The Effect of Diurnal Phase on Performance Physiology and Immune System

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Declaration

It is hereby declared that this thesis and the research work upon which it is based were conducted by the author.

Peter Tormey

Abstract

The circadian cycle, although seasonally adjusted, consists of two phases: a nocturnal period of darkness and a diurnal period of light. Many parameters of human physiology display either a nocturnal or diurnal peak as seen in athletic performance, immune, endocrine, physiology and cognitive function, respectively. The studies included in this thesis focused on the diurnal (morning or AM versus evening or PM) effect of high-intensity self-paced exercise on physiological and immunological measures and the influence of individual preference for activity or chronotype on these responses. Self-paced time-trials were completed in an environmental chamber (6°C) in the AM and PM and displayed a similar effect on physiological and biological parameters. Performance times were non-statistically quicker ($P>0.05$) at PM. Physiological parameters heart rate, rate of perceived exertion, lung function and self-paced treadmill speed were not found to be statistically different at AM or PM trial ($p> 0.05$). Core body temperature (CBT), was significantly higher ($p<0.05$) at 16.00hrs and 17.00hrs (pre-PM trial) and at the 1km stage compared to AM trial. CBT displayed the greater response at AM trials from pre to post increasing by 1.34°C. Circulating lymphocyte and neutrophil cell counts displayed an increase in response to exercise but an interaction between exercise and diurnal phase was not observed ($P>0.05$). CD8+ T-lymphocyte cell-surface markers for naïve/senescence and differentiation (CD27CD45RA and CD27CD28) reported no diurnal difference between AM and PM exercise in experienced and experienced/recreational distance runners respectively ($P>0.05$). However, a higher percentage of highly

differentiated phenotypes were found in the recreationally active population. Neutrophils displayed a pronounced elevation in response to PM exercise with the mechanism driving this response unclear at this time. Cortisol concentration displayed less inflammatory responses in the morning compared to the evening with higher values pre, post and one-hour post AM trials. Chronotype showed no effect on physiology or biology at rest or in response to exercise.

Chapter 4 presents data from a study that investigated diurnal physiology and immune response to high-intensity exercise in highly-trained men. Recreational and experienced endurance male runners at differing diurnal time-points were investigated in *Chapter 5,* while individual chronotype differences and circadian phase responses were explored in *Chapter 6*.

In summary, it is concluded from this work that there was a lack of evidence showing a diurnal effect on running performance and subsequent immune response. Elevated circulating immune counts prior to exercise, irrespective of diurnal phase, appear to govern exercise-induced responses. The effect of high intensity exercise is subject to three distinct variables: the fitness status and experience of the individual completing the exercise, the time of day at which the exercise is undertaken, and the phase response of exercise at that point of the circadian cycle. No diurnal phase mediated a divergent effect on variables examined was observed at 09.00hrs and 17.00hrs. These time-points should be considered not sufficiently dissimilar to elucidate diurnal variation in trained and healthy males.

Dedication

This thesis is dedicated to the memory of my beloved Mam, Mary Tormey. Your dignity, bravery and resolute courage inspires me to challenge myself in work and in life.

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To my family who have been a beacon of strength to me over the years. My self-enforced exile will soon be over! I look forward to catching up for lost time

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Finally, and most importantly, this thesis would not have been possible without the sacrifice, love and support of the most remarkable person I know, Sabrina. For too long you gave everything you could and requested little in return. I will always love you and try my best to make you proud.

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Abbreviations and Symbols

AHR	Airway Hyperresponsiveness
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
APC	Allophycocyanin
APCs	Antigen Presenting Cells
Bmal1	Brain and muscle aryl hydrocarbon receptor nuclear translocator like 1 (Arntl)
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CD4TL	CD4+ T-lymphocytes
CD8TL	CD8+ T-lymphocytes
CLOCK	Circadian Locomotor Output Cycles Kaput
CM	Central Memory
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disease
Cry1	Cryptochrome 1
Cry2	Cryptochrome 2
CTLs	Cytotoxic T-Lymphocytes
DBP	Diastolic Blood Pressure
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay
EM	Effector Memory
EM45RA	CD45RA+ Effector Memory
FCS	Foetal Calf Serum
FSC	Forward Scatter
FEF	Forced Expiratory Flow
FEF_{25-75}	Forced Mid-Expiratory Flow
FEV1	Forced Expiratory Volume in 1 Second
FITC	Fluorescein Isothiocyanate
FVC	Forced Vital Capacity
HPA	Hypothalamus Pituitary Adrenal
HR	Heart Rate
Hrs	Hours
IL	Interleukin
KLRG1	Killer Lectin-Like Receptor G1
Km	Kilometre
MAb	Monoclonal Antibody
MEQ	Horne-Östberg Morningness Eveningness Questionnaire
MESOR	Midline Estimating Statistic of Rhythm
MHC	Major Histocompatibility Complex
NA	Naïve

NK (cell)	Natural Killer (cell)
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cells
PE	R-Phycoerythrin
PEF	Peak Expiratory Flow
Per	Period
PerCP Cy-5	Peridinin-Chlorophyll Proteins Cyanine 5
Per1	Period Homolog 1 (Drosophila)
Per2	Period Homolog 2 (Drosophila)
Per3	Period Homolog 3 (Drosophila)
REV-ERB-α	Reverse Viral Erythroblastis Oncogene Product α (Nr1d1)
RHT	Retinohypothalamic Tract
RNA	Ribonucleic Acid
ROR	Related Orphan Receptor
RORE	Retinoic Acid-Related Orphan Receptor Response Element
ROS	Reactive Oxygen Species
RPE	Rating of Perceived Exertion
SBP	Systolic Blood Pressure
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation
SNS	Sympathetic Nervous System
Ssc	Side Scatter
T-lymphocyte	T-cell
TCR	T-cell Receptor
TRAEM	Effector-Memory T-cell Re-expressing CD45RA
Th	T-helper
TNF	Tumor Necrosis Factor
ug	Microgram
ul	Microlitre
URTI	Upper Respiratory Tract Infection
$\dot{V}O_2max/peak$	Maximal/Peak Oxygen Uptake

Glossary of Terms

(as defined by the Centre of Chronobiology, Basel)

Acrophase: Phase angle corresponding to the maximal value of the rhythmic parameter studied.

Amplitude: The measure of one half of the extent of the rhythmic change estimated by the mathematical model (e.g., cosine curve) best fitting to the data (e.g., the difference between the maximum and the rhythm-adjusted mean of the best fitting curve).

Biological clock: Self-sustained oscillators which generate biologic rhythms in absence of external periodic input (e. g., at the gene level in individual cells).

Biological rhythm: A cyclical, repeated variation in a biological function.

Chronobiology: The science of investigating and objectively quantifying phenomena and mechanisms of the biologic time structure, including the rhythmic manifestations of life. Term derived from: Chronos (time), bios (life), and logos (science).

Circadian rhythm: Circa-rhythm of metabolic, physiological or behavioral processes with a naturally synchronized period of 24 hours (the term 'circadian' is derived from the Latin *circa* meaning about and *diem* meaning day).

Cycle: Recurrence of events, without necessarily being of periodic nature (in contrast to rhythm).

Desynchronization: State of two or more previously synchronized rhythmic variables that have ceased to exhibit the same frequency and/or the same acrophase relationships and show different than usual and/or changing time relations.

Diurnal: Activity or event occurring in the day between dawn and dusk.

Endogenous rhythm: An oscillating system capable of self-sustained oscillations.

Entrainment: Coupling of an endogenous rhythm to an environmental oscillator with the result that both oscillations have the same frequency. In contrast to masking, the phase of the endogenous rhythm is affected by entrainment.

Exogenous rhythm: An oscillating system not capable of self-sustained oscillations, but passively driven by external factors.

External desynchronization: Desynchronization of a biologic rhythm from an environmental cycle.

Free-running: Frequency manifestation of self-sustained oscillations, with a periodicity deviating from (eventual) zeitgebers.

Frequency (f): The number of cycles occurring per time unit; f is the reciprocal of the period (t).

Frequency ranges: Groups of frequencies (or periods) frequently encountered in biologic rhythms. (Circadian frequency range: rhythm with periods of about one day, i. e., by definition > 20 to < 28 h).

Infradian rhythm: Non circa-rhythm with a period longer than 24 hours.

Internal desynchronization: State in which two or more previously synchronized variables within the same organism have ceased to exhibit the same frequency and/or the same acrophase relationships and show different than usual and/or changing time relations.

Masking effect: Overruling of the expression of endogenous rhythm by random or non-random environmental stimuli only temporarily, without affecting the phase of that rhythm (in contrast to entrainment).

Melatonin: A hormone produced rhythmically in vertebrates by the pineal gland, a pea sized organ at the centre of the human brain.

Oscillator: Mechanism leading to the manifestation of a rhythmic phenomenon (oscillation), that either is self-sustained (pacemaker), or depends on another oscillator (passive or slave-oscillator), or is characterized by a decrease in amplitude (damped oscillator).

Pacemaker: A functional entity capable of self-sustaining oscillations.

Peak: The highest point in a series of measurements obtained as a function of time.

Period: Time after which a defined phase of the oscillation re-occurs.

Phase: Instantaneous state of an oscillation within a period (reference point).

Phase advance: Shortening of period for one to a few cycles (denoted by a plus sign).

Rhythm: Periodic recurrence of events; not synonymous with cycle.

Suprachiasmatic nucleus (SCN): Group of neurons situated above the optic chiasm in the vertebrate hypothalamus exhibiting an endogenous circadian oscillation acting as circadian pacemaker, receiving external phase information via the retina.

Synchronization: State in which two or more oscillations have the same frequency due to mutual or unilateral influences (referring both to entrainment and to masking).

Temperature compensation: The phenomenon exhibited by biological clocks that results in them not responding to different ambient temperatures in a way that would be expected according to normal physiological Q10 principles. In simple terms Biological Clocks are not effected by different temperatures, whereas other physiological systems are.

Trough: The lowest point in a series of measurements obtained as a function of time.

Ultradian rhythm: Non circa-rhythm with a period less than 24 hours.

