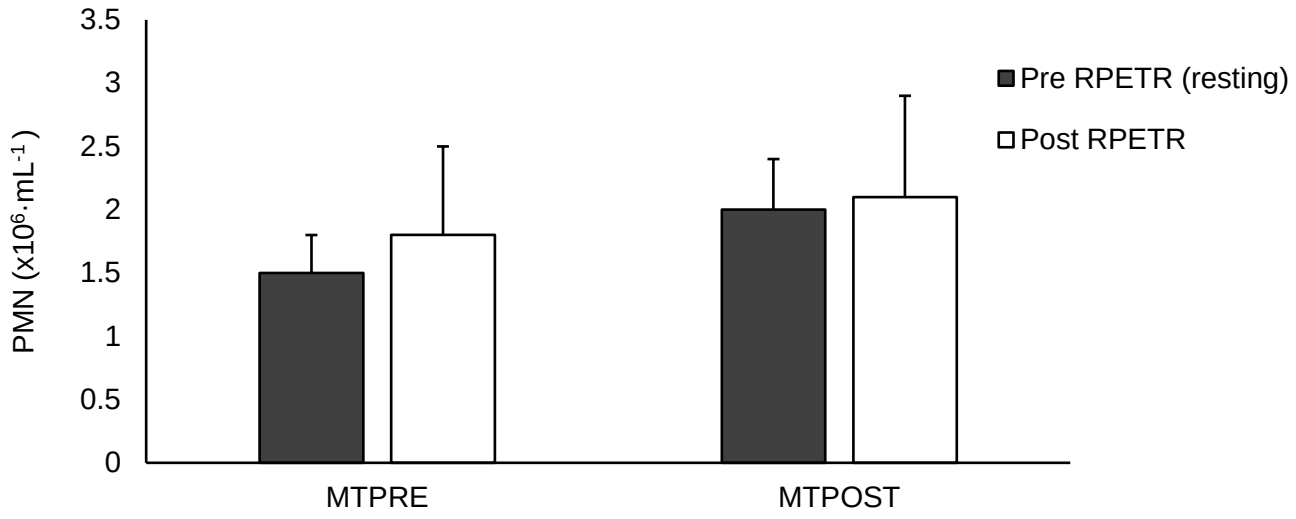

Figure 4.8: *Comparison of blood PMN concentration (cells x10^6.mL^{-1}) in response to the RPE_{TR} at MT_{PRE} and MT_{POST}.*

Values expressed as mean ± SD of 2 participants.

4.7 Tumor Necrosis Factor-α Production by Antigen-stimulated Neutrophils

No main effect for trial (MT_{PRE} x MT_{POST}) was present in measures of TNF-α concentration produced by antigen stimulated PMN ($F_{1, 1}$ = 3.974, P = 0.295). Mean TNF-α concentration was markedly lower at MT_{POST} (24 ± 5 pg.mL^{-1}) compared to MT_{PRE} (99 ± 43 pg.mL^{-1}). No significant main effect for time (Pre RPE_{TR} x Post RPE_{TR}) was reported ($F_{1, 1}$ = 30.822, P = 0.113). Mean TNF-α concentration was higher post RPE_{TR} (82 ± 21 pg.mL^{-1}) compared to pre RPE_{TR} (resting) (42 ± 28 pg.mL^{-1}). No significant interaction effect of time over trial was present ($F_{1, 1}$ = 4.921, P = 0.196) (Figure 4.9).

The absolute change of TNF-α concentration in response to the RPE_{TR} decreased from 39 ± 33 pg.mL^{-1} at MT_{PRE} (36% increase) to -12 ± 6 pg.mL^{-1} at MT_{POST} (40% decrease). (Figure 4.10).

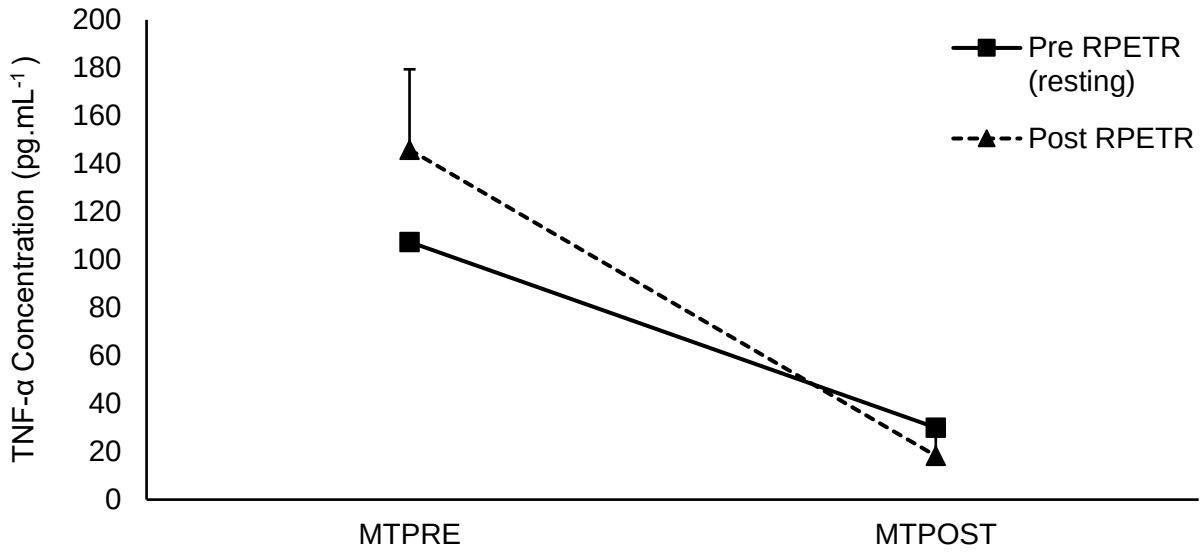

Figure 4.9: *TNF-α concentration (pg.mL^{-1}) produced by antigen-stimulated PMN in response to the RPE_{TR} at MT_{PRE} and $MT_{POST.}$*

Values expressed as mean ± SD of 2 participants.

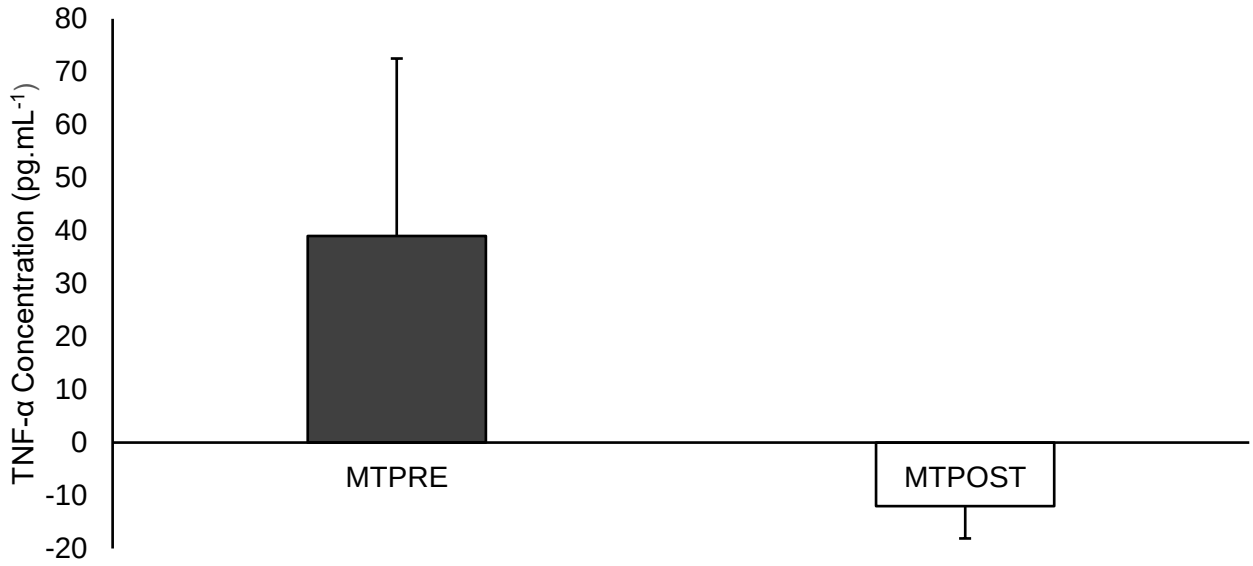

Figure 4.10: *Comparison of the absolute exercise (RPE_{TR})-induced change in TNF-α concentration (pg.mL^{-1}) at MT_{PRE} and MT_{POST}.*

All values were expressed as mean ± SD of 2 participants.

4.8 Tumor Necrosis Factor-α Production by Non-stimulated Neutrophils

Statistical analysis could not be performed on this variable due to participant 2 having undetected levels (< 4 $pg.mL^{-1}$) of TNF-α concentration at all time points. At $MT_{PRE,}$ participant 1's TNF-α production by non-stimulated PMN decreased from 19 $pg.mL^{-1}$ pre RPE_{TR} to 11 $pg.mL^{-1}$ post RPE_{TR}. A similar decrease was observed at MT_{POST}, with participant 1's TNF-α production by non-stimulated PMN decreasing from 7 $pg.mL^{-1}$ pre RPE_{TR} to 5 $pg.mL^{-1}$ post RPE_{TR}.

4.9 Interleukin-8 Production by Antigen-stimulated Neutrophils

No main effect for trial (MT_{PRE} x MT_{POST}) was present in measures of IL-8 concentration produced by antigen-stimulated PMN ($F_{1, 1} = 0.529$, $P = 0.6$). Mean IL-8 concentration was lower at MT_{POST} (831 ± 340 $pg.mL^{-1}$) compared to MT_{PRE} (3835 ± 3788 $pg.mL^{-1}$). No significant main effect for time (Pre RPE_{TR} x Post RPE_{TR}) was reported ($F_{1, 1} = 1.167$, $P = 0.475$). Mean IL-8 concentration was higher post RPE_{TR} (3417 ± 2727 $pg.mL^{-1}$) compared to pre RPE_{TR} (resting) (1249 ± 720 $pg.mL^{-1}$). No significant interaction effect of time over trial was present ($F_{1, 1} = 0.950$, $P = 0.508$) (Figure 4.11).

The absolute change of IL-8 concentration in response to the RPE_{TR} decreased from 3235 ± 6097 $pg.mL^{-1}$ at MT_{PRE} (109% increase) to 265 ± 38 $pg.mL^{-1}$ at MT_{POST} (26% increase) (Figure 4.12).

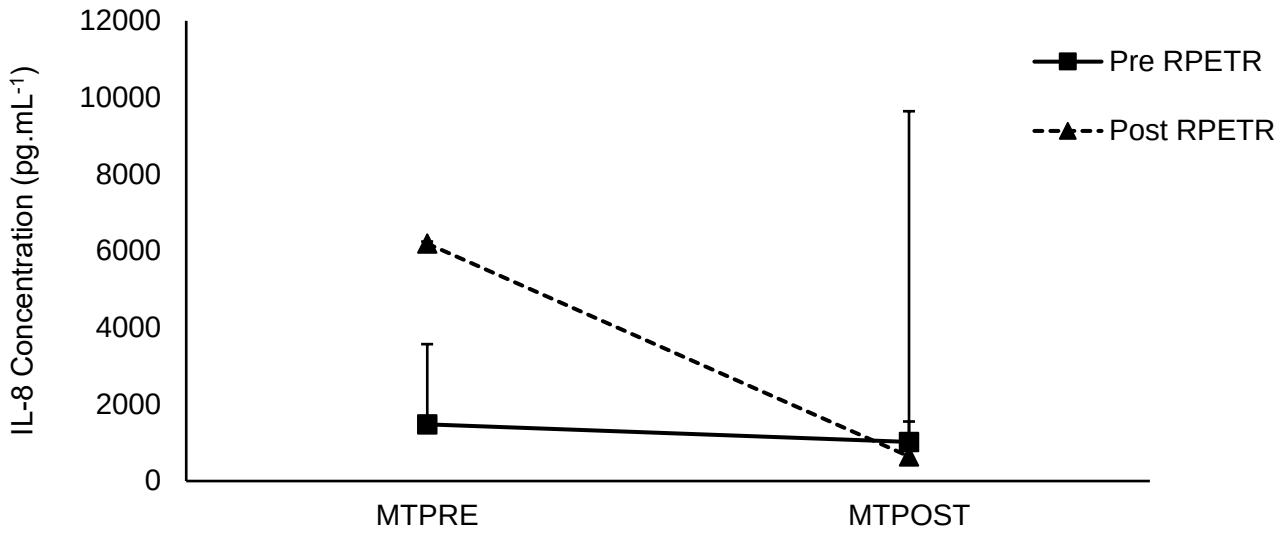

Figure 4.11: *IL-8 concentration (pg.mL^{-1}) produced by antigen-stimulated PMN in response to the RPE_{TR} at MT_{PRE} and MT_{POST}.*

Values expressed as mean ± SD of 2 participants.

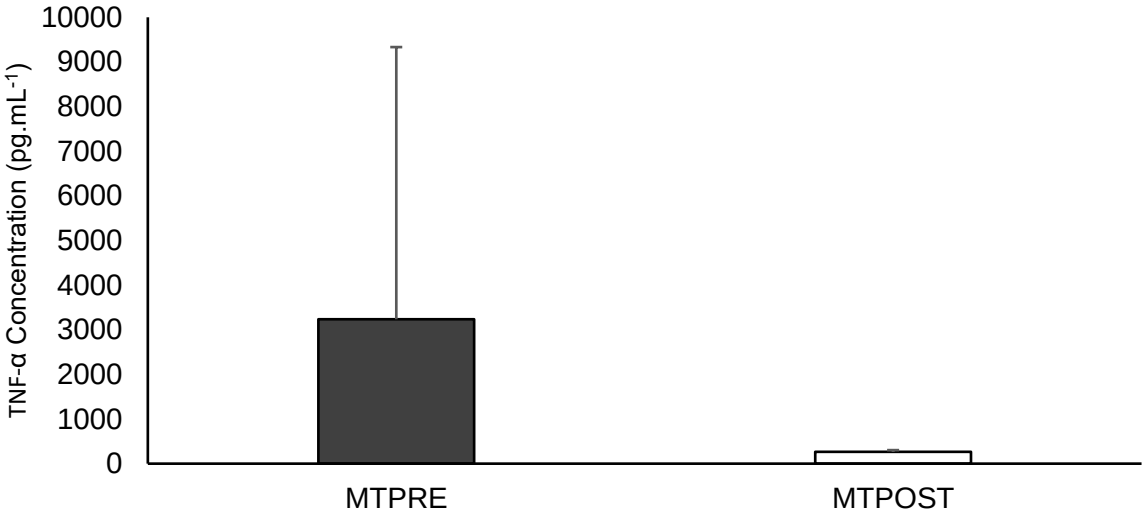

Figure 4.12: *Comparison of the absolute exercise (RPE_{TR})-induced change in IL-8 concentration (pg.mL^{-1}) at MT_{PRE} and MT_{POST}.*

All values were expressed as mean ± SD of 2 participants.

4.10 Interleukin-8 Production by Non-stimulated Neutrophils

No main effect for trial (MT_{PRE} x MT_{POST}) was present in measures of IL-8 concentration produced by non-stimulated PMN ($F_{1,1} = 1.479$, $P = 0.438$). Mean IL-8 concentration was lower at MT_{POST} (153 ± 20 $pg.mL^{-1}$) compared to MT_{PRE} (679 ± 412 $pg.mL^{-1}$). No significant main effect for time (Pre RPE_{TR} x Post RPE_{TR}) was found ($F_{1,1} = 22.281$, $P = 0.133$). Mean IL-8 concentration was higher post RPE_{TR} (501 ± 178 $pg.mL^{-1}$) compared to pre RPE_{TR} (resting) (332 ± 213 $pg.mL^{-1}$). No significant interaction effect of time over trial was present ($F_{1,1} = 0.149$, $P = 0.765$) (Figure 4.13).

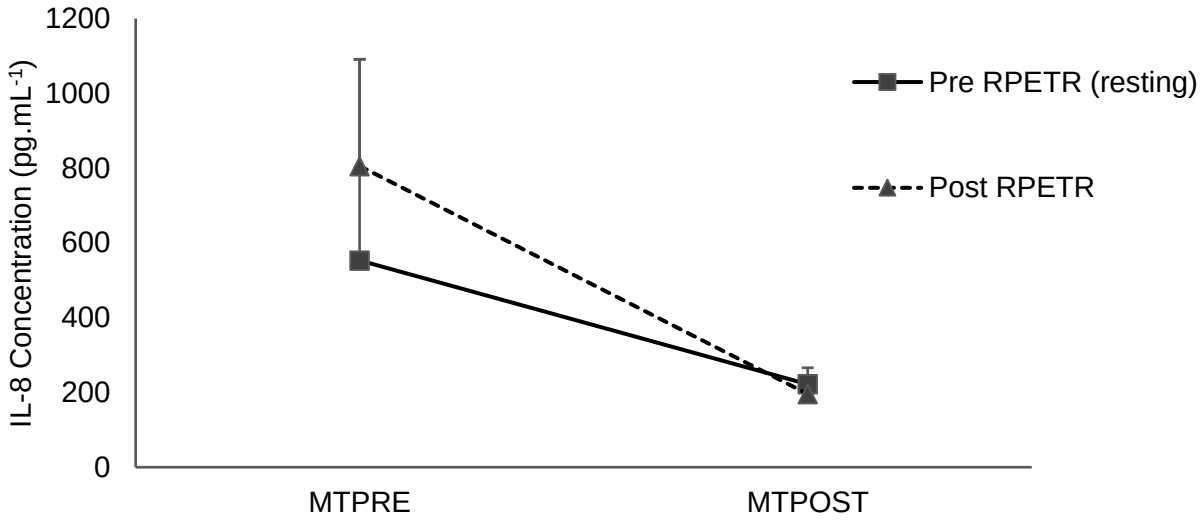

Figure 4.13: *IL-8 concentration ($pg.mL^{-1}$) produced by non-stimulated PMN in response to the RPE_{TR} at MT_{PRE} and MT_{POST}.*

Values expressed as mean ± SD of 2 participants.