Zeitgeber: From the German for meaning “time giver”. A periodic environmental signal that entrains some biological rhythm, for example light, temperature, or social zeitgebers. It is important to understand that the “zeitgeber” does not induce a rhythm but determines its arrangement in time

Chapter 1: General Introduction

All living organisms are under the influence of daily and seasonal changes from the Earth's orbit around the sun (Refinetti, 2006). The daily light-dark cycle is considered the most influential factor that synchronises biological function to the environment (Lucas *et al.*, 2014; Yamashita *et al*., 2014). This is the basis of predictive homeostasis (Moore-Ede, 1986), which evolved as an adaption to anticipate predictable changes in the environment, for example light and dark, eating times, ambient temperature or periods of heightened activity. Consequently, this predictable, periodic daily timing system, or circadian system, is essential for all living organisms populating the earth as it acts as a multifunctional system synchronising endogenous biologic and physiologic parameters from within the organism to the environment.

In humans, circadian rhythms are found in almost all systems, including the immune system (Ackermann *et al*., 2012; Bollinger *et al*., 2011; Lange and Born, 2011; Dimitrov *et al*., 2010; Keller *et al*., 2009; Suzuki *et al*., 1997), the endocrine and nervous system (Dimitrov *et al*., 2009; Levi and Schibler, 2007; Redwine *et al*., 2000), which all display distinct patterns over a 24-hour period. Circadian timing is governed by tissue-specific peripheral clocks, found, for example in the heart, liver and the digestive tract (Yamzaki *et al*., 2009; Yoo *et al*., 2004). Although peripheral clocks are self-pacing, they are synchronised to the organism via constant communication by the master pacemaker: the suprachiasmatic nuclei (SCN) (Yamazaki *et al*., 2000). The trillions of peripheral cellular clocks in primates are synchronised by a few thousand neurons located in the SCN (Berczi *et al*., 2007). The SCN, which is situated in the hypothalamus in the brain, receives continuous information via light

signalling, communicating a coherent response to the peripheral oscillators (Golombek and Rosenstein, 2010).

Since the Industrial Revolution in the 18th century, much of the world has progressively become an increasingly fast-paced, 24-hour society. The invention of the light bulb in 1879 paved the way for around-the-clock lighting, profoundly changing society's activity, work, social and sleep patterns. No longer did sunrise signify the start of the day and sunset the end. Sleep, as reported by Ekirch (2001), in the pre-industrialised world commenced after darkness fell and was seasonally-adjusted. Unlike today's sleep patterns in the industrialised world, pre-industrialised Britain slept in two phases of three to four hours of sleep separated by one to two hours of activity (Ekirch, 2001). Consequently, internal biologic rhythms were in-tune or 'synchronised' to the natural world.

Contemporary work, social interaction and activity (for example exercise) patterns have become fluid with each activity commonly occurring at all times of the day and night (Noon *et al*., 2013). Work-shift patterns in the United Kingdom represented 17.4% (The Office of National Statistics, 2014) of the work force in 2014, with employees most commonly working a morning shift (starting at 06.00 hours (hr) and finishing in the early afternoon), evening shift (typically 14.00hrs to 22.00hrs) or a variant of a nightshift (18.00hrs to 06.00hrs). Social interactions have similarly changed with pastimes and hobbies undertaken at all hours of the day and night (Noon *et al*., 2013). Exercise and sporting activities fit around work and life commitments, typically performed in the early evening or early morning. Competitive endurance

events, for example, generally start between 05.00hrs and 07.00hrs (Kunorozva *et al*., 2012). As a result of contemporary societal pressures, issues around circadian dysregulation have become prevalent.

The circadian system evolved to optimise the physiological and cellular functions, synchronising the host to their environment. Dysregulation of the circadian system increases the incidence of obesity (Di Lorenzo *et al*., 2003), cardiovascular disease (Ha and Park, 2005), diabetes mellitus (Szosland, 2010), mental illness, cancer (van Leeuwen *et al*., 2009; Shea *et al*., 2005), circulating hormone and immune levels (Arjona and Sarkar, 2008; Coogan and Wyse, 2008; Suzuki *et al*., 1997).

The immune system is a remarkably adaptive defence system from a complex network of cells and molecules capable of specifically recognising and eliminating infectious microorganisms (Gleeson, 2013). The principal function of the immune system is to preserve self-integrity and to identify and remove any foreign or infected element not recognised as self (Gleeson *et al*., 2013).

The immune system can be separated into two distinct but overlapping subsystems, the innate and the adaptive immune systems. As with biologic rhythms, the immune system has evolved over millennia, with the adaptive immune system first emerging some 300 million years ago in cartilaginous fish (Geenen and Chrousos, 2004). The immune system displays distinct circadian organisation, with the hormones melatonin and cortisol playing a pivotal role in both circadian organisation and modulating many immune parameters (Dimitrov *et al*., 2009; Kudielka *et al*., 2009; Redwine *et al.*, 2000).

Melatonin exhibits remarkable immunomodulatory properties as displayed by the synchronisation of melatonin production and circadian and seasonal adjustments in the immne system (Carrillo-Vico *et al*., 2006; 2005; Reiter, 2003). Secreted from the pineal gland, melatonin modulates an array of physiological functions including immunostimulant and anti-inflammatory actions in a time of day and light-sensitive manner. Pineal abalation, by surgical or functional means, promotes weightloss of lymphoid organs and altered immune profile in mammals with a reversal of these effects with melatonin administration. Research confirms the presence of melatonin receptors on circulating immune cells, lymphatic tissues (Nelson and Draven, 1999) with melatonin concentrations correlated with the production of pro-inflammatroy cytokines TNF-α, IL-1 and IL-12 facilitating immune cell differentiation, activation and proliferation (Radogna *et al*., 2010). Conversely, catecholamines and cortisol act in a synergistic manner to melatonin by suppressing immune function by promoting anti-inflammatory actions in a similarly circadian influenced pattern.

The neuroendocrine and autonomic nervous systems are considered the primary systems communicating circadian information generated in the hypothalamus. These two systems modulate many immune responses, for instance, the number of immune cells (e.g. lymphocytes, neutrophils and cytokines such as Il-6) in peripheral blood compartments display circadian variation (Cermakian *et al*., 2013; Logan and Sarkar, 2012) indicating that the circadian system plays a role in modulating immune function. Importantly

however, ablation of the SCN does not affect the release of all immune cells as it remains unchanged in bone marrow (Filipski *et al*., 2004).

Stimulation of the immune system by physiological (Gleeson *et al*., 2013), psychological (Segerstrom and Miller, 2004) or emotional (Damjanovic *et al*., 2007) stress displays similar temporal responses in the adaptive and innate immune systems. However, if a stressor is repeated or continuous without an adequate recovery period, for example, in caregivers (Damjanovic *et al*., 2007) or in ultra-endurance type events (Mooren *et al*., 2002) immune function may be detrimentally altered, increasing the risk of illness or lowered immunity.

Exercise immunology is a relatively recent, albeit a rapidly growing research area. This discipline was first developed from suggestions of adverse effects of prolonged high-intensity exercise on human immunity and the incidence of viral and bacterial infections in athletes, compared to those who completed moderate amounts of physical activity (Nieman, 1994). Although initially led by anecdotal reports, early researchers quickly established scientific evidence to support athletes' and coaches' experiences of greater incidences of infection post-endurance events and after periods of heavy training (Mooren *et al*., 2002; Steensberg *et al*., 2002; Peters, 1997; Nieman, 1994). In 1994, the J-shaped curve, a model suggesting that the relationship between the risk of upper respiratory tract infection and exercise intensity/volume, was first reported by David Nieman. Indeed, this discipline has developed the scientific understanding as to the effect of varying levels of physical activity on immune parameters (Matthews *et al*., 2002), repeated bouts of exercise (Ronsen *et al.,* 2001), nutritional deficiency to the exercising athlete (Gleeson, 2007; Calder

and Kew, 2002) and age-associated immunosenescence (Simpson *et al*., 2007; 2006) to provide just a few examples.

Thus far, research in exercise immunology has aimed to analyse the functionality of physiological stress on immune response. The interaction between the immune, nervous and endocrine systems appears to hold the key to fully understanding the homeostatic organisation of host defences, and indeed, the dichotomy of induced-immune response. The interaction of these three distinct systems in regulating immune response has been well established in animal models and a new emerging multidisciplinary science, neuroimmune biology, has been developed.

The majority of research to date in the area of exercise immunology focuses on the effect of exercise on immune response at a narrow circadian time point, due in most part to the practical issues of multiple sample time points. The result of which is an abundance of research at specific times of the 24-hour solar day that does not fully represent the dynamic oscillations that are present within human physiology. Research using laboratory conditions has, by manipulation of environmental and nutritional factors, investigated human physiological capacity over defined periods of time. However, thus far, to the best of the author's knowledge, no research has explicitly investigated diurnal variation in exercise-induced immune responses under free-running laboratory settings.

It has long been known that immune cells are not a homogenous group free from a circadian rhythm, but rather a complex, dynamic and distinct collection of cells synchronised to their environment to provide optimum host protection

(Logan and Sarkar, 2012). The seven subsequent chapters presented in this thesis investigate diurnal variation in physiological and immunological parameters, in response to an induced stress that is time-trial running. These chapters are detailed as follows:

- **Chapter 2:** This chapter offers a review of the pertinent literature of chronobiology and exercise immunology, in relation to performance.
- **Chapter 3:** This chapter details the general material and methods used during the experimental part of data collection and analysis.
- **Chapter 4:** This chapter presents data from a study that investigated diurnal physiology and immune response to high-intensity exercise in highly-trained men.
- **Chapter 5:** This chapter presents data from a second study that investigated physiological and immune responses in recreational and experienced endurance male runners at differing diurnal time-points.
- **Chapter 6:** This chapter presents data from study two investigating chronotype and circadian phase responses in immune and physiological parameters at diurnally different time-points.
- **Chapter 7:** This chapter presents general discussion of the main findings contained within this thesis. Conclusions, limitations and suggestions for future research are presented.