Chapter 5. Results (Retrospective Analysis of Ten Participants)

5.1 Changes to Training Load

5.1.1 Total Exercise Duration

Participant 2 and 6 did not record their exercise duration during the baseline period. Total exercise duration was significantly longer during the ITP (984 ± 356 min, $P < 0.001$) in all 10 participants compared to the total exercise duration measured during the baseline period (415 ± 418 min) (Figure 5.1) thus all recorded participants increased their training load.

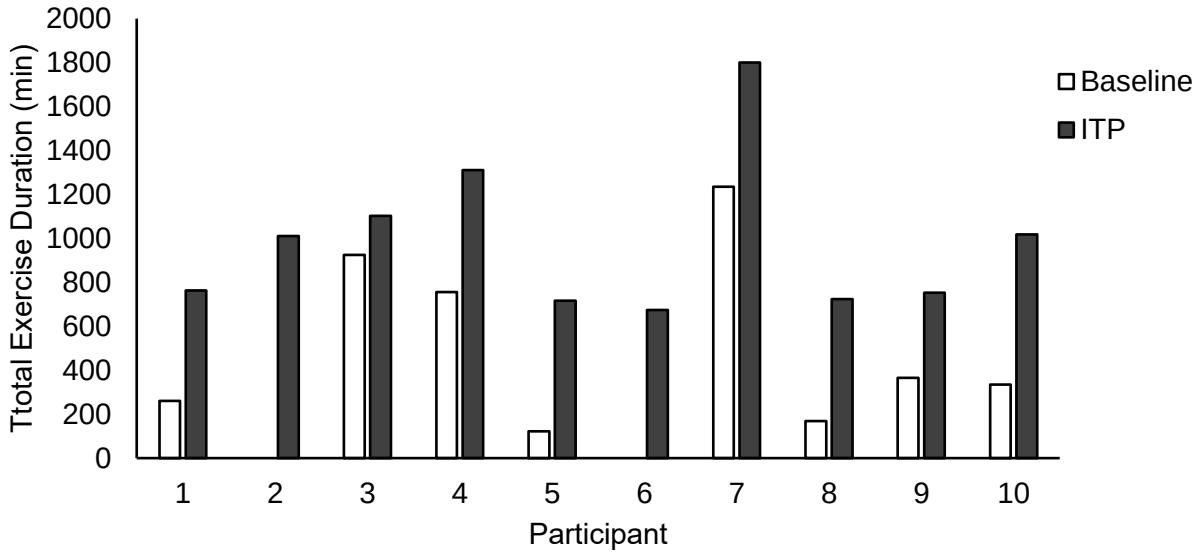

Figure 5.1: *Total exercise duration (min) measured during the ITP compared to the total exercise duration measured during the baseline period.*

5.1.2 Total Sum of TRIMP Scores

Participant 2 and 6 did not record their HR and exercise duration during the baseline period, therefore their TRIMP scores for this period could not be established. The total sum of TRIMP scores measured during the ITP was higher (1535 ± 580, $P < 0.001$) in all 10 participants compared to the sum of TRIMP scores measured during the baseline period (597 ± 580) (Figure 5.2).

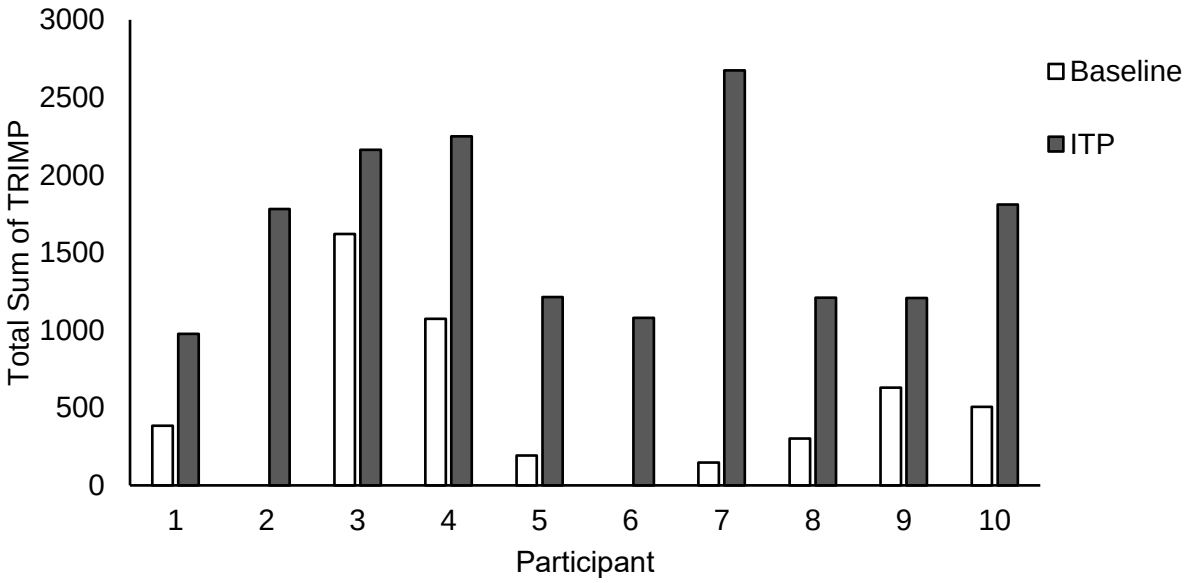

Figure 5.2*: Sum of TRIMP scores measured during the ITP compared to the sum of TRIMP scores measured during the baseline period.*

5.2 Hydration Status

There was no difference in the mean urine osmolality measured pre RPE_{TR} at MT_{PRE} (285 ± 235 $mOsmol.kg^{-1}$) compared to the urine osmolality measured pre RPE_{TR} at MT_{POST} (404 ± 271 $mOsmol.kg^{-1}$) ($P = 0.34$).

5.3 Physiological Responses to RPE_{TR}

5.3.1 Heart Rate Responses to RPE_{TR}

No trial effect (MT_{PRE} x MT_{POST}) was present in HR responses across the RPE_{TR} ($F_{1, 9} = 1.204$, $P = 0.29$) (Figure 5.3). $HR_{average}$ of the RPE_{TR} was 150 ± 4 beats.min^{-1} during MT_{PRE} and 146 ± 5 beats.min^{-1} during MT_{POST}.

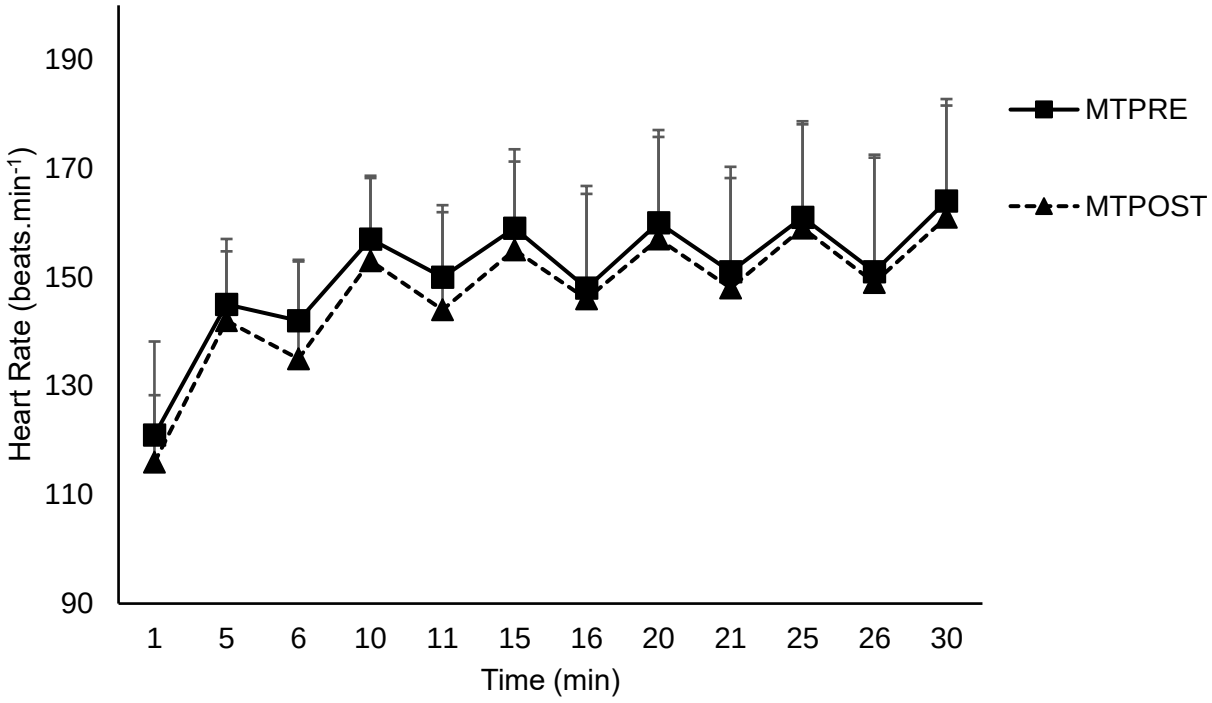

Figure 5.3: *HR responses (beats.min^{-1}) across the RPE_{TR} measured at MT_{PRE} and MT_{POST}.*

Values expressed as mean ± SD of 10 participants.

5.3.2 Treadmill Velocity Responses to RPE_{TR}

No trial effect (MT_{PRE} x MT_{POST}) was present in treadmill velocity responses across the RPE_{TR} ($F_{1, 9} = 0.659$, $P = 0.773$) (Figure 5.4). Average treadmill velocity of the RPE_{TR} was 10.6 ± 0.7 km.h^{-1} at MT_{PRE} and 11 ± 0.7 km.h^{-1} at MT_{POST}.

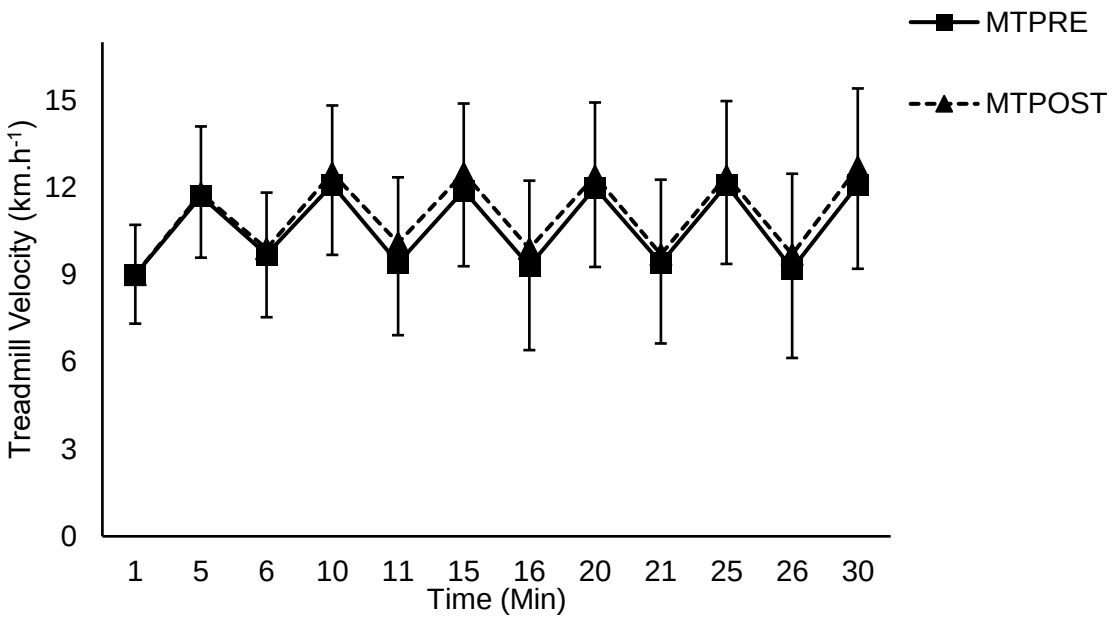

Figure 5.4: *Treadmill velocity (km.h^{-1}) measured across the RPE_{TR} at MT_{PRE} and $MT_{POST.}$*

Values expressed as mean ± SD of 10 participants.

5.4 Maximal Volume of Oxygen Uptake ($\dot{V}O_{2max}$)

There was no difference between $\dot{V}O_{2max}$ values (58.1 ± 5.2 mL.kg^{-1}.min^{-1}) at VO_{2PRE} and VO_{2POST} (57.7 ± 6.1 mL.kg^{-1}.min^{-1}) (P = 0.696) (Figure 5.5). All 10 participants $\dot{V}O_{2max}$ varied in response following the ITP (Figure 5.6).

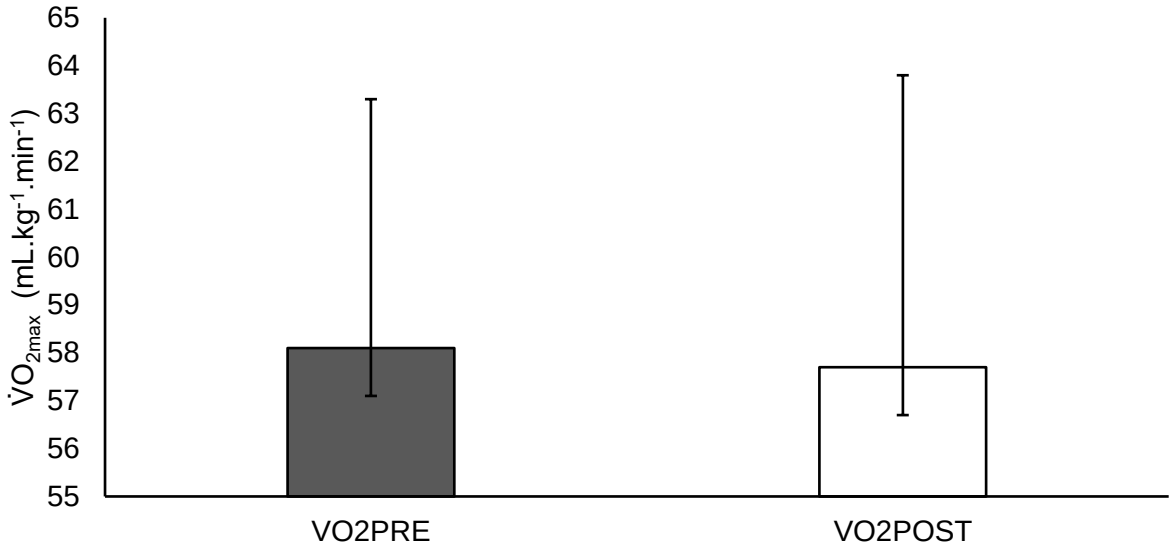

Figure 5.5: *Comparison of $\dot{V}O_{2max}$ values at VO_{2PRE} and VO_{2POST}*

Values expressed as mean ± SD of 10 participants.

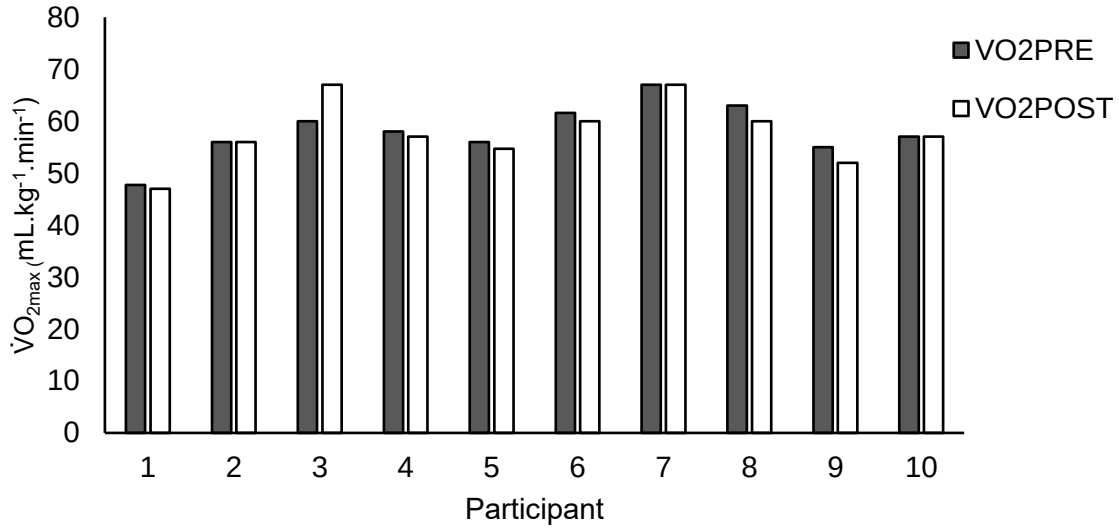

Figure 5.6*: Comparison of individual $\dot{V}O_{2max}$ values at VO_{2PRE} and VO_{2POST}.*

5.5 Markers of Overtraining

5.5.1 Changes to 10 km Time Trial Time to Completion

Participant 1, 4, and 9 did not complete the 10 km TT. The time to completion scores of the 10 km TT measured at MT_{PRE} (46:20 ± 05:39 (mm:ss)) and MT_{POST} (47:31 ± 05:12) were not significantly different (P = 0.497). A small ES (*d* = 0.32) increase in completion time was evident at MT_{POST}. Absolute change between MT_{PRE} and MT_{POST} was 01:11 ± 00:27 (Figure 5.7). Five out of the seven measured participants had slower time to completion scores at MT_{POST} (Figure 5.8).

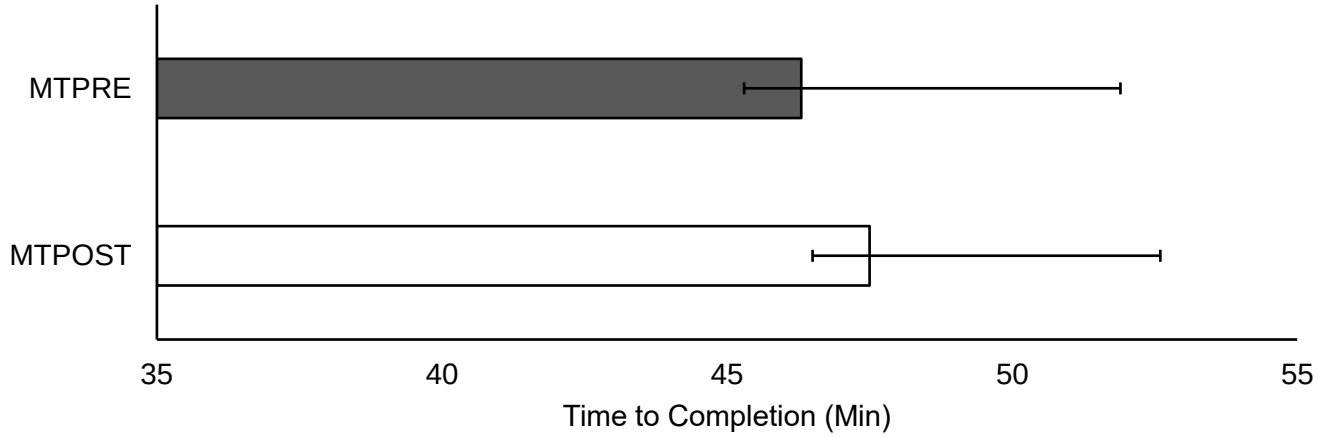

Figure 5.7: *Comparison of the 10 km TT time to completion (min) scores at MT_{PRE} and MT_{POST}.*

Values expressed as mean ± SD of 7 participants.

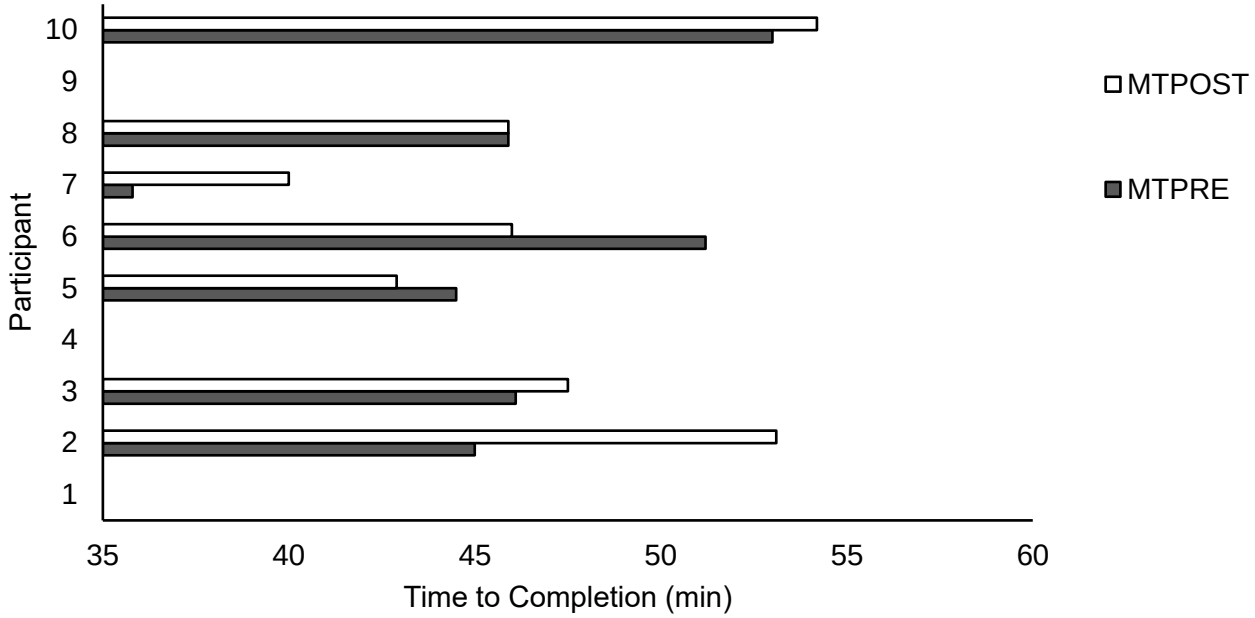

***Figure 5.8**: Comparison of individual time to completion (min) scores at MT_{PRE} and MT_{POST}.*

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5.5.2 Changes in the Severity of Upper-respiratory Tract Symptoms

The sum of URTS scores reported by participants increased in six of the ten participants at MT_{POST} compared to MT_{PRE} (Figure 5.9). No significant differences were observed between the URTS subscales '*sneezing*', '*malaise*', '*nasal discharge and obstruction'*, '*sore throat'*, '*cough*', '*ear ache'*, '*hoarseness'*, '*fever*', '*chilliness*' and '*joint aches and pains'* measured at MT_{PRE} and MT_{POST} ($P > 0.05$). The '*headache*' subscale significantly increased at MT_{POST} ($P < 0.05$) (Figure 5.10). Large ES were found between mean malaise ($d = 0.90$), nasal obstruction ($d = 1.12$), joint aches and pain ($d = 1.10$) and nasal discharge ($d = 0.89$) scores measured at MT_{PRE} and MT_{POST}.

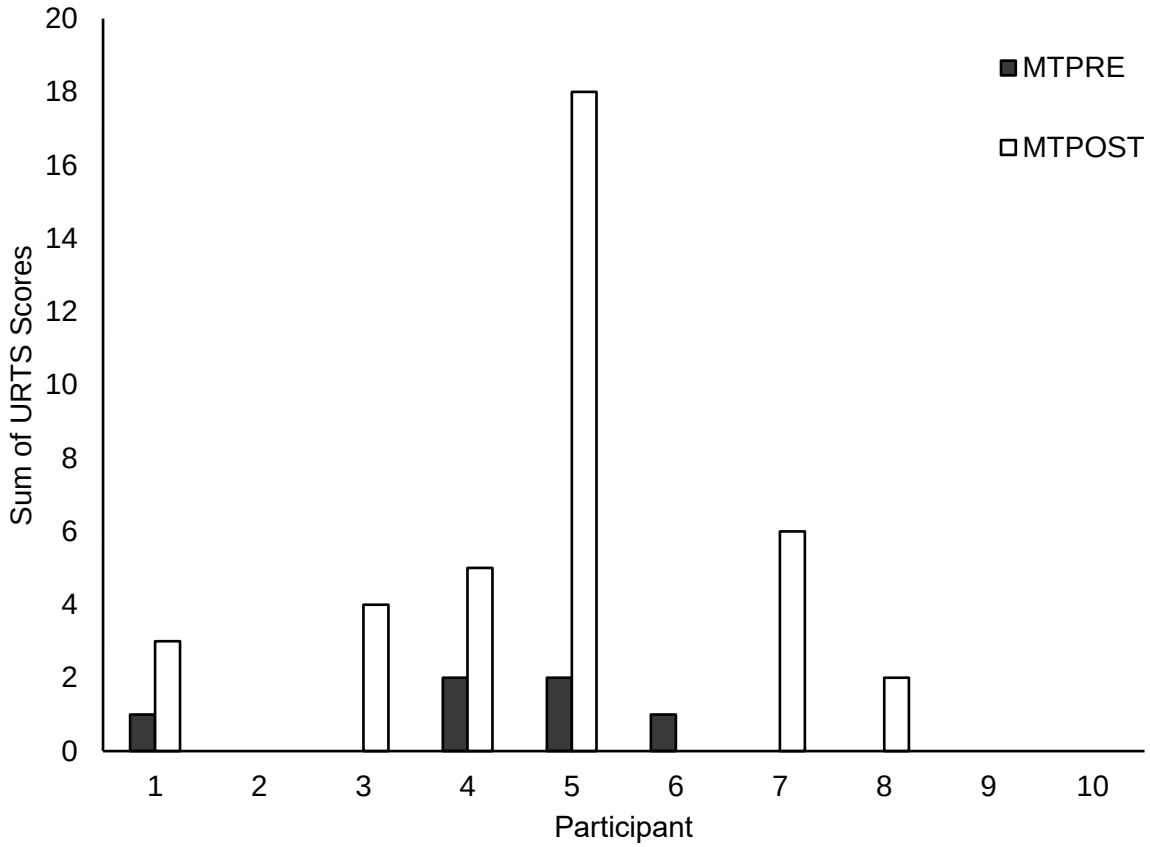

Figure 5.9: *Comparison of the total sum of URTS scores reported by participants at MT_{PRE} and MT_{POST}. Participant 2, 9, and 10 did not report any URTS at either MT_{PRE} and MT_{POST}.*

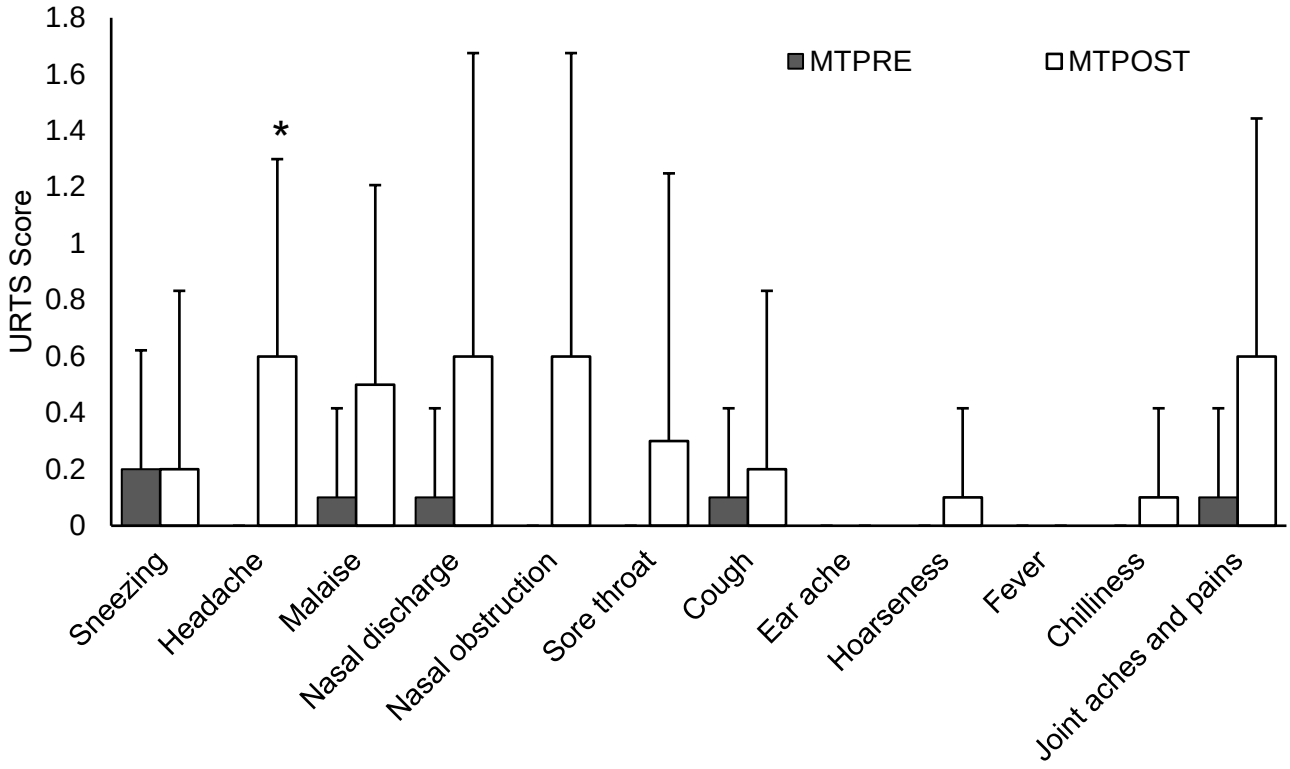

Figure 5.10: *URTS scores reported by participants for each subscale at MT_{PRE} and MT_{POST}. Participants did not report any incidences of ear ache or fever at MT_{PRE} or MT_{POST}. * = significantly different to MT_{PRE} ($p < 0.05$).*

Values expressed as mean ± SD of 10 participants.

5.5.3 Changes to Perceived Stress and Recovery

The mean '*general stress*' (P = 0.04) and '*sport stress*' (P = 0.01) scores were significantly elevated at MT_{POST} compared to MT_{PRE} (Figure 5.11). At MT_{POST}, seven participants had an increased '***general stress'*** score (Figure 5.12) and nine participants had an increased '***sport stress'*** score (Figure 5.13). The 'fitness *injury*' subscale within the '***sport stress'*** scale was significantly higher at MT_{POST} (P = 0.005) (Figure 5.14). No significant differences were observed when comparing the '*general recovery*' and '*sport recovery*' scales between MT_{PRE} and MT_{POST} (P = > 0.05).

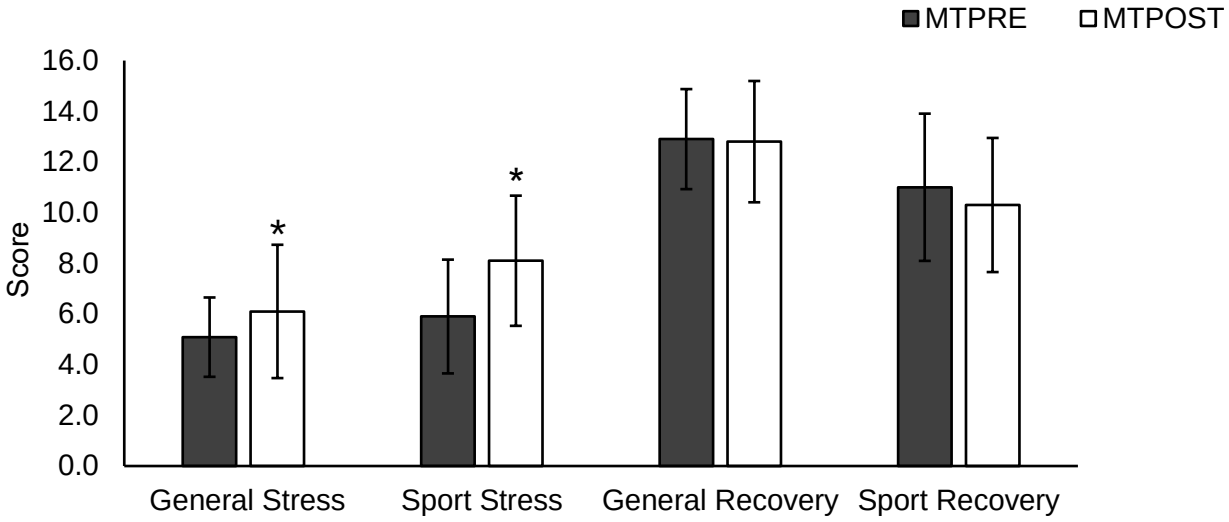

Figure 5.11: *Comparison of RESTQ-76 Sport scores measured at MT_{PRE} and MT_{POST}. * = significantly different to MT_{PRE} ($p < 0.05$).*

All values expressed as mean ± SD of 10 participants.