Chapter 2: Literature Review

This chapter provides a general overview of the relevant literature for the studies described within this thesis. Each of the subsequent chapters provide greater detail to the relevant literature in respective research areas. The literature cited in this thesis primarily focuses on studies with humans. However, research using animal or *in vitro* models is acknowledged when cited. This literature review chapter is divided into two main sections: The first part introduces chronobiology, focusing on circadian rhythms in physiology, the circadian clock and individual differences. The second part introduces the immune system, primarily focusing on leukocytes, the effect of exercise on the redistribution of immune cells and phenotype markers distinguishing T-lymphocyte subset activation status. A brief summary exploring circadian rhythms in the immune system concludes this chapter.

2.1 An Introduction to Chronobiology

For successful and efficient survival, all organisms populating the earth, including humans, require the ability to anticipate and respond to their environment. Evolution has ensured that this environmental adaption for all living organisms, and especially mammals, occurs without conscious thought or control by way of biological rhythms orchestrated from within the organism, synchronising biologic function to the environment (Lucas *et al.*, 2014; Yamashita *et al*., 2014). The most prevailing synchronising environmental factor is the light-dark cycle (Refinetti *et al*., 2006; Waterhouse *et al*., 2004). However, the synchronising of the host organism to the environment occurs

over many time periods or biologic cycles, with each rhythmic cycle constantly adjusted to maintain homeostasis (Moore-Ede, 1986).

Early work conducted in the nineteenth century by the eminent physiologist Claude Bernard (1865), *An Introduction to the Study of Experimental Medicine*, first reported the concept of homeostatic, steady-state physiology of internal regulatory systems. More latterly seminal work, *The Wisdom of the Body* (Cannon, 1932), developed Bernard's novel homeostatic approach to physiology further, emphasising an integrated holistically co-ordinated communication of bodily systems to maintain equilibrium. Contemporary understanding of physiological inputs communicating signals in a paracrine manner can be traced back to Rosenbleuth, Wiener and Bigelow (1943), which drew on the early work of homeostatic control, when the theory of continuous feedback between systems of living organisms maintaining homeostasis was presented.

In humans, the distinction of the various biologic rhythms that exist can be broadly categorised into three main domains: ultradian, infradian and circadian rhythms, respectively (Koukkari and Sothern, 2006; Reilly *et al*., 1997). Circadian rhythms are periodic cycles that present for approximately 24 hours (20 to 28 hours). Infradian rhythms, for example the menstrual cycle, occur over periods of time more than 24 hours, while cycles that last less than 21 hours are referred to as ultradian rhythms. Importantly, the rhythmic predictability of these cycles are the fundamentals of researching biological rhythms. The role of these rhythms is to adapt to the anticipated environmental demands most efficiently to preserve life and normal function. In humans, this

is generally considered good health, regular sleep patterns and social interaction with others. Chronobiology as a research area investigates these periodic biological cycles and how they are adapted to the diurnal or solar-lunar rhythms (Bron and Furness, 2009; Atkinson *et al*., 2003).

Living organisms, including human beings, respond to cyclic physiological (Drust *et al*., 2005; Atkinson and Reilly, 1996) and psychological changes (Curtis *et al.*, 2014) caused by time, known as biological rhythms (Reilly *et al*., 1997). The terms biological rhythm and especially circadian rhythm, can often be misunderstood due to the interchangeable use of terminology between research disciplines and indeed in general life. As a consequence, for example, circadian rhythm can often be confused as a synonym for 'daily' or for a period of time that is exactly 24 hours in duration. However, in the discipline of chronobiology this is not an accurate representation as 'daily', in fields out-with this discipline, may refer to day-to-day differences and not the circadian phase as defined by a rhythm that fluctuates in a regularly, albeit not an exact, manner. In this context, terms such as 'diurnal' or 'nocturnal' are used to specify day-time and night-time phases of the circadian period, where activity in cycles may peak or reach their lowest point. For example, sleeping time (Reilly and Edwards, 2007), human physiology (reviewed by Golombek and Rosenstein, 2010), haematology (Ackermann *et al*., 2012), immune (Lange and Born, 2011; Dimitrov *et al*., 2010; Suzuki *et al*., 1997; Palm *et al*., 1996) and endocrine parameters (Dimitrov *et al*., 2009; Redwine *et al*., 2000) display peaks and troughs that follow distinct diurnal or nocturnal cycles under healthy conditions. Importantly, these rhythms do not follow a very precise 24-

hour cycle, rather they are subject to the influence of both intrinsic (endogenous) and extrinsic (exogenous) factors that impact and, in many respects, synchronise the organism to their environment (Adan *et al*., 2012).

Endogenous rhythms are those structures that originate from internal channels, which contain clock-like pulsations that oscilate in a predicable, if not precise, cycle (Arendt, 2010; Weinert and Waterhouse, 2007; Reilly *et al*., 1997). An example of such includes the pacemaker cells of the heart and the suprachiasmatic nuclei (SCN) situated in the brain (Weinert and Waterhouse, 2007; Reilly *et al*., 1997). These inherent oscillations are aided to remain synchronised and entrained through external exogenous cues (Reilly *et al*., 1997), the most prevailing of which being the solar light-dark cycle (Maywood *et al*., 2007). Considering this, the amplitude of circadian phase may vary from day-to-day within individuals or between one individual and another. However, the interaction of cues emanating from external and internal synchronisers produces a rhythm that is fine-tuned to approximately a 24-hour period and can be anticipated (Adan *et al*., 2012; Rajaratnam and Arendt, 2001). Even under controlled and synchronised laboratory conditions, an identical circadian phase from one period to the next is improbable due to the many exogenous and endogenous factors that are interpreted by the central and peripheral clocks (Atkinson *et al.*, 2005).

Circadian rhythms refer to the combination of daily biological cycles and metabolisms (Maywood *et al*., 2007) and their interaction over a 24-hour period (Reilly and Waterhouse, 2009; Reilly *et al*., 1997). The existence and timing of such biological rhythms have been shown to be driven by

endogenous components which oscillate and fluctuate over a time period of approximately 24 hours (Reilly and Waterhouse, 2009; Reilly *et al*., 1997). Endogenous components are those mechanisms that originate from internal channels (Arendt, 2010; Weinert and Waterhouse, 2007; Reilly *et al*., 1997) which entrain the organism to the environment to which it is present through exogenous (external) cues (Reilly *et al*., 1997), the most prevailing of which is the solar light-dark cycle (Maywood *et al*., 2007).

The suprachiasmatic nuclei (SCN) situated in the base of the hypothalamus region of the brain is considered the central pacemaker of the circadian system (Weinert and Waterhouse, 2007; Reilly *et al.,* 1997). Light-signalling (photoperiod information received from the environment) passes continuously via the retinohypothalamic tract (RHT) to the SCN to provide temporal information for constant host entrainment (as illustrated in figure 2.1). More recently, the non-image-forming photopigment melanopsin expressed on retinal ganglion cells (comprising approximately 1-5% of total retinal tissue) has been identified to project directly to the SCN, entraining circadian rhythms (Kalsbeek *et al*., 2012a; Hattar *et al*., 2006; Melyan *et al*., 2005; Panda *et al*., 2005; Lucas *et al*., 2003; Berson *et al*., 2002; Panda *et al*., 2002; Ruby *et al*., 2002; Provencio *et al*., 2000). Unlike the vast majority of retinal tissue which is important for interpreting spatial patterns and image-forming, the role of melanopsin seems predominantly associated with photoreception and relaying information to the SCN. Importantly, melanopsin sensitivity to light is independent to image-forming retinal cells and has been found in some individuals without functional sight (Czeisler *et al*., 1995) and when these

tissues are isolated *ex vivo* (Berson *et al*., 2002). Furthermore, although external light stimuli provide supplementary information to the SCN, informing its rhythmic output, the autonomy of the structure remains absolute (as demonstrated by Clayton *et al.* (2001) with *in vitro* analysis showing circadian rhythm).

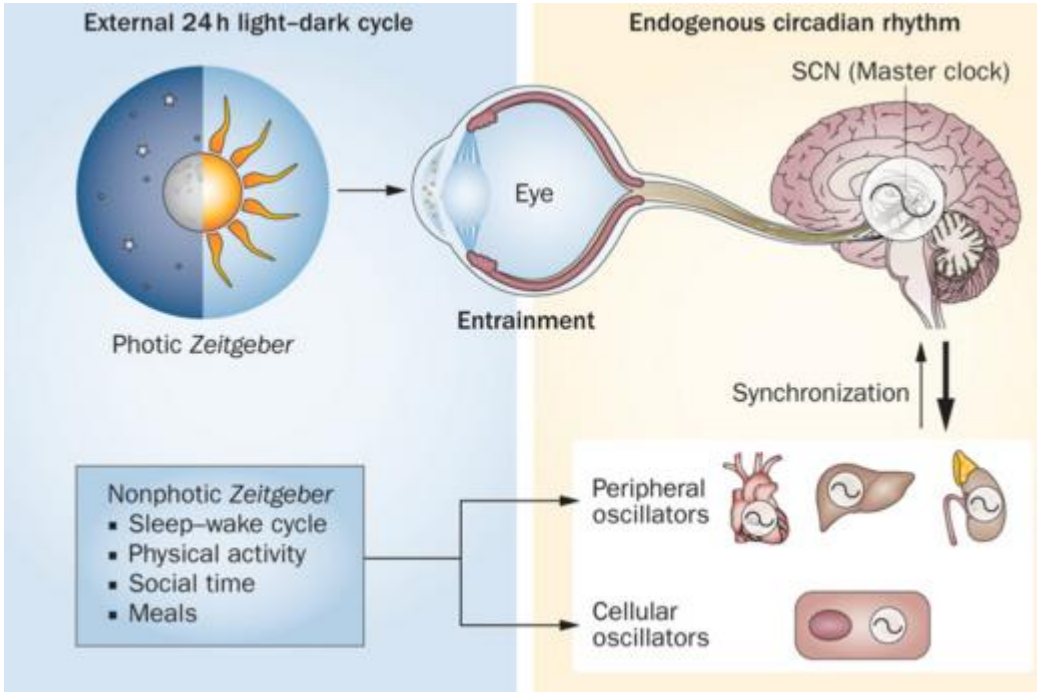


Figure 2.1. Internal circadian clocks and external zeitgebers (adapted from Buttgereit *et al*., 2015).