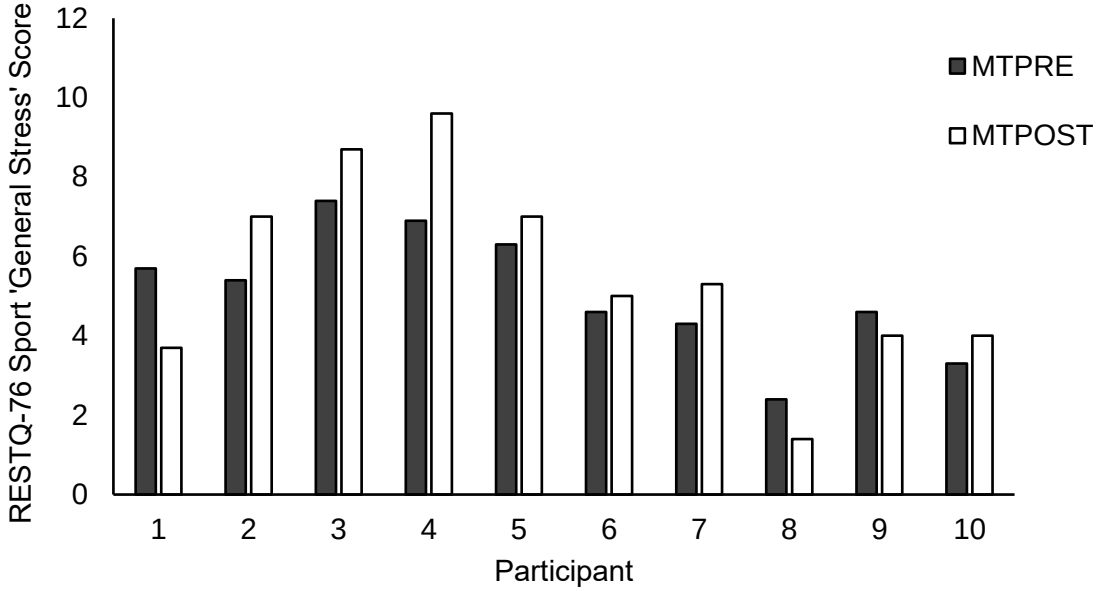

Figure 5.12: *Comparison of individual RESTQ-76 Sport 'General Stress' scores at MT_{PRE} and MT_{POST}.*

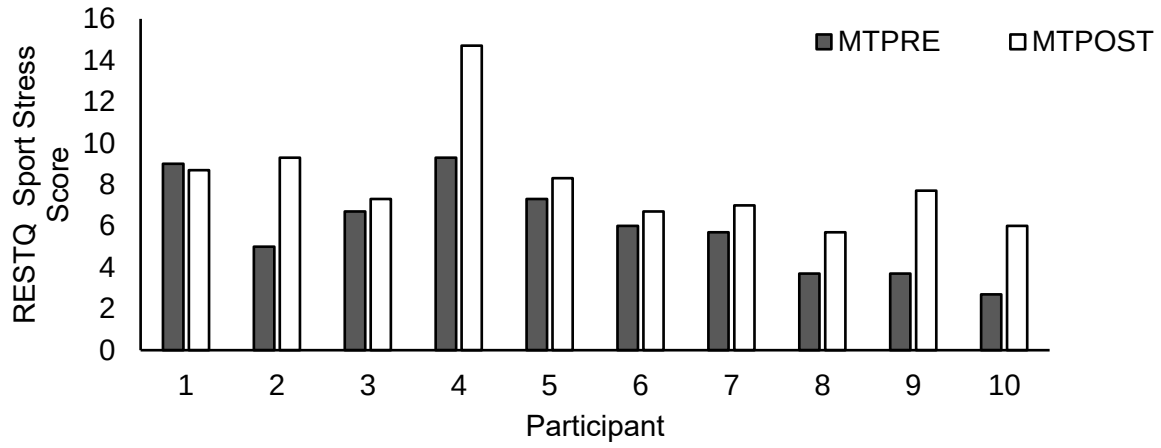

***Figure 5.13**: Comparison of individual RESTQ-76 Sport 'Sport Stress' scores at MT_{PRE} and MT_{POST}.*

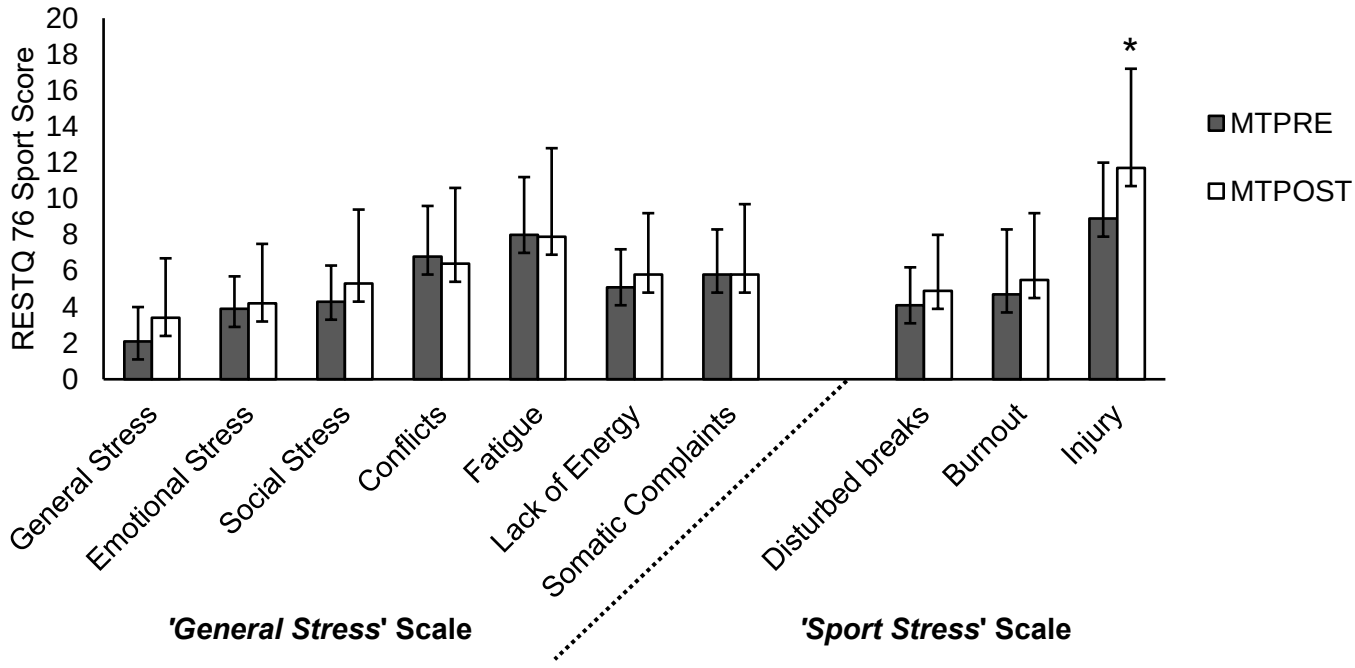

***Figure 5.14:** Comparison of each subscale within the RESTQ 76 Sport 'General Stress' and 'Sport Stress' scales at MT_{PRE} and MT_{POST}. * = significantly different from MT_{PRE} ($P < 0.05$).*

All values expressed as mean ± SD of 10 participants.

5.6 Changes to Blood Neutrophil Concentration

No main effect for trial (MT_{PRE} x MT_{POST}) was present in the concentration of blood PMN ($F_{1,9}$ = 0.461, P = 0.51) between MT_{PRE} (2.0 ± 0.1 cells $x10^6.mL^{-1}$) and MT_{POST} (2.1 ± 0.2 cells $x10^6.mL^{-1}$). There was however a significant main effect for time (Pre RPE_{TR} x Post RPE_{TR}) ($F_{1, 9}$ = 15.656, p = < 0.01)

During MT_{PRE}, blood PMN concentration significantly rose from 1.8 ± 0.5 cells $x10^6.mL^{-1}$ pre RPE_{TR} to 2.1 ± 0.5 cells $x10^6.mL^{-1}$ post RPE_{TR} (P = 0.02). During MT_{POST}, blood PMN concentration significantly rose from 1.9 ± 0.6 cells $x10^6.mL^{-1}$ pre RPE_{TR} to 2.3 ± 0.6 cells $x10^6.mL^{-1}$ post RPE_{TR} (P = 0.016) (Figure 5.15). No significant interaction effect of trial over time was present ($F_{1,9}$ = 0.447, P = 0.521).

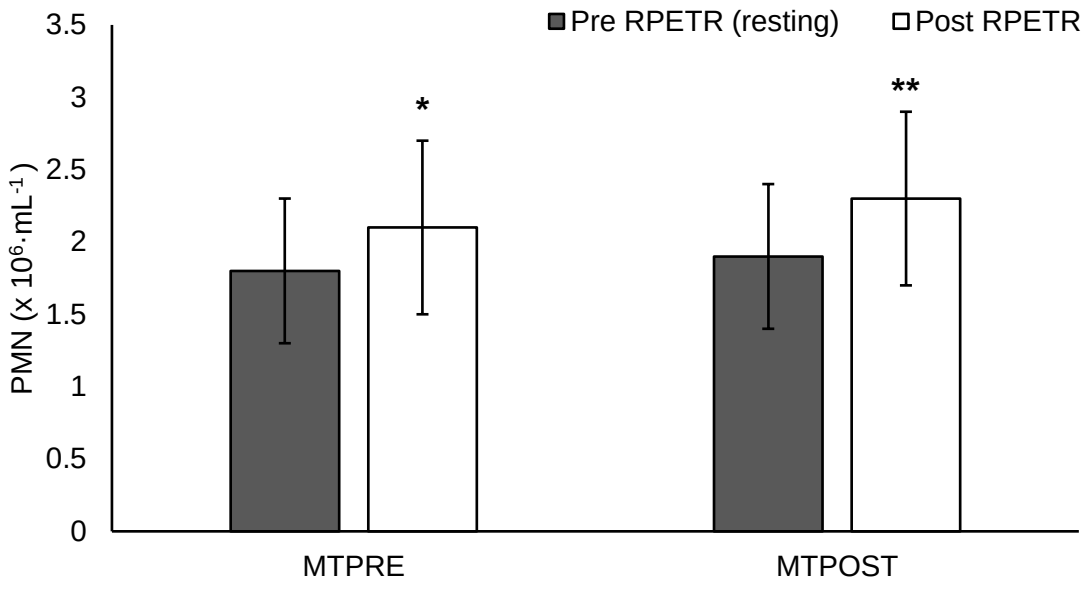

Figure 5.15: *Comparison of blood PMN concentration (cells $x10^6.mL^{-1}$) in response to the RPE_{TR} at MT_{PRE} and MT_{POST}. * = Significantly different to pre RPE_{TR} at MT_{PRE} ; ** = Significantly different to pre RPE_{TR} at MT_{POST} (P < 0.05).*

Values expressed as mean ± SD of 10 participants.

5.7 Tumor Necrosis Factor-α Production by Antigen-stimulated Neutrophils

No main effect for trial (MT_{PRE} x MT_{POST}) was present in measures of TNF-α concentration produced by antigen-stimulated PMN ($F_{1, 6} = 4.727$, $P = 0.073$). Mean TNF-α concentrations were lower at MT_{POST} (107 ± 35 $pg.mL^{-1}$) compared to MT_{PRE} (183 ± 40 $pg.mL^{-1}$). There was however a significant main effect for time (Pre RPE_{TR} x Post RPE_{TR}) ($F_{1, 6} = 10.999$, $P = 0.016$).

In response to the RPE_{TR} completed at MT_{PRE}, TNF-α concentration increased from 22 ± 35 $pg.mL^{-1}$ pre RPE_{TR} (resting) to 356 ± 206 $pg.mL^{-1}$ post RPE_{TR} ($P = 0.004$). In response to the RPE_{TR} completed at MT_{POST}, TNF-α concentration similarly increased from 28 ± 25 $pg.mL^{-1}$ pre RPE_{TR} (resting) to 162 ± 173 $pg.mL^{-1}$ post RPE_{TR} ($P = 0.047$) (Figure 5.16). No significant interaction effect of trial over time was present ($F_{1, 6} = 5.433$, $P = 0.058$).

The absolute increase in TNF-α concentration in response to the RPE_{TR} decreased from 317 ± 232 $pg.mL^{-1}$ at MT_{PRE} (789% increase) to 170 ± 191 $pg.mL^{-1}$ (639% increase) at MT_{POST} ($P = 0.056$; $d = 1.20$) (Figure 5.17). Individual TNF-α concentrations varied per individual, with concentrations ranging from 1 – 541 $pg.mL^{-1}$.

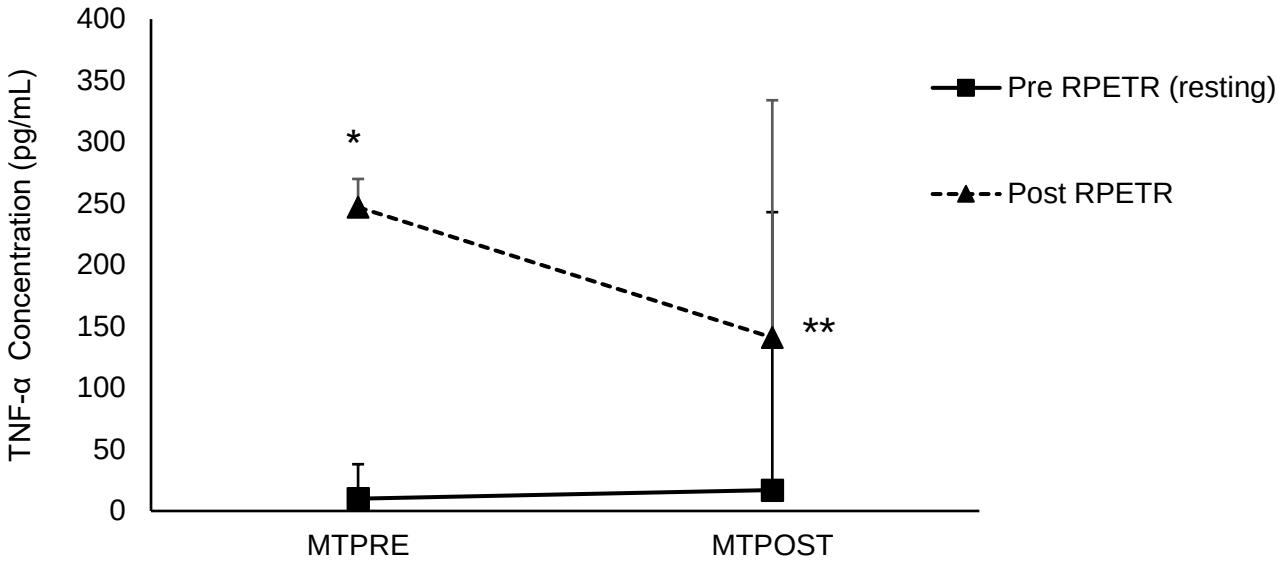

Figure 5.16: *TNF-α concentration (pg.mL^{-1}) produced by antigen-stimulated PMN in response to the RPE_{TR} at MT_{PRE} and MT_{POST}. * = significantly different to pre RPE_{TR} at MT_{PRE}; ** = significantly different to pre RPE_{TR} at MT_{POST} ($P < 0.05$).*

All values were expressed as mean ± SD of 10 participants.

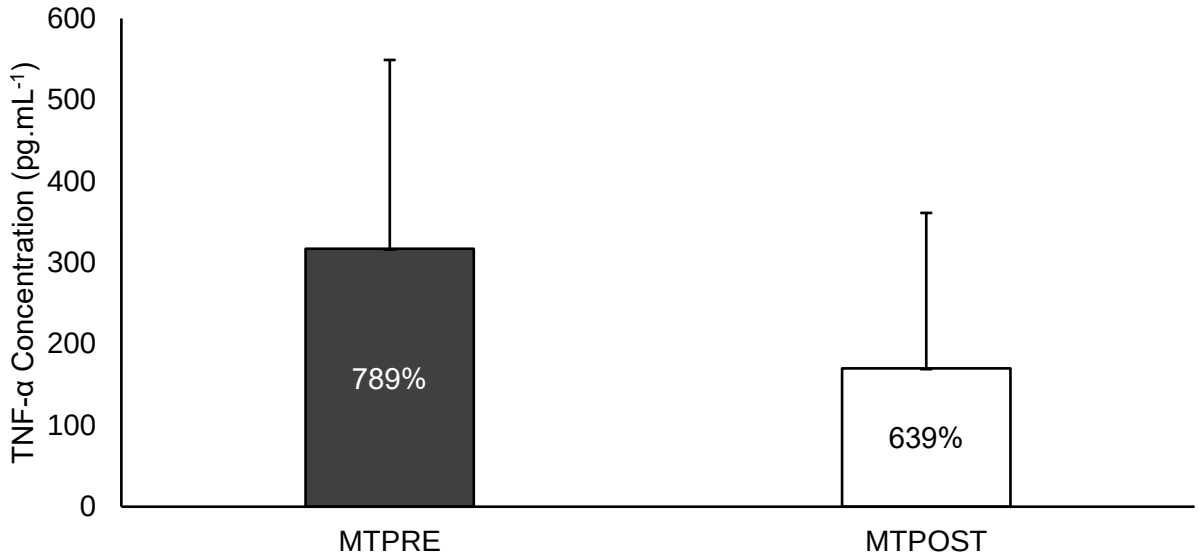

Figure 5.17: *Comparison of the absolute exercise (RPE_{TR})-induced increase in TNF-α concentration (pg.mL^{-1}) at MT_{PRE} and MT_{POST}.*

All values were expressed as mean ± SD of 10 participants..

5.8 Tumor Necrosis Factor-α Production by Non-stimulated Neutrophils

No main effect for trial (MT_{PRE} x MT_{POST}) was present in measures of TNF-α concentration produced by non-stimulated PMN ($F_{1, 3} = 1.634$, $P = 0.291$). Mean TNF-α concentration was lower at MT_{PRE} (67 ± 58 $pg.mL^{-1}$) compared to MT_{POST} (88 ± 60 $pg.mL^{-1}$). Additionally, no main effect for time (Pre RPE_{TR} x Post RPE_{TR}) was observed ($F_{1, 3} = 1.223$, $p = 0.349$). Mean TNF-α concentration was greater post RPE_{TR} (138 ± 113 $pg.mL^{-1}$) compared to pre RPE_{TR} (17 ± 5 $pg.mL^{-1}$). No significant interaction effect of trial over time was present ($F_{1, 3} = 4.480$, $P = 0.125$) (Figure 5.18).

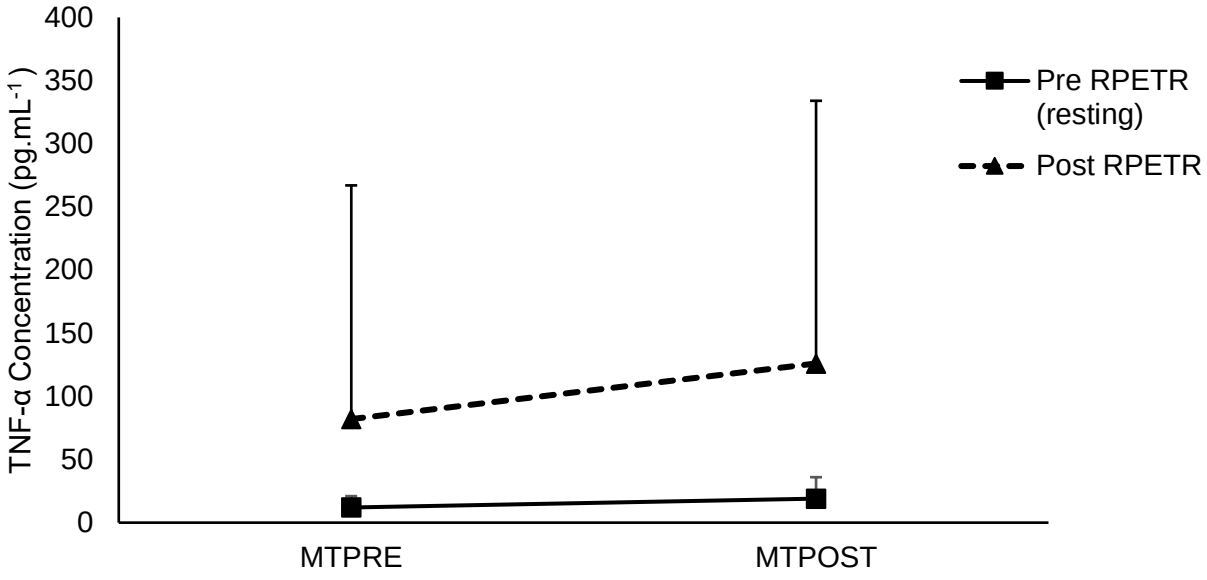

Figure 5.18: *TNF-α concentration ($pg.mL^{-1}$) produced by non-stimulated PMN in response to the RPE_{TR} at MT_{PRE} and MT_{POST}.*

All values were expressed as mean ± SD of 6 participants.