The SCN's role as the major synchroniser of the circadian system comes from its ability to orchestrate peripheral tissues, which have their own innate ability to self-sustain circadian oscillations (Yoo *et al*., 2004). For example, the phase of peripheral tissues may differ from the SCN (Lowrey and Takahashi, 2004; Balsalobre, 2002). As such, the SCN does not completely control peripheral tissues but rather provides an input to communicate the circadian phase, serving as a synchroniser without which peripheral clocks dampen (Yoo *et al*.,

2004; Yamazaki *et al*., 2000). The importance of the SCN as the dominate pacemaker controlling circadian rhythms can be seen with reference to it being "the site of the body clock in humans" (Waterhouse *et al*., 2002, pp. 104) and 'master pacemaker' (Maywood *et al*., 2007, pp. 261).

2.2 Circadian Rhythm and Physiology

Circadian rhythms rise and fall from times of minimum physiological functional ability or nadir (broadly 01.00hrs to 10.00hrs) reaching minimal responsiveness at 06.00hrs (Waterhouse *et al*., 2004), to times of maximum physiological ability or acrophase (broadly 14.00hrs to 21.00hrs), peaking at the hours between 20.00hrs and 21.00hrs (Waterhouse *et al*., 2004; Reilly *et al*., 1997). Extensive literature exists assessing the role of circadian influences on physiological performance capabilities throughout a 24-hour day (see figure 2.2, below) (Drust *et al*., 2005; Atkinson *et al*., 2003; Reilly *et al*., 1997). Circadian influenced periods of time have been shown to mirror daily oscillations in core body temperature (Benloucif *et al*., 2005; Waterhouse *et al*., 2004; Aldemir *et al.,* 2000; Reilly *et al*., 1997). Sporting or physiological performance capabilities broadly correlate with the rise in core body temperature and circadian phase (Edwards *et al.,* 2013; Florida-James and Doggart, 2000). Research demonstrates significant variations in muscular strength (Guette *et al*., 2005; Gauthier *et al*., 2001; Callard *et al*., 2000), lung function (Goel *et al*., 2015), aerobic athletic performance (Taylor *et al*., 2011; Drust *et al*., 2005) and prolonged endurance events, with amplitudes ranging from 2% to 20% (Waterhouse *et al*., 2004; Reilly *et al*., 1997).

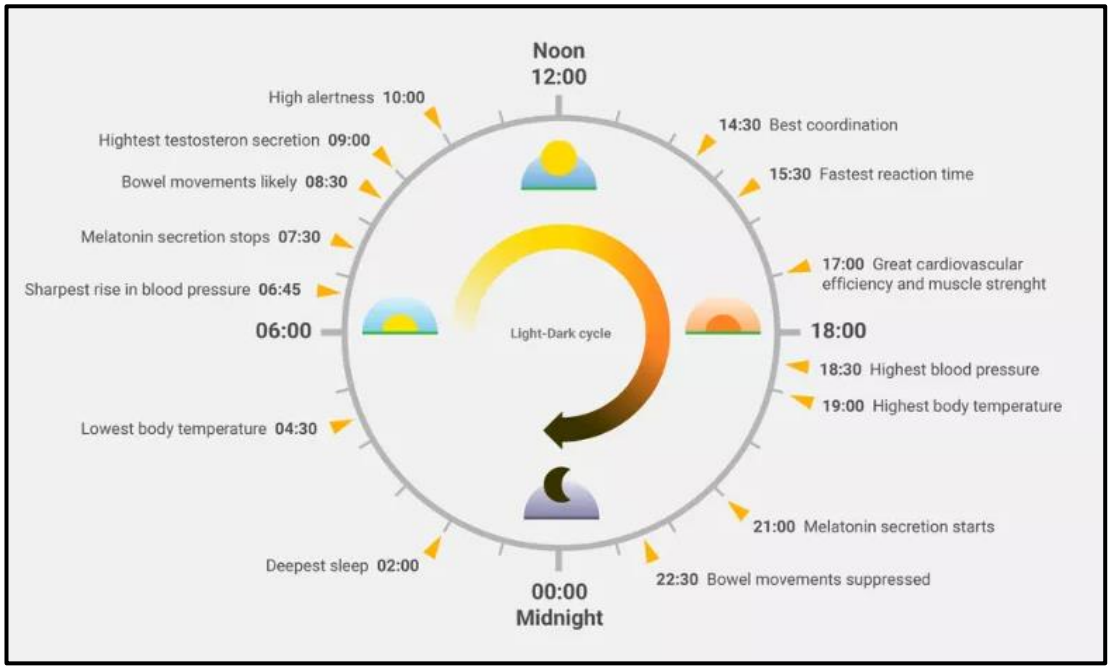


Figure 2.2. Circadian rhythms in human physiology and behaviour.

The absorption of numerous drugs, in many forms, have been demonstrated to display distinct circadian rhythms. The oral administration of the drugs temazepam (Muller *et al*., 1987), diazepam (Nakano *et al*., 1984), paracetamol (Kamali *et al*., 1987) and many non-steroidal anti-inflammatories (NSAIDs) (De Mey *et al*., 1992; Mustofa *et al*., 1991) for example, are absorbed faster in the morning-diurnal phase compared to evening efficacy. Gastric acidity (pH) (Moore and Englert, 1970), enzyme activity and emptying (Reinberg *et al*., 1982), respectively, all show pronounced circadian rhythmicity affecting the absorption rates of drugs administered orally further emphasising the predictability of human physiology under healthy sleep-wake conditions (Figure 2.2). Similarly, the transport of drugs from within the cardiovascular system, to the target sites of absorption, also show a predictable chronobiological cycle by way of a 30% increase in cardiac output and greater blood flow in renal circulation partly facilitating the circadian rhythm reported

(Fagiolino *et al*., 2006). In addition to the effect of the respective circadian rhythms within the gastric and cardiovascular systems, the binding of a drug to an unbound plasma protein similarly demonstrates robust diurnal peaks (16.00hrs) and nocturnal (04.00hrs) throughs (Angeli *et al*., 1978) further augmenting circadian rhythmicity.

The terms amplitude and phase are used commonly in chronobiology and require clarification. Using mathematical modelling, a cosine curve is formulated with the magnitude of the minimum to peak (usually the mean) values used to report the amplitude of a rhythm (Reilly *et al*., 1997). Importantly, the calculation of an amplitude, by way of a cosine curve, is essential as without it, a rhythm cannot be reported (Koukkari and Sothern, 2006). Furthermore, a phase, which is a repeatable state of a cycle, is a characteristic of varying stages of a rhythm, as defined above. Using precise measures such as the phase and cosine curve mathematical model, a Midline Estimating Statistic of Rhythm or MESOR (Halberg *et al*., 1979) allows the researcher to quantify the rhythmic nature of variables, i.e. bathyphase being the lowest point on a cosine model and acrophase the highest, for comparison and analysis.

In mammals, the major external environmental factor for synchronising or entraining the biologic clock is light (photic information) (Corbett *et al*., 2012; Panda *et al*., 2002; Freedman *et al*., 1999) (figure 2.2). Often referred to as a zeitgeber (a German word for ‘time giver’), external cues, for example light, provide an input that stimulates the SCN, via the RHT found in the eyes (Koukkari and Sothern, 2006; Refinetti *et al*., 2006; Reilly *et al*., 1997). As

such, photic information constantly flows to the SCN, fine-tuning and entraining the host to the environment continuously (Roenneberg and Merrow, 2002). Thus, photic information received by the RHT is relayed to the SCN and transduced (converted) to output signals, for example hormonal or neural (Lowrey and Takahashi, 2004). This adjustment of the human body clock by way of zeitgebers occurs partially due to the body clock's poor time-keeping ability and the ever changing environmental conditions (Duffy *et al*., 2001; Reilly *et al*., 1997).

As such, exposure to light, and especially sunlight, in the morning or at the start of the circadian phase in humans (or diurnal mammals) entrains the internal rhythm to the environment subtly. Early research suggested illuminance of 2500 lux was necessary to suppress nocturnal levels of melatonin and alter circadian phase (Moore-Ede *et al*., 1980) with exposure to light of 3000 lux resulting in a maximal alteration to endogenous rhythmicity (Kripke *et al*., 2007). However, more recent research has demonstrated that exposure to as little as 1 to 1000 lux can suppress melatonin in humans (Wright *et al*., 2013; Glickman *et al*., 2002). Melatonin (synthetic) has been used for therapeutic purposes to alleviate the phenomena of jetlag by inducing sleep and 'resetting' the circadian clock caused by desynchronisation to the natural environment, associated with long-haul travelling through time-zones (Fowler *et al*., 2015). Conversely, the absence of zeitgebers in a controlled environment, such as a darkened room, would have the opposite effect to light exposure therapy (Appleman *et al*., 2013; Corbett *et al*., 2012). Notwithstanding a change to time-zones, indoor lighting and electronic devices

(blue light) with a lux of less than 40 to 200 also display the propensity to alter the circadian phase (Najjar *et al*., 2014; Wood *et al*., 2013).

Although daylight is considered to be the strongest and most influential of zeitgebers, many other external environmental cues can also play a significant role. Examples of such include levels of physical activity, social interactions and habitual meal times (Drust *et al*., 2005; Damiola *et al*., 2000; Reilly *et al*., 1997). Ambient air temperature has also been shown to play a considerable role as a zeitgeber adjusting the circadian phase (Waterhouse *et al*., 2004). The hypothalamus regulates a temporal compensatory mechanism where environmental conditions stimulate a coherent response mechanism. Under thermalneutral conditions, homeostatic control of body temperature follows a regular circadian pattern, whereas heat-loss due to cooler environmental conditions promotes energy-conserving mechanisms such as vasoconstriction to limit the rate of heat leaving the body (Refinetti *et al*., 2006; Reilly *et al*., 1997). Conversely, vasodilation occurs in response to higher body temperatures with increased blood flow to the skin and periphery, facilitating the promotion of heat-loss (by sweating) (Waterhouse *et al*., 2004; Reilly *et al*., 1997). The balance between the mechanisms that preserve the thermal state in humans relies on constant communication via the cells of the hypothalamus and the SCN with the consequential outputs from the SCN synchronising a holistic integrated response.