5.9 Interleukin-8 Production by Antigen-stimulated Neutrophils

No main effect for trial (MT_{PRE} x MT_{POST}) was present in measures of IL-8 concentration produced by non-stimulated PMN ($F_{1, 7} = 1.555$, $P = 0.252$). Mean IL-8 concentration was lower at MT_{POST} (6100 ± 1756 $pg.mL^{-1}$) compared to MT_{PRE} (7761 ± 1735 $pg.mL^{-1}$). There was however a significant main effect for time (Pre RPE_{TR} x Post RPE_{TR}) ($F_{1, 7} = 6.548$, $P = 0.038$).

In response to the RPE_{TR} completed at MT_{PRE}, IL-8 concentration significantly increased from 4884 ± 5290 $pg.mL^{-1}$ pre RPE_{TR} (resting) to 10607 ± 5025 $pg.mL^{-1}$ post RPE_{TR} ($P = 0.033$). In response to the RPE_{TR} completed at MT_{POST}, IL-8 concentration did not significantly increase from pre RPE_{TR} (4650 ± 4539 $pg.mL^{-1}$) to post RPE_{TR} (6455 ± 5956 $pg.mL^{-1}$) ($P = 0.199$) (Figure 5.19). No significant interaction effect of trial over time was present ($F_{1, 7} = 1.301$, $P = 0.292$).

The absolute increase of IL-8 concentration in response to the RPE_{TR} decreased from 5753 ± 7419 $pg.mL^{-1}$ at MT_{PRE} (117% increase) to 2003 ± 4083 $pg.mL^{-1}$ at MT_{POST} (51% increase) ($P = 0.261$; $d = 2.25$) (Figure 5.20). Individual IL-8 concentrations by antigen-stimulated PMN significantly varied per individual, with concentrations ranging from 95 – 18908 $pg.mL^{-1}$.

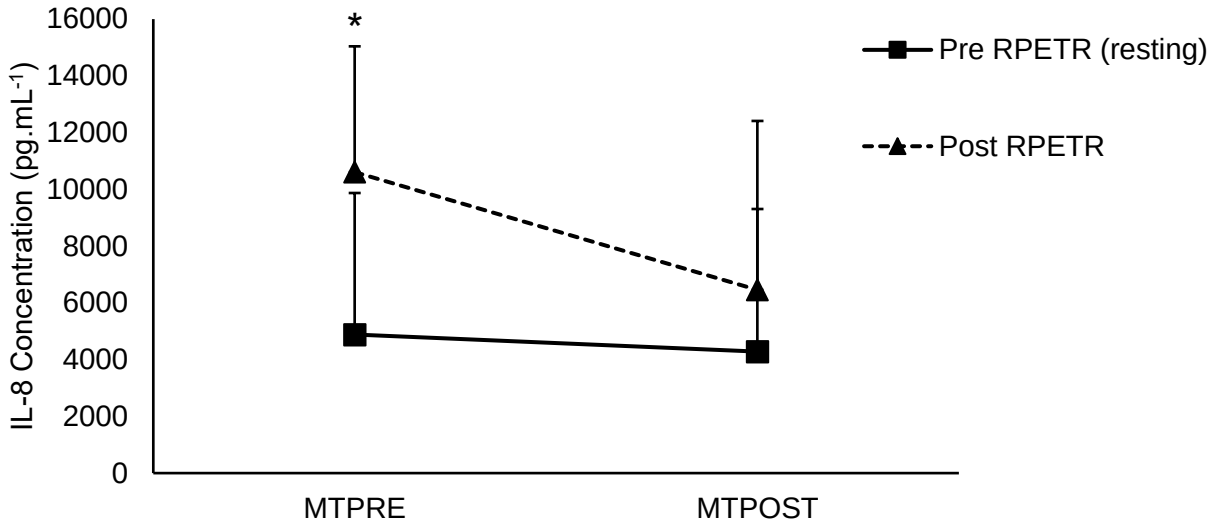

Figure 5.19: *IL-8 concentration (pg.mL^{-1}) produced by antigen-stimulated PMN in response to the RPE_{TR} at MT_{PRE} and MT_{POST}.* * = Significantly different to pre RPE_{TR} at MT_{PRE} ($P < 0.01$). All values were expressed as mean ± SD of 10 participants.*

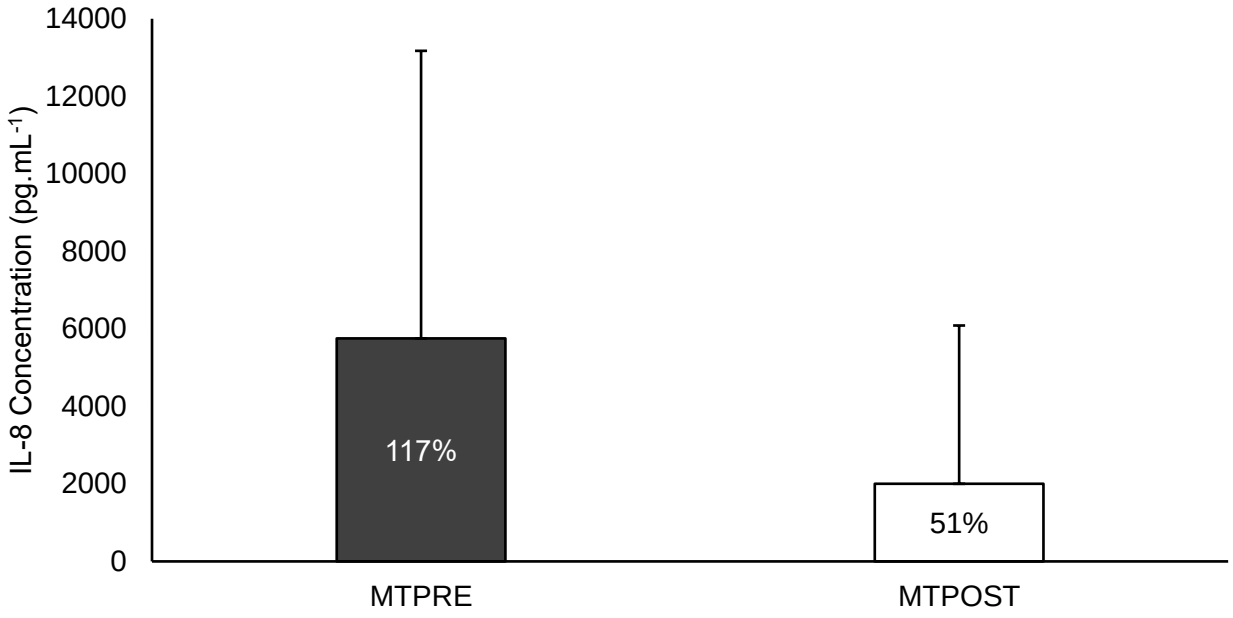

Figure 5.20: *Comparison of the absolute exercise (RPE_{TR})-induced increase in IL-8 concentration (pg.mL^{-1}) at MT_{PRE} and MT_{POST}.*

All values were expressed as mean ± SD of 10 participants.

5.10 Interleukin-8 Production by Non-stimulated Neutrophils

No main effect for trial (MT_{PRE} x MT_{POST}) was present in measures of IL-8 concentration produced by non-stimulated PMN ($F_{1, 6} = 2.465$, $P = 0.167$). Mean IL-8 concentration was lower at MT_{PRE} (3736 ± 1367 $pg.mL^{-1}$) compared to MT_{POST} (6945 ± 2488 $pg.mL^{-1}$). Additionally, there was no main effect for time (Pre RPE_{TR} x Post RPE_{TR}) ($F_{1, 6} = 0.500$, $p = 0.506$). Mean IL-8 concentration was greater post RPE_{TR} (5819 ± 1899 $pg.mL^{-1}$) compared to pre RPE_{TR} (resting) (4862 ± 1812 $pg.mL^{-1}$). No significant interaction effect of trial over time was present ($F_{1, 6} = 0.236$, $P = 0.645$) (Figure 5.21).

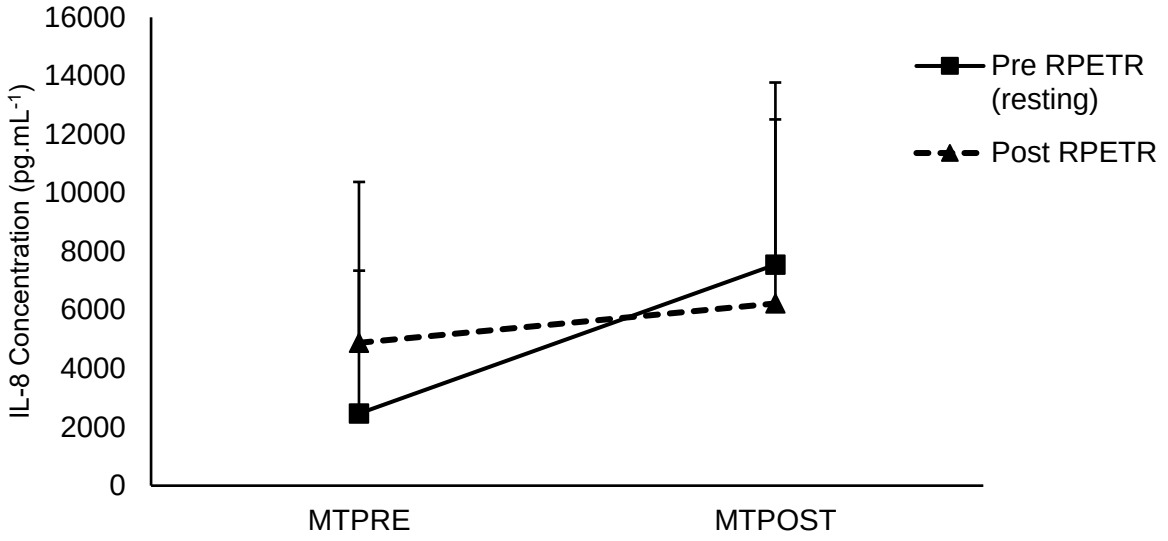

Figure 5.21: *IL-8 concentration ($pg.mL^{-1}$) produced by non-stimulated PMN in response to the RPE_{TR} at MT_{PRE} and MT_{POST}.*

All values were expressed as mean ± SD of 10 participants.

Chapter 6. Discussion

The purpose of this present study was to investigate whether a 12-day ITP would alter resting or exercise-induced TNF-α and IL-8 production by antigen-stimulated PMN. The main finding from this study is that in response to the RPE_{TR} at MT_{POST}, the absolute increase in exercise-induced TNF-α and IL-8 concentration produced by antigen-stimulated PMN was lower compared to the absolute exercise-induced rise in response to the RPE_{TR} at MT_{PRE}. As a result, the experimental hypothesis of this present study was accepted – pro-inflammatory cytokine production by antigen-stimulated PMN decreased following the ITP.

6.1 Markers of Overtraining

Many symptoms associated with states of FOR, NFOR, and the OTS have been reported in previous literature (Fry *et al*., 1991; Robson-Ansley and Costa, 2014). One of the challenges in completing good quality research in the area of overtraining is that it requires the confirmation that markers of overtraining are present. In this study, various physiological and psychological markers were implemented to identify apparent markers of overtraining in participants. The use of the 10 km TT performance test would confirm whether a decline In physical performance capacity was present at MT_{POST}. The RESTQ-76 Sport questionnaire would identify whether participants had become more fatigued and developed more physiological and psychological stress at MT_{POST}. The Jackson URTS questionnaire would identify whether participants had developed new URTS or had increased the severity of existing URTS at MT_{POST}.

General and Sport-related Stress

Following the 12-day ITP, general and sport-related stress scores had significantly increased in all ten participants, including the two participants who were specifically recruited for this thesis. In particular, the subscale '*fitness/injury'* was significantly higher at MT_{POST}. For this subscale to have increased, participants had experienced the following scenarios more

frequently; ‘parts of my body were aching’; ‘my muscles felt stiff or tense during performance’; ‘I had muscle pain after performance’; and ‘I felt vulnerable to injuries’. An increased perception of stress following an ITP was similarly reported by Hough *et al.* (2013) who used a shorter version of the RESTQ-76 Sport questionnaire (RESTQ-52) and found that ‘fatigue’ and ‘burnout’ scores were significantly higher after an 11-day ITP.

The muscle discomfort and pain reported by the end of the ITP may have resulted from skeletal tissue microtrauma. Skeletal tissue microtrauma is a common biological adaptation in response to exercise that incorporates eccentric activity such as the treadmill running incorporated throughout this study (MacIntyre *et al*., 1995). In addition, the lack of recovery time between exercise sessions (24 hours) would have developed an imbalance between training stress and recovery. In line with Selye’s (1936) GAS theory, a lack of recovery following exposure to novel stressors with insufficient recovery will result in the acquirement of biological maladaptation such as muscle fatigue and injury.

The incidence of injury and increased muscular discomfort are commonly reported symptoms by athletes who have developed NFOR or the OTS (Fry *et al*., 1991; Birrer *et al*., 2013). Birrer *et al.* (2013) measured the prevalence of key clinical symptoms in 127 Swedish athletes who had been diagnosed with either NFOR or the OTS. It was reported that 30% of NFOR/OTS athletes experienced more than 5 injuries per year and 73% had experienced feelings of exacerbated muscular fatigue. The findings in this present study that highlights an increased perceived injury risk and greater muscular fatigue in participants could therefore support the notion that these participants may had become overreached by the end of the ITP.

Physical Performance Capacity

One of the primary outcomes following the development of NFOR or the OTS is a decrease to physical performance capacity (Jeukendrup *et al*., 1992; Urhausen *et al*., 1998; Halson *et al*., 2002; Meeusen *et al*., 2006; Dupuy *et al*., 2010; La Meur *et al.,* 2014; Aubrey *et al.,* 2015). In this present study, a 10 km TT performance test was completed at MT_{PRE} and MT_{POST} to

identify whether the ITP had impacted participants' physical performance capacity. No significant differences between time to completion scores measured at MT_{PRE} and MT_{POST} were observed, however further investigation highlighted that a small ES in completion time was evident at MT_{POST}. The completion time measured at MT_{POST} was 2.5% slower, or 01:11 ± 0:27 (mm:ss) longer in duration than the time measured at MT_{PRE}. This decrease in completion time was evident in five out of the seven recorded participants. In addition, one out of the two participants recruited for this thesis also recorded a slower time to completion at MT_{POST}. Two out of the ten participants' time to completion scores improved by MT_{POST}, however this may simply be due to a learning process whereby the participants became more familiar with treadmill running, or running for a more consistent period of time.

It is widely understood in elite sport that small worthwhile changes of 1% to physical performance capacity can be a decisive factor in determining competitive success (Whyte, 2006). Cohen *et al*. (1988) claimed that an ES of 0.2 equates to a variance explained by 1%. In this present study, it was determined that a small ES (d = 0.32) was observed between the mean time to completion scores at MT_{PRE} and MT_{POST}. This suggests that the decreased physical performance capacity at MT_{POST} in the seven participants would have significantly compromised competitive success. To put the 01:11 mean difference in completion time into perspective, such a time difference during the 2012 10 km final at the Rio de Janeiro Olympics would have separated 1st place with 32nd place (Olympic, 2016). A decreased physical performance capacity following the ITP may have resulted from the development of more severe headaches which may have impaired cognitive function that further impacted work capacity (Martins de Araújo *et al.,* 2012). This relationship however was only found in two (participant one and seven) out of the ten participants. Alternatively, elevated levels of sport-related stress, in particular a heightened perception of muscular discomfort and injury, may have negatively impacted participants' ability to complete the TT. A clear example of this can be observed in participant two, their '*general stress'* scores from the RESTQ 76 Sport Questionnaire increased from 5.4 to 7 MT_{POST}. This increase in perceived stress following the

ITP was coupled with a significantly longer time to completion score, which increased from 44:59 at MT_{PRE} to 53:13 at MT_{POST}. This trend was similarly observed in three other participants (participant three, seven, and ten).

A decrease to TT performance following a similar period of intensified training has previously been reported by Jeukendrup *et al*. (1992) and Halson *et al.* (2002). These studies demonstrated that a 2-week period of intensified training completed by 7 competitive cyclists and 8 endurance cyclists, respectively, resulted in a significant increase in time to completion in response to a simulated TT event. Both Jeukendrup *et al*. (1992) and Halson *et al*. (2002) reported that this reported decrease in TT performance was indicative that participants had become overreached. If true, then this may suggest that seven out of the nine recorded participants may have become overreached following the ITP.

Upper-respiratory Tract Symptoms

An increased susceptibility to URTS is often reported by athletes who have developed NFOR or the OTS (Fry *et al*., 1991; Robson-Ansley and Costa, 2014). Following the ITP, six out of the ten participants had developed more severe URTS. In contrast, the two participants who were recruited for this thesis did not develop any URTS by MT_{POST}. The difference in results may be explained by the seasonal variations between the two cohorts. The eight previous participants primarily undertook this study design during autumn and winter where the incidence of URTS development is far greater in individuals (Atkinson and Drust, 2005). Contrary to this, the two participants who were recruited for this particular thesis undertook this study design during spring and summer where the risk of URTS development is much less (Atkinson and Drust, 2005). This is a clear limitation to the study, however due to the time restrictions enforced during the Masters by Research degree, it was unpreventable as data collection could only begin in early spring.