The release of the hormone melatonin (synthesised from serotonin) by the pineal gland is also viewed as an important zeitgeber contributing to the timing of the circadian clock (figure 2.2) (Atkinson *et al*., 2003). Melatonin is released

from the pineal gland during the first two to four hours of sleep in the absence of natural daylight (Waterhouse *et al*., 2004; Redwine *et al*., 2000), while Atkinson *et al*. (2003) report that the secretion of this hormone is strongly correlated to the decline of body temperature, which also occurs at a similar stage of the light-dark cycle (Waterhouse *et al*., 2004; Reilly *et al*., 1997). In addition, melatonin displays many immunomodulating characteristics essential for maintaining host homeostasis (reviewed by Hardeland *et al*., 2011; Bitzer-Quintero *et al*., 2005). Any one, or more commonly a combination of, zeitgebers may influence or mask core mechanisms (Drust *et al*., 2005). Zeitgebers influence circadian phase by 'resetting' or synchronizing the SCN, through constant input, ensuring a maximally adaptive system structured in a cyclic fashion to both endogenous and exogenous factors (Reilly *et al*., 1997). While not completely understood, the SCN controls biologic rhythms, including circadian rhythms, through direct and indirect mechanisms. For example, the SCN regulates the synchronisation of the clock-cells of the periphery through the autonomic nervous system (ANS) and the hypothalamus pituitary adrenal (HPA) axis via their respective catecholamines (adrenaline and noradrenaline) and glucocorticoids (i.e. cortisol) hormones, amongst others (reviewed in Berczi, 2007). These hormones act as zeitgebers, providing messages to the peripheral clocks synchronising them to the central orchestrated response (Kalsbeek *et al*., 2012b; Curtis *et al*., 2007). Circulating hormones such as catecholamines, glucocorticoids and melatonin, which interact with and influence the immune system, peak either at diurnally or nocturnally different time-points of the circadian day (Lange *et al*., 2010). Consequently, the SCN-

controlled endocrine and autonomic nervous systems outputs organise the peripheral clocks, for example of immune cells, for a temporal circadian rhythm (Dimitrov *et al*., 2009; Guo *et al*., 2006; Haus and Smolensky, 1999).

The SCN uses external stimuli to fine tune biologic rhythm responses by way of exogenous zeitgebers as discussed previously. This integrated and coherent network of external and internal factors synchronising the circadian phase can be identified by a number of proxy markers, namely core body temperature (Easton *et al*., 2007; Waterhouse *et al*., 2005; Waterhouse *et al*., 2004), blood pressure (Shea *et al*., 2011; Atkinson *et al*., 2006), heart rate (Armstrong *et al*., 2011; Reilly *et al*., 1997) and a plethora of hormones (Paul *et al*., 2010; Dimitrov *et al*., 2009; Bitzer-Quintero *et al*., 2005; Redwine *et al*., 2000). Indeed, under normal sleep-wake conditions core body temperature rises in the early morning and reaches a peak in the evening between 18.00hrs and 22.00hrs, with minimal values reported between the hours of 03.00hrs to 06.00hrs (Redwine *et al*., 2000; Reilly *et al*., 1997). The secretion of the pineal gland hormone melatonin displays a distinct circadian rhythm, with circulating levels of the hormone inversely correlated to the rhythm of core body temperature (Burgess and Eastman, 2005; Burgess *et al*., 2003; Buxton *et al*., 2003; Redwine *et al*., 2000; Brun *et al*., 1998). Moreover, so closely correlated are these respective circadian rhythms that the decrease in core body temperature and the rise in melatonin levels during the hours of night-time sleep occur in almost harmony in a quid pro quo manner, with melatonin mediating the control over this cycle (Redwine *et al*., 2000). Importantly, this melatonin driven response can only take place under defined nocturnal sleep

conditions without the presence of the robust (exogenous cue) zeitgeber of sunlight (Higuchi *et al*., 2014; Paul *et al*., 2010; Buxton *et al*., 2003). The effect of melatonin administration on core body temperature response during times when endogenous levels are low, i.e. during the diurnal phase, results in a reduction in core body temperature ranging from 0.01 ºC to 0.3ºC in a dose-dependent fashion (Redwine *et al*., 2000). Furthermore, Redwine *et al*. (2000) reported that the suppression of nocturnal endogenous melatonin by administering atenolol (β1-adrenergic antagonist) resulted in an atypical increase in nocturnal core body temperature. However, the administration of exogenous melatonin (to endogenous levels) reversed this process, returning core temperature to nocturnal nadir levels. Similarly, exposure to zeitgebers, i.e. light, out-with the normal circadian phase in the evening, extends the diurnal acrophase of elevated temperature delaying nocturnal bathyphase by some hours (Kubota *et al*., 1998).

Core body temperature, due to a distinct 24-hour phase, has been used as a proxy for physiological arousal (Reilly *et al*., 1997; Moore, 1992). Due to the distinctive variant throughout the 24-hour solar day (light-dark cycle) and the close relationship with melatonin secretion, body temperature has been used as an indicator to measure endogenous components of biological rhythms (Reilly *et al*., 1997). As a result, evaluation of body temperature may be used as a reference point to gauge circadian phase on an individual-specific basis (Waterhouse *et al.,* 2004; Moul *et al*., 2002).

2.3 Molecular Clock and Circadian Rhythm

At a molecular level, circadian biological clocks are centred on the activity of clock genes, which encode proteins able to feedback and inhibit their own transcription (expression) (Ferrante *et al*., 2015; Ko and Takahashi, 2006; Reppert and Weaver, 2002). The core oscillator contains three interrelating feedback loops (reviewed by Curtis *et al*., 2014). The positive drive of the daily biological clock consists of two transcriptional factor genes, CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle Arnt-like protein), which together comprise the central feedback loop (Curtis *et al*., 2014; Balsalobre *et al*., 1998). The protein products of these genes control the transcription of other clock genes, notably three Period (Per1, Per2 and Per3) genes and two Crytochrome (Cry1 and Cry2) genes (Balsalobre *et al*., 1998). Importantly, Period and Crytochrome complete the negative feedback drive, limiting their own expression of BMAL1/CLOCK genes, ceasing activity to complete the circadian cycle (~24 hours). Per and Cry CLOCK gene messenger ribonucleic acid (RNA) peaks in the SCN during the midpoint of the circadian day respectively, again demonstrating the inherent circadian nature (Curtis *et al*., 2014).

Nuclear receptors RAR-related orphan receptor (ROR) (α,β,γ) and REV-ERB (α,β), comprise the second feedback-loop. The ROR and REV-ERB feedback loops rely on activation via BMAL1:CLOCK expression also. However, unlike the Per-Cry feedback loop, this loop can translocate back into the nucleus and promote BMAL1 expression via binding of receptor-related orphan receptor response elements (ROREs) (Sato *et al*., 2004). Importantly, this second

feedback loop is counterbalanced as REV-ERBs expression repress BMAL1 gene expression whereas ROREs play an opposite role and activate. This tightly regulated feedback loop interlocks with not only the Per-Cry loop but also the third, more integrated feedback loop (Ko and Takahashi, 2006). This feedback loop involves albumin D-box binding protein (DBP) which activates transcription expression and is regulated through Per-Cry (feedback loop 1) and the RORE regulated repressor interleukin 3 (feedback loop 2) (Curtis *et al*., 2014). This three-way system controls the molecular clock genes which comprise the foundation of the molecular oscillator in mammals (Curtis *et al*., 2014, see figure 2.3).

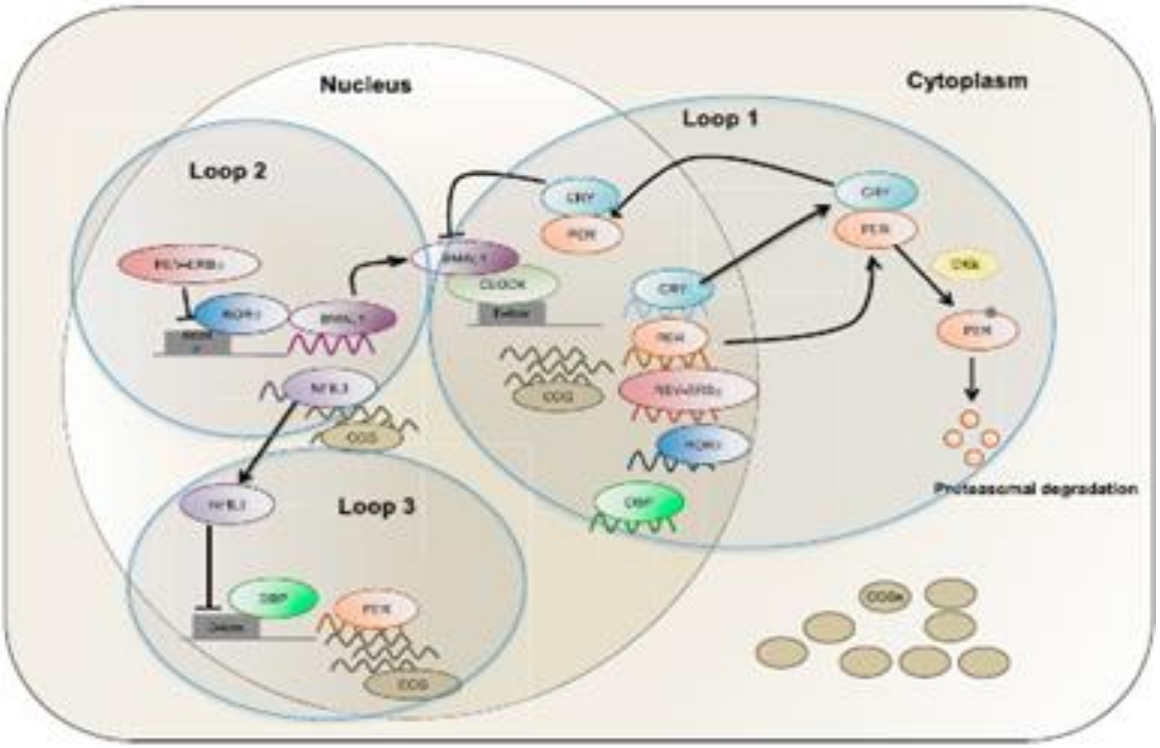


Figure 2.3. The Molecular Clock Feedback Loops (taken from Curtis *et al*., 2014, pp.179).