After further investigation into which particular URTS had increased in the six participants, it was apparent that the '*headache*' subscale had significantly elevated by MT_{POST}. Large ES

between malaise, nasal obstruction, joint aches and pain and nasal discharge score were also reported between MT_{PRE} and MT_{POST}. It has been proposed that the increased susceptibility to URTS following intensified training is caused by impaired immune function (Peters and Bateman, 1983; Neiman *et al*., 1990; Verde *et al*., 1992; Gomez-Merino *et al.* 2005; Morgado *et al*., 2012). This could therefore imply that the six participants had developed possible immune impairment(s) by the end of the ITP that permitted the development and increased severity of URTS.

Collectively, only one participant (participant eight) exhibited all of the measured symptoms of NFOR/OTS following the ITP, however as highlighted by Meeusen *et al*. (2012), symptoms of overtraining will vary from individual to individual and that all of the reported symptoms may not be exhibited all at the same time. All of the participants in this study however had reported at least one symptom of NFOR/OTS following the ITP which is suggestive that it was likely all ten participant were close to developing, or had fully developed a state of overreaching. This present study was unable to truly determine whether participants were overreached as it did not retrospectively measure the time it took participants to recover and for their symptoms to return to baseline following MT_{POST}. This would have meant implementing more TT's following the ITP which could have proved unethical considering the vast difficulty and longevity of the initial study design.

6.2 Aetiology of Upper-respiratory Tract Symptoms

The significant development of headaches reported at MT_{POST} may have occurred due to the onset of dehydration (Blau *et al*., 2004), however no changes in hydration status was observed over the ITP. Alternatively, the reported increased general- and sport-related stress scores may indicate that the accumulation of training and/or non-training stress could have triggered the development of headaches (Theeler *et al*. 2010).

It is unlikely that the developed URTS reported by six participants was caused by the infiltration of foreign infectious or non-infectious antigens into the body. If infectious, allergenic, or

pollutant antigens were present at the time of blood extraction, then it would have markedly elevated the pre RPE_{TR} (resting) blood PMN concentration at MT_{POST} as exposure to such antigens during the ITP would have induced a resting state of neutrophilia (Dias *et al.,* 2011; Xu *et al*., 2013; Hosoki *et al.,* 2015). Furthermore, there were no significant changes to TNF-α and IL-8 concentrations that were produced by non-stimulated PMN over the ITP. The production of pro-inflammatory cytokines from non-stimulated PMN may have reflected whether PMN were stimulated by the presence of PAMP *in vivo* prior to harvest. Overall, the production of IL-8 by non-stimulated PMN was generally higher in comparison to the IL-8 production by antigen-stimulated PMN which may imply that PMN were stimulated whilst they were still circulating within the blood. In contrast, the production of TNF-α by non-stimulated PMN was low throughout the training period. A low production of TNF-α would have not occurred if antigens were present within the blood stream as it has been demonstrated that antigen-stimulated PMN will produce significant concentrations of TNF-α. The reported high level of IL-8 production from non-stimulated PMN may have therefore occurred due to incorrect handling techniques during the completion of the ELISA or during the initial harvest of PMN which could have caused the PMN to undergo necrosis. Alternatively, the lack of stimulation *in vitro* whilst performing the isolation and washing procedures may have caused PMN to undergo apoptosis as a regulatory mechanism to prevent the cytotoxic nature of inactivated PMN from harming healthy tissue (Nathan, 2002). It is understood that PMN can store pre-formed IL-8 within cytoplasmic bound vacuoles (Pellmé *et al.,* 2006). If PMN therefore became necrotic or underwent apoptosis, then this could have released these vacuoles containing IL-8 into the culture medium. Nevertheless, it appears that the aetiology of URTS in this present study is unknown and could ultimately only be determined through invasive methods of which this research project was unable to use.

6.3 Blood Neutrophil Count

In this present study, neutrophilia was evident following the RPE_{TR} at MT_{PRE} and MT_{POST} in the two participants specifically recruited for this thesis and also in the ten combined participants.

Resting or exercise-induced blood PMN concentrations at MT_{PRE} and MT_{POST} however were not significantly different. Neutrophilia in response to a high-intensity exercise bout alike the RPE_{TR} has previously been reported by Field *et al*. (1991), Neiman *et al*. (1994) and Quindry *et al*. (2003) and is thought to be provoked by an elevation in glucocorticoids such as cortisol, and cytokines such as granulocyte-macrophage colony-stimulating factor, TNF-α and leukotriene B_4. These stimulants have been reported to downregulate the expression of ICAM upon endothelial cells, thus preventing PMN migration into tissue (Griffin *et al*., 1990). This present study established that TNF-α production by antigen-stimulated PMN will increase over the course of a high-intensity exercise bout such as the RPE_{TR}. This elevated TNF-α production by antigen-stimulated PMN may therefore partially account for the exercise-induced neutrophilia by downregulating ICAM upon endothelial cells and thus limiting PMN mobilization during exercise. Alternatively, the reported neutrophilia could be due to an elevated production of cortisol that similarly downregulated participants' ICAM upon their endothelial cells. No measure of cortisol was completed during this present research, however the cortisol concentrations of eight participants used within this study was measured within the preceding study by Leal (2017; *unpublished*) who reported that both salivary and plasma cortisol concentrations did not alter over the RPE_{TR}. This suggests that TNF-α release from activated PMN within the blood stream may potentially be an important mechanism that influences neutrophilia during exercise in the absence of elevated cortisol production. Further research could investigate whether glucocorticoid receptor knockout mice may display similar levels of neutrophilia during exercise compared to a control group. This may emphasize the effectiveness of cytokines such as TNF-α and other mechanisms in the appearance of neutrophilia within the blood during exercise.

It must also be highlighted that during the harvesting of PMN from the isolation medium post-centrifuge, there may have been contamination from other granulocytes such as basophils and eosinophils which could have been present within the harvested granulocyte band. As these cell types have been reported to rise in response to a 6-minute cycle ergometer test

(Schwindt *et al*., 2007), then such cells may have accounted for the reported exercise-induced neutrophilia. While possible, it is unlikely that sufficient numbers of eosinophils and basophils, which have a comparatively lower concentration within the blood than PMN (Abbas *et al*., 2014), could account for this neutrophilia.

Previous research by Robson-Ansley *et al*. (2007) has similarly reported no changes to resting blood PMN concentration following a 12-day ITP. In contrast, Svendsen *et al*. (2016) and Hooper *et al*. (1995) reported a decreased resting blood PMN concentration following an 8-day ITP and a 6-month competition period, respectively. It was proposed by both authors that this reduction in resting blood PMN concentration was caused by the development of chronic inflammation within skeletal tissue resulting from prolonged exercise with limited recovery. A hallmark sign of chronic inflammation is the shift from short-lived PMN to longer-lasting macrophages and lymphocytes (Allison *et al.,* 1978). The onset of chronic inflammation may ultimately lead to a shift in the type of leukocyte present within the inflamed tissue, thus reducing circulating PMN concentration and subsequently increasing lymphocyte and macrophage concentration. The finding that resting PMN concentration did not change following the ITP within this present study may therefore suggest that any inflammation developed over the ITP was acute in nature and did not progress into a chronic state.

6.4 Pro-inflammatory Cytokine Production by Antigen-stimulated Neutrophils in Response to the RPE_{TR}

This present study reports that an acute bout of short-duration, high-intensity exercise such as the RPE_{TR} can significantly elevate TNF-α and IL-8 production by antigen-stimulated PMN, thus upregulating a greater inflammatory response to exercise. TNF-α is an important mediator of the immediate inflammatory response by initiating the mobilization of phagocytes into compromised tissue (Walsh, 2013), increasing the release of cytokines from cells such as IL-1, IL-6, IL-8 and colony-stimulating factors (Walsh, 2013), promoting vasodilation (Johns and Webb, 1998) and increasing endothelial permeability (Hoffman *et al*., 2002). Alternatively,

IL-8 is another important inflammatory mediator by attracting leukocytes such as T cells, basophils and PMN into tissue, upregulating ICAM on endothelial cells to promote a greater transmigration of leukocytes (Bickel, 1993), stimulating histamine release (Koch *et al*., 1992) and increasing angiogenesis (Koch *et al*., 1992).

An increased inflammatory response during and following exercise may be useful in clearing foreign antigens that have been inhaled through the mouth or nose due to a greater exercise-induced respiratory rate (Burton *et al*., 2004). Additionally it may also emphasize the recovery process of exercise-induced skeletal tissue microtrauma (Coico, 2015). A heightened inflammatory response during or following exercise may however have negative implications to an individual's health if left untreated. The accumulation of exercise-induced microtrauma has been theorised to lead to the onset of acute systemic inflammation which can cross-talk with the brain to develop sickness behaviours (Smith, 2000). Sickness behaviours such as a loss of appetite, increased fatigue, increased sensitivity to pain, and a loss of motivation are symptoms associated with states of NFOR and the OTS (Fry *et al*., 1991). Pro-inflammatory cytokine production may therefore be implicated in the development of sickness behaviours associated with NFOR and the OTS. As presented within the literature review, considering PMN are the primary leukocyte mobilized during the immediate inflammatory response, an exercise-induced increase in pro-inflammatory cytokine production by antigen-stimulated PMN may, over periods of continuous exercise training, mediate the activation of blood-brain mechanisms which leads to the development of the sickness behaviours that are often reported by athletes who have developed NFOR or the OTS.

An increased perception of stress was evident at MT_{POST} which implied the potential development of sickness behaviours. Further investigation of the RESTQ 76 Sport Questionnaire shown that participants also developed minor ($P > 0.05$) psychological and behavioural impairments such as a heightened perception of general, social, and emotional stress, a lack of energy, a decreased sleep quality, increased sensation of burnout, and lower self-regulation and efficiency. It could therefore be theorised that the reoccurring exercise-

induced rises in pro-inflammatory cytokine production from stimulated PMN over the ITP initiated cross-talk with the central nervous system that contributed to the development of minor psychological and behavioural impairments that were present at MT_{POST}.

The development of acute systemic inflammation due to elevated pro-inflammatory cytokine production by PMN may trigger a corresponding rise in anti-inflammatory cytokines such as IL-10 (Opal and DePalo, 2000). Anti-inflammatory cytokines such as IL-10 are the primary inhibitors of the inflammatory response within the body and have subsequently been reported to inhibit pro-inflammatory cytokine production by PMN (Wang *et al*., 1994). Inhibition of pro-inflammatory cytokine production by PMN may lead to a greater susceptibility of URTS by reducing the inflammatory responses ability to clear antigen challenge from the body. As presented, pro-inflammatory cytokine production by antigen-stimulated PMN increased in response to the RPE_{TR}. Hypothetically, repeated production of pro-inflammatory cytokines by antigen-stimulated PMN over an ITP may lead to a concurrent increase in anti-inflammatory cytokines such as IL-10 that effectively inhibits pro-inflammatory production by PMN – of which this thesis has demonstrated following the ITP.

The reported neutrophilia following the RPE_{TR} may have been responsible for the rise in TNF-α and IL-8 concentrations by antigen-stimulated PMN. An increased number of circulating PMN post RPE_{TR} mean that there would have been more cells harvested and activated by LPS *in vitro,* thus potentially constituting to a greater TNF-α and IL-8 concentration following exercise. Furthermore, as previously stated, harvested PMN may have been contaminated with a small number of basophils and eosinophils. It has previously been reported that eosinophils are able to produce TNF-α when activated by antigen challenge (Finotto *et al.,* 1994). The exercise-induced rise in TNF-α concentration by antigen-stimulated PMN could therefore have been caused in part by the rise and activation of eosinophils. Neither basophils or eosinophils however have been reported to produce IL-8, which may indicate that the rises in pro-inflammatory cytokine production derived solely from PMN. Additionally, eosinophils

and basophils are minority cell types in blood and not mobilised in large numbers during exercise.

The increased production of TNF-α and IL-8 by antigen-stimulated PMN after the RPE_{TR} may also be as a result of TLR4 upregulation upon PMN. It has been reported that acute bouts of moderate-to-high intensity aerobic exercise such as the RPE_{TR} can increase the mRNA expression and protein level of TLR4 upon cells such as monocytes, PMN, endothelial cells and adipose tissue (Booth *et al*., 2004; Rosa *et al*., 2011; Zbinden-Foncea *et al*., 2012; Rodriguez-Miguelez *et al*., 2015). PMN can express cytokines following the ligand-receptor interaction with TLR4 which activates the transcription factor NF-kB (Kawait and Akira, 2010) to transport to the cell nucleus where it triggers the expression of cytokines (Means *et al*., 2000). The RPE_{TR} performed during this study may have increased the expression of TLR4 on PMN which in turn permitted a greater expression of TNF-α and IL-8 by PMN when stimulated by LPS *in vitro*.

6.5 Pro-inflammatory Cytokine Production by Antigen-stimulated Neutrophils Following 12-days of Intensified Training

A reduced exercise-induced increase in TNF-α and IL-8 production by antigen-stimulated PMN was observed at MT_{POST} in both the two participants recruited specifically for this thesis and also in the ten combined participants. These findings were not statistically significant, however a large ES was found between the mean exercise-induced change reported at MT_{PRE} and MT_{POST} which indicates that the reduced exercise-induced increase at MT_{POST} is still a significant finding and worthy of investigation.

Previous research suggests that athletes with NFOR or the OTS report of an increased susceptibility to URTS (Heath *et al*., 1992; Nieman, 1994; Pyne and Gleeson, 1998; Tiollier *et al*., 2005; Gleeson, 2006a; Neville *et al.,* 2008; Hausswirth *et al*., 2014). The greater susceptibility to URTS is associated with several immune impairments that have been reported occur following periods of intensified training (Peters and Bateman, 1983; Neiman *et al*., 1990;

Verde *et al*., 1992; Gomez-Merino *et al.* 2005; Morgado *et al*., 2012). It is yet to be concluded however which particular immune impairment, or the effect of multiple impairments, may lead to the pathogenesis of reported URTS.

This present study reports that after a period of intensified training, a lower pro-inflammatory response by antigen-stimulated PMN is evident following the completion of exercise such as the RPE_{TR}. A reduced inflammatory response by antigen-stimulated PMN following exercise should hypothetically impair the clearance of foreign antigens from the body that may become prevalent during exercise. An impaired clearance of antigens within the body may subsequently manifest and could lead to the development of URTS. It is particularly important that there is an adequate inflammatory response during exercise as the risk of exposure to foreign antigens may be particularly higher due to the increased exercise-induced respiratory rate that permits a greater inhalation through the mouth and nose (Burton *et al*., 2004). Therefore, a reduced pro-inflammatory response by PMN during exercise may be significantly detrimental to athletes, particularly to those who are performing in potentially noxious environments such as highly polluted areas which are becoming increasingly more common. A reduction to pro-inflammatory cytokine production by antigen-stimulated PMN may therefore be one of several dysregulated immune functions responsible for the development of URTS in NFOR/OTS athletes.

A reduced pro-inflammatory cytokine production by a leukocyte has similarly been reported by Morgado *et al*. (2012) who observed a reduced pro-inflammatory cytokine response (IL-1, IL-6, IL-12, TNF-α, and MIP-1) from dendritic cells and monocytes following a 6-week increase to training load in elite swimmers. Interestingly, this reduction was also associated with greater episodes of URTS alike this present study, thus furthering the hypothesis that a reduced pro-inflammatory cytokine production by particular leukocytes may be heavily responsible in the development of URTS in athletes. It is not fully understood what particular mechanisms caused this apparent reduction in pro-inflammatory cytokine production by antigen-stimulated

PMN (and other leukocytes in previous research), however the following hypotheses have been considered.

It has been postulated that a decreased PMN function following an ITP may result from an influx of immature, sub-functional PMN into the circulation (Robson-Ansley *et al*., 2007). At MT_{POST}, there was a small raise ($P > 0.05$) in the resting and exercise-induced concentration of PMN in response to the RPE_{TR} compared to the concentrations measured at MT_{PRE}. This finding may highlight that a greater number of immature PMN were being released from bone marrow reserves and into the periphery. Previous research conducted by Drifte *et al*. (2013) reported that immature PMN were less efficient at mediating important innate immune functions such as bacterial phagocytosis and killing via the production of reactive oxygen species. A possible influx of immature PMN at MT_{POST} may therefore be inefficient at producing pro-inflammatory cytokines compared to the mature PMN that were potentially the more dominant cell-type present at the time of harvest during MT_{PRE}. In addition, the cytokine IL-10 has been reported to inhibit pro-inflammatory cytokine production by PMN (Wang *et al*., 1994). A bout of high-intensity cycling has been reported to significantly elevated plasma IL-10 concentrations in healthy, physically active participants (Dorneles *et al*., 2016). The effect of cumulative exercise with repeated spikes in IL-10 production may therefore have inhibited IL-8 and TNF-α production from neutrophils. Unfortunately, no measure of plasma IL-10 was completed during this present study and therefore it is difficult to determine whether elevations in IL-10 were responsible for the inhibition.

Whether or not reduced pro-inflammatory cytokine production by antigen-stimulated PMN during exercise is capable of increasing the susceptibility of URTS is undetermined. In theory, a significant decrease in pro-inflammatory cytokine production by antigen-stimulated PMN following exercise may perhaps indicate that PMN are less responsive in mediating the immediate inflammatory response against foreign or self-antigens that may become prevalent during exercise, thus increasing the risk of infection or cellular damage. This vulnerability however may only occur when pro-inflammatory cytokine production by stimulated PMN falls

below a certain threshold. Even so, the immune system boasts an impressive armoury of functions to rid antigen challenge from the body even if TNF-α and IL-8 production from stimulated PMN is compromised. TNF-α can otherwise be produced by monocytes, T cells and natural killer cells (Walsh, 2013) whereas IL-8 can be produced by epithelial cells, fibroblasts, endothelial cells, macrophages, lymphocytes, and mast cells (Bickel, 1998). Nevertheless, as PMN are the first (before macrophages, lymphocytes, and mast cells) and most abundant leukocyte mobilized during the immediate inflammatory response (Freire and Van Dyke, 2013), their ability to produce pro-inflammatory cytokines may significantly compromise the subsequent mobilization and activation of leukocytes to produce more pro-inflammatory cytokines to evoke an adequate inflammatory response against antigen challenge. This apparent reduced exercise-induced increase in pro-inflammatory cytokine production by antigen-stimulated PMN after a period of intensified training may ultimately mediate a less effective inflammatory response against antigen challenge which could be one of several immune impairments responsible for the pathogenesis of reported URTS in NFOR/OTS athletes.

The reduced exercise-induced increase of pro-inflammatory cytokine production by antigen-stimulated PMN also emphasizes that it may perhaps not only be the PMN microbicidal function which decreases following a period of intensified training, but also PMN ability to mediate an inflammatory response against antigen challenge. This present study therefore emphasizes that if PMN function was to be used as a sensitive biological marker to detect the onset of NFOR or the OTS, as suggested by Gleeson *et al*. (2002) and Yaegaki *et al.* (2008), then a comprehensive overview of all of its functions could be used.

6.6 Future Research Considerations

In light of the findings from this present study, many questions which have been left unresolved could be addressed in future research. To understand the mechanisms that regulate exercise-induced neutrophilia, future research may want to consider the role of TNF-α produced by

PMN in regulating ICAM expression. To determine the mechanisms that are responsible for the altered pro-inflammatory cytokine response by PMN following an ITP, future research could determine whether a concurrent rise in the production of IL-10 over an ITP is associated with the decline in PMN function. In addition, future research may also want to determine whether immature or sub-functional PMN are less responsive to antigen challenge compared to mature functional PMN. As presented, TLR4 upregulation may be responsible for the increased production of pro-inflammatory cytokines by antigen-stimulated PMN following a bout of high-intensity exercise. Future research could use flow cytometry or quantitative polymerase chain reaction (qPCR) analysis to determine whether TLR4 is upregulated on PMN following a bout of high-intensity exercise.

6.7 Conclusion

In conclusion, a 12-day ITP reduced exercise-induced pro-inflammatory cytokine production by antigen-stimulated PMN. As a decreased pro-inflammatory cytokine production may impact the body's ability to successfully clear antigen challenge, this finding may be one of several immune impairments that could lead to the pathogenesis of reported URTS in NFOR and OTS athletes.

6.8 Limitations

The following limitations were present during this study:

1. High intra-assay coefficient of variance values from the TNF-α ELISA tests would have produced less accurate concentrations, therefore the absolute rise in antigen-stimulated TNF-α concentrations could have been statistically significant between MT_{PRE} and MT_{POST}.
2. The harvesting of PMN using the Lympholyte-poly^{TM} method may have been contaminated by a small number of basophils and eosinophils which may have contributed to the total blood PMN counts. Purification of PMN using flow cytometry or magnetic bead-based cell sorting could have prevented this.

3. 25 samples violated the standard curve of the TNF-α or IL-8 ELISA tests. The optical densities of these samples were consequently extrapolated which could make the concentration of these samples less accurate.

Chapter 7. References

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Chapter 8. Appendices

8.1 Ethical Approval Document

11/07/16

Ethical Approval Confirmation

Proposer: Diogo Vaz Leal

Proposal title: Exercise-induced stress hormones and immunological responses before and after a 12-day period of intensified, physiological stress.

Dear Proposer

Your research proposal has now received ethical approval from the Institute for Sport and Physical Activity Research Ethics Committee.

Approval number: 2016ISPAR007

Please note that if it becomes necessary to make any substantive change to the research design, the sampling approach or the data collection methods a further application will be required.

Please be advised that your research project may be subject to an ethical audit at any given time. If you require any further information please contact the ISPAR Ethics Chair, Dr Laura Charalambous.

You are now clear to proceed with the data collection for this project.

Kind Regards

Dr Laura Charalambous (ISPAR Ethics Chair)

8.2 Participant Information Sheet

University of Bedfordshire

Participant Information Sheet

Study Title: Polymorphonuclear-derived Interluekin-8 and Tumour Necrosis Factor-alpha Profiles Following a 12-day Period of Intensified Training

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Supervisors: Dr John Hough, Dr. Jeffery Aldous, Dr. Anna Furmanski

Dear participant, thank you for showing an interest in participating in this research study. This document will emphasize the study aim, the participant requirements, safety precautions and the protocol structure to be followed by potential participants. Please read this information sheet carefully before deciding whether to participate. Individuals who accept our invitation to participate in this study will be treated with the utmost care, ensuring your safety and wellbeing is our priority during the entirety of the study period. If you decide at any stage throughout the study to withdraw from participation, your wish will be honoured without reprisal. Participant's privacy shall be respected throughout the study. Personal information shall remain anonymous and collected data from the study will be kept confidential.

What is the aim of the study? The purpose of the study is to analyse whether a 12-day period of intensified training may induce a state of overreaching, and to also examine the resting and exercise-induced immune responses to a short-duration, self-paced running bout before and after this 12-day intensified training period. The potential alterations in immune markers may be a good method to predict and/or prevent the incidence of overreaching or the overtraining syndrome (OTS).

Who are we looking for? We are seeking to recruit physically active, healthy male volunteers aged between 18-40 years old who exercise at least three times per week. Participants must not be smokers, alcoholic or night-shift workers.

Is it safe for you to participate in the study? Prior to any kind of participation in the study, you will be asked to complete a participation consent form and a blood screening analysis questionnaire. Your safety is our upmost priority, and will ensure that your safety is not at risk during the entirety of the study.

What will you need to participate in? 16 consecutive laboratory sessions in total – 1 familiarisation session of the main trial. 2 main trials completed before and after the training period. 1 maximal oxygen uptake (VO_{2max}) test. 12 days of intensified training.

Main trials

The main trials will be completed before and after the 12-day intensified training period. Participants are required to attend the University of Bedfordshire laboratories at 11:30 am. Each main trial will take on average ~2 hours 30 minutes to complete. The first main trial, otherwise known as the 'pre-training main trial', has to be completed on either a Monday, Tuesday or Wednesday to ensure that the remaining trials do not fall upon a weekend. The participant will choose which day they prefer to begin their main trial.

Once participants have arrived to the University of Bedfordshire laboratories at ~11:30 AM, they will be presented with a plastic container in which they will be asked to urinate into. Participants are required to attend the laboratory in a hydrated state. Failure to do so will result in postponement of the study. A heart rate monitor will be fitted around the participants chest. Participants will then be required to complete several documents:

- **Consent form:** To ensure that the participant has given consent to be tested upon.
- **Upper Respiratory Tract Infection Questionnaire:** To measure the incidence of upper respiratory tract infection before and after the study protocol.
- **REST-Q 76 Sport Questionnaire:** To measure the participants mental, emotional and physical well-being prior and during the study protocol.
- **Health questionnaire**: To provide evidence that the participant is in a healthy condition prior to completing the main trial.

The completion of the documents will take ~10 minutes to complete. During this period, participants are refrained from consuming any water. This will prevent saliva samples from becoming diluted. A resting heart rate value will also be determined during this time. Upon completion of the documents, participants height and weight will be collected. Bloods via venepuncture will then be taken at the antecubital fossa. The participants shall be sat (or led) down whilst a trained phlebotomist checks for available insertion sites upon the arm. A small butterfly needle will be inserted into an available vein and blood shall be extracted. Immediately following blood collection, participants will be escorted to have their saliva sampled. Participants will be asked to passively drool without any orofacial movements into a small container for 3 minutes.

At ~12:00, participants will complete a short-duration, self-paced treadmill-running bout (RPEtreadmill). The RPEtreadmill consists of a continuous 30-min treadmill run. The 30 minutes will be split up into 1 minute running at a self-perceived rate of perceived exhaustion (RPE) score of 11 (light) and 4 minutes running at 15 (hard). As this is a self-paced treadmill bout, participants are able to alter the treadmill speeds to correspond to their perceived intensity. 20 minutes into the RPEtreadmill protocol, participants will again be refrained from consuming any liquids to prevent salivary dilution. 15 seconds prior to the completion of each stage, participants will be asked to give their self-perceived RPE. Heart rate and treadmill speed will also be collected.

Upon completion of the RPEtreadmill, a second post-exercise blood and saliva sample shall be taken in an identical manner to the first. Participants will then be given 1 hours rest. For the first 30 minutes of the 1 hour break, participants are required to remain seated. Participants are permitted

to bring in devices to keep them occupied during this period (i.e. laptops, books). 30 minutes into the 1 hour period, a third and final blood and saliva sample shall be taken. For the remaining 30 minutes of the rest period, participants are permitted to exit the laboratories for fresh air or go to the toilet. A 10 km time trial will then be completed. This will be a determinant of the participants endurance performance and should thus be completed as fast as possible. This protocol will be self-paced and thus the participant will have full control of the treadmill speeds. Water is allowed to be freely consumed during this protocol. Following completion, the participant will be asked to give their average RPE. Participants are prohibited from leaving the laboratory until their heart rate falls below 100 beats per minute.

Before each main trial, the following instructions need to be met:

- 500mL of water in the morning prior to each session to ensure euhydration status.
- Standard breakfast that can be reproduced before each main trial (food diaries shall be completed to ensure reproducibility)
- Fasted state from breakfast to main exercise trial (>4hrs before main trials)
- No alcohol or intense physical activity 24 hours prior to the main trials. No caffeine during the morning of the trial.

VO_{2max} Test

The VO_{2max} test is a gold-standard method of measuring physical fitness. The VO_{2max} test is completed 24 hours following the 'pre-training' main trial and 24 hours before the beginning of the 12 day intensified training period.

Participants will initially complete a 16-minute submaximal treadmill run. This test is divided into 4 x 4 minute running blocks. The participant will choose which speed they begin the test with. After the completion of each stage, the treadmill speed increases by 1 $km.h^{-1}$. Participants will then be provided with a 15 minute rest period. Following rest, an incremental oxygen uptake test shall begin. The test begins at the speed in which was ran during the submaximal test when the participants heart rate reached 150 beats per minute. Each stage of this protocol is 1 minute of length. 15 seconds prior to each stage, the participants will be required to point at their self-perceived RPE score. After the completion of each stage, the inclination of the treadmill will increase by 1% whilst the treadmill speed remains the same. Participants will continue to complete stages until exhaustion or if they exceed the threshold criteria set by the researcher.

It is optional to have a 2^{nd} VO_{2max} test following the post-training main trial if participants are interested to see whether their physical fitness has improved.

12 day Intensified Training Period

During the 12-day period of training, participants will be required to attend the University of Bedfordshire laboratories every day (*weekend training can be performed at a local gym, this can be discussed further upon acceptation*) to complete one of three different exercise protocols. Participants can choose the time of day in which they complete each protocol, permitting it is between 9:00 – 16:00. Under special circumstances, participants can complete these training days at a local gym or leisure facility (under provision from the researcher). **UPDATE 01/09/18:** Participants will be allowed to complete each training session at a local leisure facility, permitting they are unable to travel to the University of Bedfordshire laboratories each day. Participants choosing this route will be required to track their resting, average and max heart rate of each session. A heart rate monitor will be offered to the participant to complete this.

Protocols will be interposed so participants do not complete the same exercise protocol on two consecutive days, the protocols are as follows:

1. 90-minute continuous treadmill-run, subdivided in one block of 70 min at 55% of your maximal speed in the VO_{2max} and one 20-min block at 75% of that same maximal speed.
2. 5-km time-trial.
3. 70-min, self-paced, continuous treadmill-run at a speed corresponding to a rating of perceived exertion (RPE) of 12 (light) on the 6-20 Borg scale for the first 30 min, at 13 (somewhat hard) during the following 30 min, and at 15 (hard) for the final 10 min.

Each session will be completed 4 times in total over the 12-day period. This training program will be classified as an intensification of your normal training sessions.

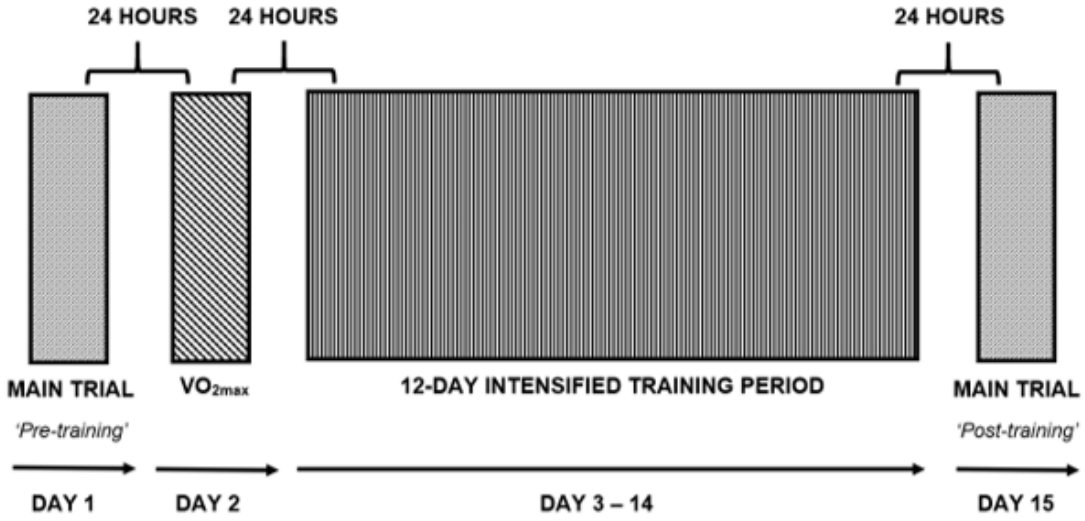

Figure 1: Study design schematic (excluding familiarization session).

After each training session, we will collect participants maximal and mean heart rate of the session. Participants will also give a RPE score following the session to interpret its difficulty.

Diaries

Food Diary

24 hours prior to the 'pre-training' main trial, participants will be asked to keep a food diary of everything they had eaten. 24 hours prior to the 'post training' main trial, participants will be asked to replicate the same food diary that was established prior to the 'pre-training' main trial. A food scale will be given to participants to weigh out all consumed foods. Participants weighing habits must be replicated to ensure the correct amount of food is being consumed. For example, if consuming rice – the rice must be measured uncooked both 24 hours before and after the training period.

External Training Diary

During the 12-day intensified training period, participants will be required to complete a training diary if they intend on completing any exercise outside of the set study protocol. Participants will be given a heart rate monitor, a copy of Borg's RPE scale and a training diary to complete this process. To complete the training dairy, participants are required to fit their heart rate monitor prior to exercise to measure their resting heart rate. Following exercise, participants would need to write down their average RPE and average/maximal heart rate of the session (*participants will be shown how to complete this step).*

What will you gain from this experience? Not only will your involvement in this study be crucial in advancing the research surrounding overreaching and the OTS, you will also gain valuable experience training in an elite sport science setting, with contemporary laboratory equipment and techniques which could further enhance your skills and knowledge within the industry. This study will also offer you the opportunity to partake in a VO_{2max} test which is the gold standard measurement tool for aerobic fitness. Likewise, the application of an individualised training programme throughout the study can be alternatively viewed as a training camp, where the cumulative effect of the exercise bouts will aid in improving physical fitness levels and potentially help you to lose a few of those unwanted pounds!

Interested? Please get in touch with us! If you have any questions whatsoever regarding the study, do not hesitate to ask. You can contact us via email at:

Lead researcher: josh.thorley@study.beds.ac.uk

Lead supervisor: john.hough@beds.ac.uk

FAQs

- **Will a state of functional overreaching be detrimental to my physical performance?** It is likely that over the 12 days of intensified training, performance will likely decrease. This is a normal maladaptation of intensified training over a short period. Following an adequate rest period following the intensified training period, participants may observe a 'super-compensatory' increase in physical performance levels. Coaches use a state of functional overreaching to improve physical performance levels several weeks before competition.
- **Do I have to give blood and saliva every day?** No. The only times blood and saliva shall be sampled is during the 'pre-training' and 'post-training' main trials. During these trials, bloods and saliva shall be taken at 3 time points – before exercise, after exercise, and 30 minutes after exercise.
- **Do I need to do anything before beginning the study?** It is recommended that the participant meets with the researcher prior to the study. This is so that the participant fully understands the magnitude of the study. Participants will also be given a folder containing 1) food diary 2) training diary 3) participant information sheet 4) study timetable. A heart rate monitor and food scales will also be given to the participant during this period. Calibration of the heart rate monitor will be shown to participants so they understand how to find their resting, average and maximal heart rate variables.
- **Can I exercise outside of the study?** Yes. If you are completing exercise outside of the study protocol, please complete the training diary you received within your folder. For each session completed outside of the study, participants are required to fill out the following information: date of session, type of exercise, exercise completed, duration of session, resting, average and maximal heart rate. Heart rate information will be calculated using the heart rate monitor you will be given. If you are completing exercise that prohibits the use of heart rate monitors, please do not wear it.
- **What is an RPE scale?** RPE = rate of perceived exhaustion. During this study, we will be using Borg's scale of RPE. You can find an image of this scale here. Participants will be often asked for your average RPE following the completion of each session, if you thought the session was 'hard', then the RPE of this self-perceived difficult is 15, as shown on the chart. During the study, participants will also complete 2 different types of RPE-based exercise sessions – the RPEtreadmill completed on the main trials and the 70-minute self-paced, continuous run. During these sessions, participant will have complete control over the treadmill speed so that the speed correlates to a particular perceived exhaustion. For example, during the RPEtreadmill, we ask participant to run for 1 minute at RPE 11 (light) and 4 minutes at RPE 15 (hard). Participants will adjust the treadmill speed during these times so that it corresponds to the correct difficulty.
- **Will I be reminded every day of what I'm doing?** Yes. 24 hours prior to each session, participants will be reminded of what session they will be running the following day. Participant will also receive a study timetable.
- **What if I can't commit to attending the laboratories for 12-consecutive days?** Participants are able to complete the 12-days of intensified training at a local leisure facility if they are unable to attend all sessions at the University of Bedfordshire laboratories. If participants choose this option, then participants will be required to wear their heart rate monitor for the duration of the study. Resting heart rate will be needed prior to each session, so

participants will need to find a comfortable area to rest for ~ 3 minutes so that their heart rate falls to its lowest value. Participants will note down/photograph this value upon the heart rate monitor interface and inform the researcher of this value following the session. After the completion of the exercise period, average and maximal heart rate, plus the information variables upon the treadmill interface (i.e. distance covered, time) will also be needed to be noted down/photographed and relayed back to the researcher. Finally, an average RPE of the session will be asked from the participant.
NOTE: We are aware that many of the treadmills at leisure facilities only run for a maximal time of 60 minutes. As some of the exercise protocols are longer than 60 minutes, participants must restart the treadmill and continue with the remaining exercise. During this restart period, it is crucial that participants do not stop for a long period of time. It is also necessary for participants to note down/photograph the information variables upon the treadmill interface before restarting the treadmill.