Similarly, circadian rhythmicity is underpinned with expression of genes forming a cyclic molecular clock, where upregulation and downregulation

results in peaks and troughs of 12 hours apart, completing a full 24-hour circadian cycle (Curtis *et al*., 2014). Importantly, individual differences at a molecular level exist resulting in distinct phase differences (Ferrante *et al*., 2015) and the phenomena of individual specific 'chronotypes' (Henst *et al*., 2015; Archer *et al*., 2003).

2.4 Chronotype and Individual Difference

As discussed above, research has consistently reported that a plethora of physiological and biological functions display distinct circadian rhythms (Ackermann *et al*., 2012; Dimitrov *et al*., 2010; 2009; Drust, 2005; Waterhouse *et al*., 2005). Indeed, human behaviour is similar in this regard with inherent differences in the population as to the preference for physical or intellectual activity (Bailey and Heitkemper, 2001). This innate circadian phenotype for activity, and indeed inactivity (rest), is specific to an individual and is referred to as chronotype (Horne and Ostberg, 1976). There are three broad categories of chronotype; morning-type, intermediate and evening-type, with the intermediate category further divided into subsets of moderately morning and moderately evening-types, respectively.

Morning-types, or 'larks' as they are commonly referred to, share distinct behavioural traits, namely retiring to bed in the early evening, rising in the early hours of morning feeling refreshed and alert (Horne and Ostberg, 1976). Conversely, evening-types, or 'owls', display the opposite preferences; rising later in the morning, going to bed later into the night and are active later in the day compared to morning-types. Extensive research suggests that the vast

majority of the population fall into the intermediate categories (moderately morning or moderately evening-types) of chronotype with approximately 10% of the population evenly distributed between morning or evening phenotypes, respectively (Henst *et al*., 2015; Kabrita *et al*., 2014; Kudielka *et al*., 2006; Zavada *et al*., 2005; Smith *et al*., 2002).

As an innate phenomena, and not a factor adjusted to temporal activity, chronotypes have a profound effect on psychological and physiological phases. For example, morning chronotypes show a two-hour phase advancement in core body temperature (higher values) compared to evening chronotypes in the hours of the morning (Henst, 2015; Baehr *et al*., 2000). As discussed previously, it is within this time of the day that morning chronotypes have a preference for activity and evening-types prefer to rest. Although chronotypes are an inherent trait, there is plasticity in how they develop and change over time, with exogenous factors influencing adjustment (reviewed by Vitale *et al*., 2014). Considering that newborn babies are born without circadian rhythmicity (arrhythmic) and display ultradian rhythms instead (Tenereiro *et al*., 1991), chronological age is a predominant factor influencing chronotype (Kim *et al*., 2010). An abundance of literature suggests, as with a wide range of parameters of human physiology and biology, advancing age is correlated with a shift towards morningness phenotype (Kim *et al*., 2010; Cavallera and Giudici, 2008; Carrier *et al*., 1997), whereas adolescence and early adulthood has a preference for eveningness phenotype (Kim *et al.,* 2010*;* Smith *et al.,* 2002). Moreover, Taillard *et al.* (2004) investigated chronotype in the middle-aged, finding that over 60% self-reported as morning-types, 36%

as intermediates, and only 2.2% as evening-types. To emphasis the relationship of age and chronotype, research with a cohort of university students (≤30 years of age) and working adults (≥31 years of age) found a propensity for morningness with advancing age (Diaz-Morales and Sanchez-Lopez, 2004). Invariantly, research investigating chronotype spanning a wide age range reported a greater proportion of intermediate chronotypes (Kostovic *et al*., 2001; Ishihara *et al*., 1992).

In addition to age, global environmental factors such as longitude, latitude and climate have been suggested to influence chronotype. Some evidence reports that climate zones (tropic, subtropic and temperate) affect chronotype development within an adolescent population. Borchers and Randall (2012) found a divergence between adolescents in the tropic and subtropic climate zones with a greater percentage of morning-types in the tropic region. In many ways this is unsurprising as distinct cultural and climatic factors, such as rising hour, work/school patterns, religious customs, in addition to circadian factors such as light exposure (natural and artificial), feeding times and ambient temperature, over many generations may affect chronotype inclination due to geographic location. Considering earlier school and work starting hours, extreme midday heat and a greater number of sunlight hours throughout the seasons, as well as the influence of climatic and especially photoperiod on circadian rhythms via zeitgebers, morning chronotypes may be conditioned to occur earlier than in other more temperate environments. Thus possibly reinforcing the evolutionary aspect of entrainment to environmental conditions. Similarly, a comparison study of young adults in relatively cold (The

Netherlands, United Kingdom and United States of America) and relatively warmer (Colombia, India and Spain) environs found that those in cooler geographic locations were more inclined to be evening-types than those who lived in warmer climates (Smith *et al.,* 2002).

As alluded to briefly, circadian rhythmicity from a very young age and over a generation may play a role in chronotype phenotype development when the light-dark cycle and ambient environmental temperature differs (Wright *et al*., 2013; Duffy and Czeisler, 2008). From example, Tonetti and associates (2012) found that students in India, where seasonal and annual mean temperatures are greater, displayed a preference for morningness compared to Italian students. As such, it is clear that environmental, cultural and circadian factors over generations are pivotal in chronotype disposition and development. Wehr's (1992) findings support this assertion, with biphasic, symmetrical sleep of four-hour duration and one to three hour waking interval (mid-sleep) after a photoperiod was shortened from the contemporary 16 hours to 10 hours per 24-hour day. Similar biphasic sleep patterns (to Wehr's methodology) can be seen in many of the tropical climates discussed above and also in pre-industrialised Europe (Ekirch, 2001).

It is evident that the complexity surrounding chronotype is vast. However, what is clear is that advancing age, irrespective of climate or geographical location, is a constant influencing factor. Newborn babies are arrhythmic and without a specific chronotype, once children reach adolescence an evening chronotype becomes dominate (Borisenkov, 2010) and as young adults mature into middle-age and older, intermediate chronotypes move towards a morning

phenotype. However, the above model describing a gradual change of chronotype is too simplistic as a population of extreme phenotypes (morning and evening-types, respectively) do remain unchanged throughout life (Reilly *et al*., 1997). In addition to age, climate and global geographic position, gender differences (Collado *et al*., 2012) have been reported consistently, with males tending to be more evening chronotypes (Randler, 2011; Tonetti *et al*., 2008) and females displaying a higher propensity for morning-types (Collado *et al*., 2012; Randler, 2011; Zimmermann, 2011; Tonetti *et al*., 2008).

2.5 Chronotype and Athletic Performance

Chronotype, the preference for activity in the morning or evening, has been found to influence sport or exercise participation and has been linked to personality-trait-like individual differences (Henst *et al*., 2015). Diurnal fluctuations are associated with lower sport participation and shorter exercise duration when chronotype and circadian phase for activity are incompatible, commonly due to work/lifestyle commitments (early morning or late evening activity) (Smith *et al*., 2002). Conversely, enhanced quality of sleep, better performance and increased exercise-load has been reported when chronotype, exercise and circadian phase are in unison (Kuffer *et al*., 2015).

The distribution of chronotypes in the global population is heterogeneous but broad common trends exist, as discussed in detail earlier in this chapter. Generally, the vast majority of individuals fall into the intermediate or neither category of chronotype (Smith *et al*., 2002). However, with regards to an athletic population there is a propensity towards a higher representation of

morning-types (Henst *et al*., 2015; Kunorozva *et al*., 2012; Lastella *et al*., 2010), particularly in sports such as distance running (Henst *et al*., 2015) and aerobic events (Silva *et al*., 2012).

2.6 Lung Function, Exercise and Environmental Considerations

Evidence suggests that circadian rhythms exist in measures of pulmonary function. Peak expiratory flow (PEF) capabilities display a diurnal rhythm with evening values elevated in comparison to the morning (Goyal *et al*., 2008; Casale and Pasqualetti, 1997), with augmented variability in smokers and those suffering from pulmonary disease (Casale and Pasqualetti, 1997). Lung function is typically assessed by spirometry, which allows for accurate measurement of several airflow parameters (Wagner *et al.,* 2006). For example, airflow characteristics, such as forced expiratory flow (FEF), forced mid-expiratory flow (FEF_{25-75}), forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1) and PEF (Blomberg *et al*., 1999).

FEV1 demonstrates the rate of flow in large and small airways, whereas large airways are represented by PEF values (Mead, 1979). FEV1 is a more accurate indicator of airflow function, than PEF (Thiadens *et al*., 1999). FVC measures respiratory stroke volume of air expired. FEV_1 assesses the volume of expired air in the initial first second of FVC. Both FVC and FEV_1 lung function values, in healthy individuals without chronic obstructive pulmonary disease (COPD), are predicted to be 80% of normative predicted value (Booker, 2005). The ratio FEV_1/FVC is commonly used to assess if an airflow obstruction is present, with a healthy ratio for FEV_1/FVC set at 80% (West,

2003). A value less than 80% for this index may imply that COPD, i.e. bronchitis or asthma to name just two, may be present (Celli *et al.,* 2004).

PEF is the maximum exhalation flow, measured in the first 100 milliseconds of forced expiration (West, 2003). PEF shows diurnal variation reaching peak values in the afternoon at around 16.00hrs (Goel *et al*., 2015). Importantly, unlike the FEV_1/FVC ratio for example, due to circadian rhythm and large variation in individuals in PEF, values outside normal range may not indicate respiratory-pulmonary dysfunction (Gregg and Nunn, 1973).

In athletic populations, environmental conditions, such as heat, cold and humidity, can have a detrimental effect on exercise performance (Bougault *et al*., 2009; Nybo and Nielsen, 2001a; Brenner *et al*., 1999; Galloway and Maughan, 1997). Airway hyperresponsiveness (AHR) and asthma are the most common chronic medical conditions in elite athletes, with 7% to 8% of athletes suffering from symptoms (Kippelen *et al*., 2012). These conditions are commonly a consequence of exercise completed in polluted (noxious or ozone) or cold environments. Outdoor sports or activities that require athletes to inspire large volumes of air, such as distance or endurance type events, are at risk of developing AHR or asthma. Nordic skiers, who commonly have ventilation rates (flow) (> 200l/min) over many hours in sub-zero (<0 degress Celsius) temperatures have an increased incidence of AHR by around 5% (Fitch and Andersen, 2012). Conversely, the incidence of exercise-induced asthma or AHR in alpine ski jump athletes who train and compete in the same environs is much lower at 4% (Kippelen *et al*., 2012). This is possibly due to the large (> 200l/min) volumes of cold air inhaled by the cross-country Nordic

skiers resulting in inflammation of the airways. Inflammation of the upper respiratory tract in athletic populations will be discussed in greater detail subsequently.