This method is not recommended, however if participants feel they are unable to commit to 12 consecutive days, then please get in touch with the researcher.

- **What foods should I eat when completing my food diary 24 hours prior to the main trial?**
  This is entirely up to you. However, as this would need to be replicated 24 hours prior to the 'post-training' main trial, it is recommended that you consume foods which are easily prepared and not too complex.
- **How do I get the heart rate monitor to show my heart rate variables?**
  This is a step-by-step process of fitting and acquiring heart rate data from the monitor.
  1. The device comes with a sensor and a watch. First, fit the sensor and strap around your chest (so the sternum is placed upon the sternum – just below the chest).
  2. Bring the watch close to the sensor and press the big button in the centre of the watch, this will sync the 2 devices together.
  3. You will know that the watch is synced to the sensor when a flashing heart at the bottom of the water appears. Also data will begin to display on the watch interface.
  4. Once it is synced, you can begin to exercise.
  5. Following completion of exercise, you will press the big button upon the watch's interface to stop recording heart rate variables.
  6. To acquire resting, average and maximal heart rate values – once you have pressed the button to stop recording, press it once again until the device scrolls to system (or SYSTM). Once you have scrolled to system, do not press anything for a few seconds – this will open up the system page automatically. In the systems page, you can scroll along by pressing the interface button. Scrolling along will allow you to find your measured heart rate variables.

8.3 Consent Form

University of Bedfordshire

Participant Consent Form

Exercise-induced alterations to neutrophil function in response to a 12-day period of intensified training

I have read and understand the Participant Information Sheet for the above study and have had the opportunity to ask questions.	☐
I have received enough information about the study and satisfactory answers to all of my questions.	☐
I understand I am free to withdraw from the study at any time, without having to give a reason for doing so.	☐
I understand what is required of me for the duration of the study.	☐
I consent to completing a health-screening questionnaire.	☐
I consent to having my weight and height measured.	☐
I consent to having bloods taken by venepuncture for immune/hormonal/haematology analysis	☐
I consent to having saliva samples taken throughout the study	☐
I understand that all results will be anonymous and that only the direct research team will be aware of my individual results.	☐
I understand that all results from the study may be published, however these results will be group scores not individual results.	☐
I agree to take part in this study.	☐

Participant:	Date:	Signature:
Researcher:	Date:	Signature:

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8.4 Health Screen and Physiological Questionnaire

Name/Student Number .....……

Sex:.....…….

Date of Birth .....…….

Health Screen and Physiological Testing Questionnaire

As an individual participating in physical activity, it is important that you are currently in good health. This is to ensure your well-being and to try and prevent confounding data. This completed questionnaire will be held in a locked filing cabinet in the Sport and Exercise Science Laboratories for a period of three years. After this time it will be shredded. Please ask for a photocopy of this questionnaire if you require one.

Please complete this brief questionnaire to confirm your ability to participate:

1. At present, do you have any health problem for which you are:

(a)	on medication, prescribed or otherwise	Yes		No		
(b)	attending your general practitioner	Yes		No		
(c)	on a hospital waiting list for an injury	Yes		No		
(d)	recovering from an illness or operation.....	Yes		No		

2. In the past two years, have you had any illness or injury which required you to:

(a)	consult your GP.....	Yes		No	
(b)	attend a hospital outpatient department	Yes		No	
(c)	be admitted to hospital	Yes		No	

3. Have you ever had any of the following:

(a)	Convulsions/epilepsy	Yes		No	
(b)	Respiratory conditions such as asthma/bronchitis/ Turburculosis……..	Yes		No	
(d)	Eczema	Yes		No	
(e)	Diabetes	Yes		No	
(f)	A blood disorder (including infections/viruses)...	Yes		No	
(g)	Head injury including concussion	Yes		No	
(h)	Digestive/ Gastrointestinal problems	Yes		No	
(i)	Heart problems/chest pains/ angina/heart attack/varicose vein/ embolism/aneurysm…….	Yes		No	
(j)	Problems with muscles, bones or joints (for example arthritis/back pain).....	Yes		No	
(k)	Disturbance of balance/coordination	Yes		No	
(l)	Dizziness / black outs / fainting	Yes		No	
(m)	Disturbance of vision	Yes		No	
(n)	Ear/hearing problems	Yes		No	
(o)	Thyroid problems	Yes		No	
(p)	Kidney or liver problems	Yes		No	

(q)	Problems with blood pressure (low or high).....	Yes		No	
(r)	A pacemaker	Yes		No	
(s)	Chronic obstructive pulmonary disease (COPD)	Yes		No	
(t)	Anaphylactic shock symptoms to needles, probes or other medical-type equipment	Yes		No	
(u)	Any allergies or food intolerances	Yes		No	
(v)	A history of heart disease in the family	Yes		No	
(w)	Been pregnant or given birth in the last 6 months	Yes		No	
(x)	Rectal problems	Yes		No	

If YES to any question, please describe in more detail if you wish (for example, was the problem short lived, if it is controlled, if it is re-occurring, if your doctor has given you specific information/instructions regarding the problem).

.....

.....

.....

4. Please state what medication (if any) you are currently taking, explain briefly what the medication is for and how long you have been taking it.

……

5. Do you have any other condition or disability that you feel we should be aware of?

Yes ☐ No ☐

If yes, please briefly explain below:

……

6. Are you currently involved in any other lab activity at the University or elsewhere?

Yes ☐ No ☐

If yes, please provide details.

……

…

7. Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name: Relationship to Participant:

…… ……

Telephone Number: Work ☐ Home ☐ Mobile ☐……

I declare that this information is correct, and is for the sole purpose of giving the tester guidance as to my suitability for the test.

As far as I am aware, there is nothing that might prevent me from successfully completing the tests that have been outlined to me. For any issues raised in sections 1-5 the appropriate precautions (Doctors check, medication to hand) have been taken and I am therefore still willing and able to participate in the current laboratory session as a participant. Whilst also being aware of the risks associated with the current protocol.

8.5 Jackson Upper-Respiratory Tract Symptom Questionnaire

JACKSON SCORE UPPER RESPIRATORY TRACT ILLNESS QUESTIONNAIRE

Name ..... Subject Number..... Date.....

Do you think that you are suffering from a common cold or flu today?
Fill in the circle if your answer is YES O

If yes please complete all the questions below-
Are any of the following symptoms of the common cold or flu present today? Please indicate your response by filling in one circle for each of the following symptoms:

SYMPTOM	DEGREE OF DISCOMFORT			
	None at all	Mild	Moderate	Severe
Sneezing	O	O	O	O
Headache	O	O	O	O
Malaise (feeling of being generally unwell, run down or out of sorts)	O	O	O	O
Nasal discharge (runny nose)	O	O	O	O
Nasal obstruction (blocked nose)	O	O	O	O
Sore throat	O	O	O	O
Cough	O	O	O	O
Ear ache	O	O	O	O
Hoarseness	O	O	O	O
Fever	O	O	O	O
Chilliness	O	O	O	O
Joint aches and pains	O	O	O	O

GENERAL QUESTIONNAIRE

Have any of these symptoms affected your ability to train this week?
Yes o

No o

If yes, indicate which of the following applies:

Training increased above normal level o

Training maintained at same level o

Training reduced below normal level o

Training stopped altogether o

Have you taken any over-the-counter medication this week to alleviate respiratory illness or gastrointestinal discomfort symptoms?

Yes o **If yes, name of medication.....**

No o

Have you been to see you doctor about your illness symptoms this week?

Yes o

No o

If yes, have you taken any prescribed medication this week?

Yes o **If yes, name of medication.....**

No o

8.6 RESTQ 76 Sport Questionnaire

R E S T Q - 76 Sport

Single Code: _____ Group Code: _____

Name (Last): _____ (First): _____

Date: _____ Time: _____ Age: _____ Gender: _____

Sport / Event(s): _____

This questionnaire consists of a series of statements. These statements possibly describe your mental, emotional, or physical well-being or your activities during the past few days and nights.

Please select the answer that most accurately reflects your thoughts and activities. Indicate how often each statement was right in your case in the past days.

The statements related to performance should refer to performance during competition as well as during practice.

For each statement there are seven possible answers.

Please make your selection by marking the number corresponding to the appropriate answer.

Example:

In the past (3) days/nights

... I read a newspaper

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

In this example, the number 5 is marked. This means that you read a newspaper very often in the past three days.

Please do not leave any statements blank.

If you are unsure which answer to choose, select the one that most closely applies to you.

Please turn the page and respond to the statements in order without interruption.

In the past (3) days/nights

1) ... *I watched TV*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

2) ... *I did not get enough sleep*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

3) ... *I finished important tasks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

4) ... *I was unable to concentrate well*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

5) ... *everything bothered me*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

6) ... *I laughed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

7) ... *I felt physically bad*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

8) ... *I was in a bad mood*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

9) ... *I felt physically relaxed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

10) ... *I was in good spirits*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

11) ... *I had difficulties in concentrating*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

12) ... *I worried about unresolved problems*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

C.2 From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

13) ... *I felt at ease*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

14) ... *I had a good time with friends*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

15) ... *I had a headache*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

16) ... *I was tired from work*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

17) ... *I was successful in what I did*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

18) ... *I couldn't switch my mind off*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

19) ... *I fell asleep satisfied and relaxed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

20) ... *I felt uncomfortable*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

21) ... *I was annoyed by others*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

22) ... *I felt down*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

23) ... *I visited some close friends*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

24) ... *I felt depressed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics. CS

In the past (3) days/nights

25) ... *I was dead tired after work*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

26) ... *other people got on my nerves*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

27) ... *I had a satisfying sleep*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

28) ... *I felt anxious or inhibited*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

29) ... *I felt physically fit*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

30) ... *I was fed up with everything*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

31) ... *I was lethargic*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

32) ... *I felt I had to perform well in front of others*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

33) ... *I had fun*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

34) ... *I was in a good mood*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

35) ... *I was exercised*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

36) ... *I slept restlessly*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

C4 From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

37) ... *I was annoyed*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

38) ... *I felt as if I could get everything done*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

39) ... *I was upset*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

40) ... *I put off making decisions*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

41) ... *I made important decisions*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

42) ... *I felt physically exhausted*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

43) ... *I felt happy*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

44) ... *I felt under pressure*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

45) ... *everything was too much for me*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

46) ... *my sleep was interrupted easily*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

47) ... *I felt content*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

48) ... *I was angry with someone*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

In the past (3) days/nights

49) ... *I had some good ideas*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

50) ... *parts of my body were aching*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

51) ... *I could not get rest during the breaks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

52) ... *I was convinced I could achieve my set goals during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

53) ... *I recovered well physically*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

54) ... *I felt burned out by my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

55) ... *I accomplished many worthwhile things in my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

56) ... *I prepared myself mentally for performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

57) ... *my muscles felt stiff or tense during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

58) ... *I had the impression there were too few breaks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

59) ... *I was convinced that I could achieve my performance at any time*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

60) ... *I dealt very effectively with my teammates' problems*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

C.6 From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

61) ... *I was in a good condition physically*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

62) ... *I pushed myself during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

63) ... *I felt emotionally drained from performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

64) ... *I had muscle pain after performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

65) ... *I was convinced that I performed well*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

66) ... *too much was demanded of me during the breaks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

67) ... *I psyched myself up before performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

68) ... *I felt that I wanted to quit my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

69) ... *I felt very energetic*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

70) ... *I easily understood how my teammates felt about things*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

71) ... *I was convinced that I had trained well*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

72) ... *the breaks were not at the right times*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

From *Recovery-Stress Questionnaire for Athletes: User Manual* by Michael Kellmann and K. Wolfgang Kallus, 2001, Champaign, IL: Human Kinetics.

In the past (3) days/nights

73) ... *I felt vulnerable to injuries*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

74) ... *I set definite goals for myself during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

75) ... *my body felt strong*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

76) ... *I felt frustrated by my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

77) ... *I dealt with emotional problems in my sport very calmly*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

Thank you very much!

8.7 Training Diary

Polymorphonuclear-derived Interluekin-8 and Tumour Necrosis Factor-alpha Profiles Following a 12-day Period of Intensified Training

Rating	Perceived Exertion
6	No exertion
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion

EXTERNAL TRAINING LOG

Name:	Age:
Height (cm):	Weight (kg):

PLEASE COMPLETE THIS TRAINING LOG IF YOU PARTICIPATE IN EXERCISE WHICH IS NOT ASSOCIATED WITH THE STUDY. A HEART RATE MONITOR SHALL BE GIVEN TO YOU TO MONITOR HEART RATE DURING SESSIONS, PLEASE FILL IN AS MUCH INFORMATION AS YOU POSSIBLY CAN.

Date:	Type of Exercise (i.e. running/football):

Exercise completed	Intensity & Heart Rate		
	Resting HR		bpm
	Max HR		bpm
	Average HR		bpm
	Duration of session		mins
	RPE of session		

Date:	Type of Exercise (i.e. running/football):

Exercise completed	Intensity & Heart Rate		
	Resting HR		bpm
	Max HR		bpm
	Average HR		bpm
	Duration of session		mins
	RPE of session		

Date:	Type of Exercise (i.e. running/football):		
Exercise completed	**Intensity & Heart Rate**		
	Resting HR		bpm
	Max HR		bpm
	Average HR		bpm
	Duration of session		mins
	RPE of session		

Date:	Type of Exercise (i.e. running/football):		
Exercise completed	**Intensity & Heart Rate**		
	Resting HR		bpm
	Max HR		bpm
	Average HR		bpm
	Duration of session		mins
	RPE of session		

Date:	Type of Exercise (i.e. running/football):		
Exercise completed	**Intensity & Heart Rate**		
	Resting HR		bpm
	Max HR		bpm
	Average HR		bpm
	Duration of session		mins
	RPE of session		

8.8 Food Diary

PARTICIPANT·FOOD·INTAKE·LOG¤				
Name:¤		Height·(cm):¤		
Age:¤	Body·Mass·(kg):¤		¤	
PLEASE·COMPLETE·THE·FOOD·LOG·24HRS·PRIOR·TO·THE·COMPLETION·OF·EACH·MAIN·TRIAL.¶ THIS·LOG·WILL·HELP·YOU·TO·TRACK·CALORIC·INTAKE·AND·TO·REPRODUCE·YOUR·DIET·PRIOR·TO· EACH·MAIN·TRIAL.·PLEASE·WEIGH·ALL·FOOD·PRIOR·TO·CONSUMPTION.¤				
24HRS·BEFORE·MAIN·TRIAL·2¤				
¶ ¶ ¶ ¶ ¶ ¶ Dinner·&· Snacks¶ ¶ ¶ ¶ ¶ ¶ ¶ ¶ ¶ ¤	Time·of· consumption¤	Food·consumed¤	Food·weight·(g)¤	
	¤	¤	·····¤	g¤
			¤	g¤
			¤	g¤
			¤	g¤
			¤	g¤
	¤		¤	g¤
			¤	g¤
			¤	g¤
¶ ¶ ¶ ¶ Lunch¤	¤	¤	¤	g¤
			¤	g¤
			¤	g¤
	¤		¤	g¤
			¤	g¤
¶ ¶ ¶ ¶ Breakfast¤	¤	¤	¤	g¤
			¤	g¤
			¤	g¤
	¤		¤	g¤
			¤	g¤

¶

8.9 Main Trial Data Sheet

RPE$_{TREADMILL}$ DATA SHEET			
PARTICIPANT NUMBER:		**DATE:**	
TRIAL: (Pre training, post training, 4-days post)			
URINE OSMOLARITY: (Below 600 mOsmol·kg^{-1})		**RESTING HR:** (Completed during forms)	
PRE WATER VOLUME: (Total weight – water bottle weight)		**ROOM TEMPERATURE/ HUMIDITY:**	
HEIGHT (cm):		**WEIGHT** (kg):	

Time	RPE (6-20)	Speed (Km/h^{-1})	HR (beats.min^{-1})
1			
5			
6			
10			
11			
15			
16			
20 *No water*			
21			
25			
26			
30			

POST WATER VOLUME:	
POST WEIGHT (kg):	

FORM CHECKLIST Tick box if completed	
Health questionnaire	
Blood consent form	
REST-Q 76	
Jackson URTI questionnaire	

10 KM time trial	