2.7 Exercise and the Inflammatory Response

Neutrophils comprise between 60% to 70% of circulating leukocyte cell counts and are one of the first leukocytes to migrate to a site of injury, infection or damage (Mackinnon, 1999). They produce reactive oxygen species (ROS) that are pivotal in challenging and destroying pathogens, however ROS, if overproduced as seen in chronic health conditions, can damage healthy tissues (Sen, 2001). With regards to circadian rhythm, acrophase for neutrophils within a healthy population occurs between 15.00 and 16.00 hours with nadir at ~03.00 hours (Ackermann *et al*., 2012). Neutrophils are highly mobilised due to exercise and stress with an acute bout of exercise increasing peripheral neutrophil cell count numbers (Kakanis *et al*, 2010; Sureda *et al*, 2009; Umeda *et al,* 2008). Research shows that neutrophils increase exponentially (by as much as 500%) in peripheral circulating numbers post-exercise (Kakanis *et al*., 2010), with circulating adrenaline and cortisol considered influential in stimulating the release of neutrophils from the bone marrow into the periphery (Hack *et al*, 1994; McCarthey *et al*., 1992). Neutrophils continue to increase from the onset of exercise reaching a peak approximately three hours into the recovery period (Steensberg *et al.,* 2002; Robson *et al*., 1999). However, prolonged exercise of more than two hours duration and moderate intensity results in a post-exercise peak in neutrophils

which subsides over the following hours returning to pre-exercise levels (Robson *et al*., 1999). Furthermore, this post-exercise neutrophilia is sensitive to many factors including exercise stimulus: mode, duration and intensity, fitness status of exerciser and over-reaching training periods (Umeda *et al.,* 2008).

Athletes generally display normal levels of blood circulating neutrophil numbers in comparison to the wider population, however trained athletes have exhibited neutrophil counts lower than the clinical norm (Blannin *et al.,* 1996; Hack *et al.,* 1994). Elite endurance competitors have been shown to display neutrophil counts lower than healthy non-athletes at rest (Hack *et al.,* 1994). However, acute exercise mobilises neutrophils in proportion to a bout of exercise intensity and is considered a marker for inflammatory response (Peake and Suzuki, 2004). This increase mainly occurs due to the detachment of neutrophils from the lining of vascular endothelium. Contrary to circulating peripheral blood neutrophil counts, neutrophils residing in the airways of highly-trained athletes are commonly higher than age matched non-athletes (Belda *et al.,* 2008; Morice *et al.,* 2004), due to the high rate of ventilation damaging the epithelial lining (Denguezli *et al.,* 2008; Morice *et al.,* 2004).

Elevated peripheral neutrophil counts post-exercise are considered to be due to muscle damage or damage to the bronchials of the lungs caused by exercise and influenced by the environmental conditions exercise is undertaken in (Brancaccio *et al*., 2010). High-intensity exercise results in an increase of stress hormones (catecholamines and cortisol) (Lovallo *et al*., 2006) and cytokines (Laing *et al*., 2008; Peake *et al*., 2007; Starkie *et al*., 2005;

Steensberg *et al*., 2003) resulting in the mobilisation of neutrophils from endothelium tissue and bone marrow (Hack *et al*, 1994; McCarthey *et al*., 1992).

Some evidence suggests that neutrophils are sensitive to rises in core body temperature, with higher values correlated to increased neutrophil counts in the circulatory system (Laing *et al*., 2008). A relationship between body temperature and neutrophil counts has been found due to passive increasing of body temperature and in response to exercise (Rhind *et al*., 1999). Furthermore, neutrophils display a robust circadian rhythm with acrophase in the early evening (Ackermann *et al*., 2012; Lange and Born, 2011). Potentially a multi-faceted mechanism may exist between circulating neutrophil numbers at rest, in response to exercise with circadian phase, core body temperature, and stress hormone concentrations.

Cytokines are signalling molecules released from a variety of cells which induce a response on the target cell and are part pivotal mediators of the inflammatory response (Gleeson, 2007). Two main cytokine populations exist, pro-inflammatory (IL-1, IL-6, IFNγ, TNF-α,) and anti-inflammatory (IL-4, IL-5, IL-10, IL-13) (Gleeson, 2007; Elenkov and Chrousos, 2002). Pro-inflammatory cytokines mediate an inflammatory response activating cytotoxic immune cells and granulocytes (predominantly neutrophils). This response triggers a subsequent apoptosis of virus infected cells (Gleeson, 2007). Anti-inflammatory cytokines suppress the pro-inflammatory cytokine production limiting potential damaging effects of over-exposure or production (Gleeson,

2007; Elenkov and Chrousos, 2002). Cytokines and cytokine response to exercise are reviewed in detail elsewhere (Suzuki *et al*., 2003).

Airway inflammation, and indeed many other forms of inflamed tissues (injury), can be characterised by recruitment in inflammatory cell types, for example monocytes, macrophages, cytokines or neutrophils (Gleeson *et al*., 2013; Gleeson, 2007). Neutrophils are of particular interest in exercise-induced inflammation of the respiratory system. Neutrophils, as constituents of the innate immune system, have the ability for non-specific immune response against pathogen. Unlike cells of the adaptive immune system (for example lymphocytes), neutrophils do not have the ability to recognise pathogen due to a resolution of a prior encounter or the development of memory specific antigen (Gleeson *et al*., 2013).

2.8 The Immune System, T-Lymphocytes and Exercise-Induced Response

The immune system is a remarkably adaptive defence system from a complex network of cells and molecules capable of specifically recognising and eliminating infectious microorganisms (Gleeson *et al*., 2013). The principal function of the immune system is to preserve self-integrity and to identify and remove any foreign or infected element not recognised as self (Gleeson *et al*., 2013).

Defence against microorganisms is mediated by two distinct but over-lapping components, which work together in synergy to comprise the immune system, namely, the innate and adaptive systems respectively (Gleeson *et al*., 2013). Innate immunity is often referred to as the first line of defence, comprising of

epithelial barriers, phagocytes and natural killer (NK) cells which lack specificity but provide immediate resistance. Using a network of soluble proteins and cell surface receptors, the innate immune cells recognise non-self-invading agents and look to neutralise or identify them to commence an immunological response, but this is done without specificity to the agent. As a consequence, once an invading or foreign agent is recognised the innate immune system predominantly relies on an inflammation response until the agent is removed or before the second component of the immune system is recruited, the adaptive immune system (Janeway and Medzhitov, 2002). For the purpose of this thesis, which primarily focuses on the adaptive immune system, an in-depth review on the innate immune system can be found in Janeway and Medzhitov (2002).

Unlike the innate system, the adaptive system can offer specificity against invading agents or microbes but this is dependent on time and/or prior exposure to the pathogen (Abbas *et al*., 2000). Both the adaptive and the innate systems comprise of respective cellular and soluble elements. The adaptive system, sometimes referred to as acquired immunity, comprises of T and B-lymphocytes which, although initially alike, the site of maturation determines their development (Abbas *et al*., 2000). Upon maturation within the thymus, T-lymphocytes express a unique cell surface antigen-binding molecule called the T-cell receptor (TCR). This antigen binding molecule consists of two transmembrane molecules, the TCR-α and the TCR-β, that are the result of rearrangement of firstly the TCR β and then the TCR-α gene. In contrast to membrane-bound antibodies on B-cells, which can recognise

antigen alone, the vast majority of TCRs recognise a complex ligand that includes an antigenic peptide bound to a major histocompatibility complex (MHC)-derived molecule (Gleeson, 2006).

The MHC is a cluster of genes which plays a central role in intercellular recognition and discrimination between self and non-self. Two forms of MHC exist, class I and class II molecules. Class I and class II molecules interact with different co-receptors on the T-lymphocytes, that is CD8 and CD4 respectively (Appay *et al*., 2009). Whereas class I molecules are expressed by nearly all nucleated cells, class II molecules are expressed only by antigen-presenting cells (APCs); however, they can be induced in the majority of cells (Abbas *et al*., 2000).

In general, mature T-lymphocytes express either CD4 or CD8 molecules, hence allowing for identification of CD4+ T-helper (Th) cells and CD8+ cytotoxic T-lymphocytes (CTLs). Th cells produce cytokines, inducing humoral and cell mediated immune response. In this regard, activation of Th cells is carefully regulated, and naïve CD4+ cells become activated only upon encountering antigen presented by MHC class II complexes in the context of appropriate co-stimulatory molecules on the surface of APCs (Duddy *et al.,* 2004). The role of the CD8+ T-lymphocytes is to monitor all the cells of the body, ready to destroy any that are considered to be a threat to the integrity of the host; for example, CTLs kill virally infected cells, stopping the cell from replicating. Collectively, cell types that display killing capabilities (NK cells, CD8 T-lymphocytes, gamma delta T-lymphocytes) are often referred to as CTLs.

2.9 T-Lymphocyte Differentiation and Phenotypes

Activation of CTLs results not only in proliferation but also in differentiation of the activated cell. Circulating CD8+, and indeed CD4+, T-lymphocytes can be divided into four groups; naïve, effector, effector/memory and memory cells, each of which signify a distinct differentiation or activation status of a given T-lymphocyte clone (Appay *et al*., 2009; 2008; Romero *et al*., 2007). These different states of T-lymphocyte activation are associated with distinct functional and phenotypic characteristics. For instance, naïve T-lymphocytes circulate only between the peripheral blood and lymphatic tissues, whereas some of the antigen-experienced sub-populations, such as with the effector phenotypes, are able to enter other tissues (Romero *et al*., 2007; Hamman *et al*., 1997). Furthermore, effector T-lymphocytes display a strong cytolytic activity, expressing high levels of perforin, while effector/memory cells display moderate levels of the cytolytic perforin (protein granules found in CD8 and NK cells), allowing only a limited cytotoxic activity, although greater levels of cytokines are produced (Bigley *et al*., 2012; Appay *et al.,* 2009). In contrast, memory CD8+ T-lymphocytes fail to kill target cells but can proliferate and produce cytokines in response to antigen stimulation (Kaech *et al.*, 2002).

In addition to functional characterisation, phenotypic characteristics of cell surface markers can be helpful in the classification of the different T-lymphocyte populations. Thus, figure 2.4 (below) illustrates, phenotypic classification of human CD8+ T-lymphocytes using the co-stimulatory receptors CD27 and CD28 as well as CD45RA has been validated as useful for the distinction of naïve, memory and effector CD8+ T-lymphocytes

(Romero *et al*., 2007; Appay *et al*., 2002). In this regard, naïve CD8+ T-lymphocytes express CD27, CD28, and CD45RA, whereas memory cells lose the expression of CD45RA. Effector CD8+ T-lymphocytes are CD27+CD28+CD45RA+, and effector/memory cells have a CD27–CD28–CD45RA– phenotype (Appay *et al.,* 2009). In addition, it has been suggested that CD27+CD28–CD45RA– CD8+ T-lymphocytes are effector/memory cells as well, as they display cytotoxic activity and can effectively produce cytokines (Appay *et al.,* 2009).

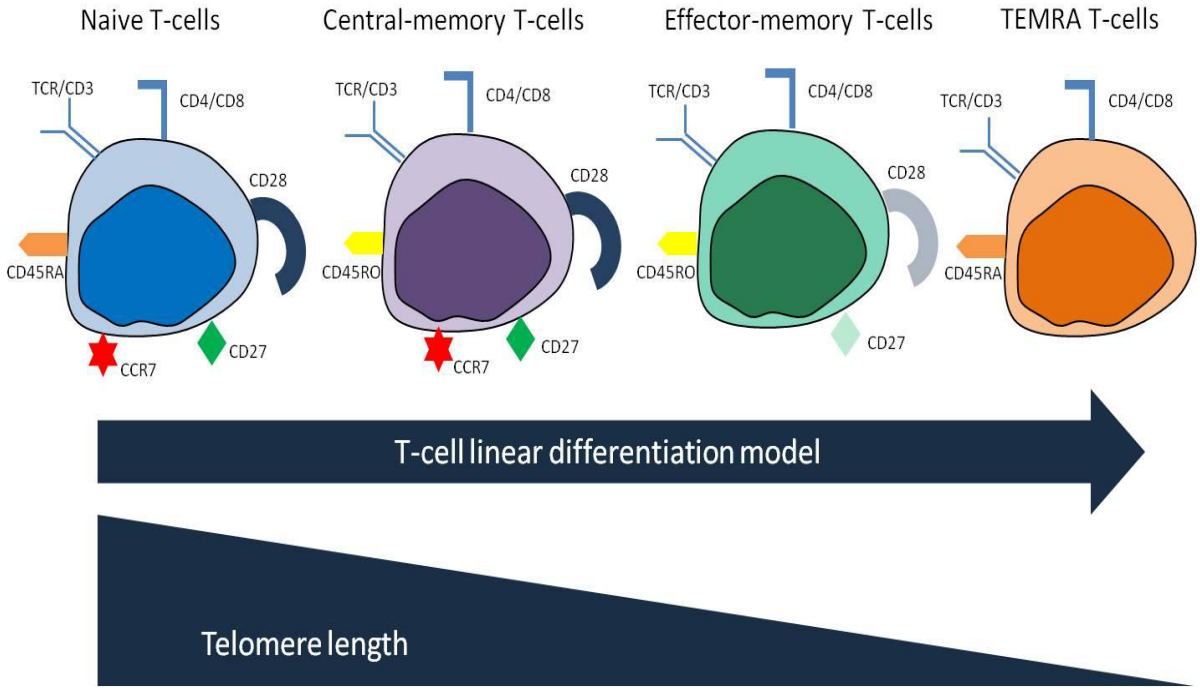


Figure 2.4. Phenotypic changes on T-lymphocytes during linear differentiation (adapted from Appay *et al*., 2009, pp.978).

The chemokine receptor CCR7 is a particularly useful marker for discriminating naïve and memory CD8+ T-lymphocytes from effector memory and effector CD8+ T-lymphocytes. The CCR7 receptor functions as a homing receptor to lymphoid tissue and is expressed on naïve CD8+ T-lymphocytes

and a subset of memory CD8+ T-lymphocytes. Thus, naïve (CCR7+CD45RA+), central/memory (CCR7+CD45RA–) and effector memory (CCR7–CD45RA+/–) cells can be distinguished. It has been suggested that CD8+ cells switch from naïve to memory status in response to antigen exposure and then gradually transform into an effector type (Appay *et al.,* 2009). The effector phenotype is CD45RAhighCD27–CD28–CCR7– and expresses high amounts of perforin, IFN-γ and granzyme. Furthermore, it has been shown that the majority of circulating immunisation-induced CD8+ T-lymphocytes display the intermediate effector/memory CD45RAhighCD27– phenotype. These cells are capable of expressing IFN-γ but demonstrate little or no expression of perforin.

Similarly, TCR γδ T-lymphocytes display both adaptive and innate characteristics, and respond to a wide variety of antigens in a manner not too dissimilar to innate immune cells, in addition to processing the ability to develop memory specific to individual antigen similar to the trait found in the adaptive immune system (Girardi, 2006). Although comprising only approximately 5% of total circulating blood T-lymphocytes, gamma delta T-lymphocytes play an influential role in the defence against bacterial infection (Wang *et al*., 2001), wound repair (Girardi, 2006), and provide cytotoxic potential during periods where exposure to antigen has a greater likelihood to occur, i.e. during periods of prolonged stress (Anane *et al*., 2009).

2.10 An Introduction to Exercise Immunology

In the last two decades or so, an abundance of research has been undertaken to investigate the role exercise-induced stress exhibits on the immune system. Responding to anecdotal evidence, early research investigated the increased incidences of upper respiratory tract infections (URTI) in athletes (Nieman *et al*., 1990) as a result of an increased exercise load. Nieman (1994) first proposed the J-shaped model (figure 2.5), asserting a relationship between activity level and exercise load and the incidence of URTI followed this trend. Sedentary lifestyles display a moderate risk, moderate physical activity display a below average risk and an ever greater volume and bouts of exercise of a prolonged and high-intensity nature are associated with a greater than average risk of experiencing an URTI (Nieman, 1994).

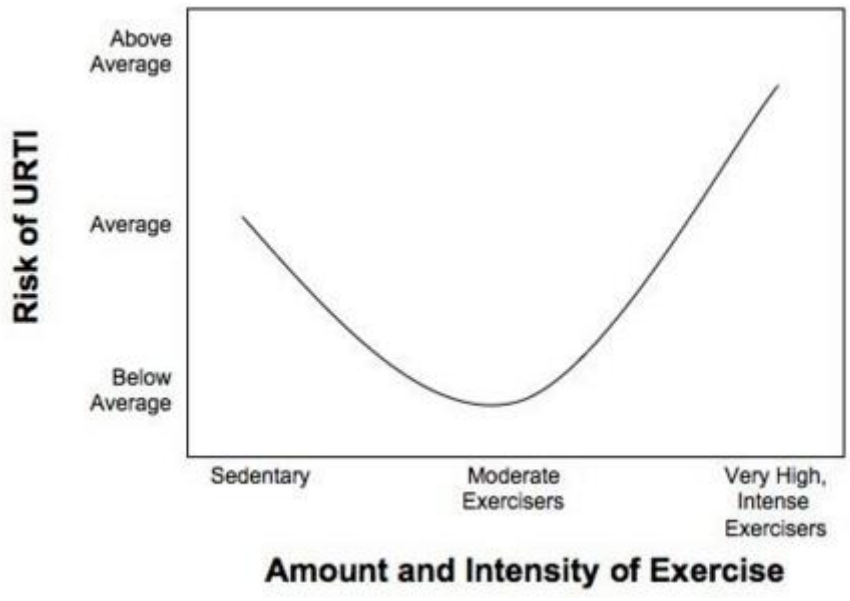


Figure 2.5. The J-shaped curve of the relationship between risk of URTI and exercise intensity/volume (adapted from Nieman, 1994).

Early research reported an increased prevalence of self-reported incidences of URTI seven to 14 days post-competitive distance running (Nieman *et al*.,1990), with a positive linear relationship found between decreasing time to complete the event (i.e. quicker performance) and increased URTI episodes in the days that followed (Peters and Bateman, 1983). The most frequent outcome in research examining the relationship between heavy training volume and an increased incidence of URTI strongly supports Peters and Bateman's (1983) findings (Cunniffe *et al*., 2011; Fahlman and Engels, 2005; Gleeson *et al*., 1999). However, it is important to consider that these studies report a relative increase in URTI events after an unaccustomed, acute bout of prolonged exercise, for example a marathon, or as a consequence of repeated bouts of high-intensity training without adequate periods of rest (Nieman *et al*., 1990). Although seminal work, methological concerns limit interpretation of Nieman's (*et al*., 1990) findings. Travel to and from the event commonly by flights in enclosed cabins, nutritional status and athletic experience of the participants pose many challenges in interpreting the data.

In addition to the duration of an exercise event, the relative distance covered in an exercise bout appears not to significantly influence the incidence of post-URTI episodes, with no apparent relationship reported in 5 kilometre (km), 10km and 21km distance events (Nieman *et al*., 1989). Furthermore, Ekblom and colleagues (2006) reported that an increased incidence in URTI episodes post-marathon type events may be due to recurrence of a pre-race infection with one-third of athletes reporting symptoms in the three weeks prior and three weeks post event. More recently, debate concerning the J-shaped model

of URTI incidences has focused on whether it accurately distinguishes between the fitness levels of athletes and especially ‘elite’ performers. Data collected in exercise immunology research aptly characterises sedentary, moderately and highly trained cohorts, however, elite athletes report fewer URTI incidences than the other populations and as such an S-shaped model has been proposed, as displayed in figure 2.6 (below) (Malm, 2006).

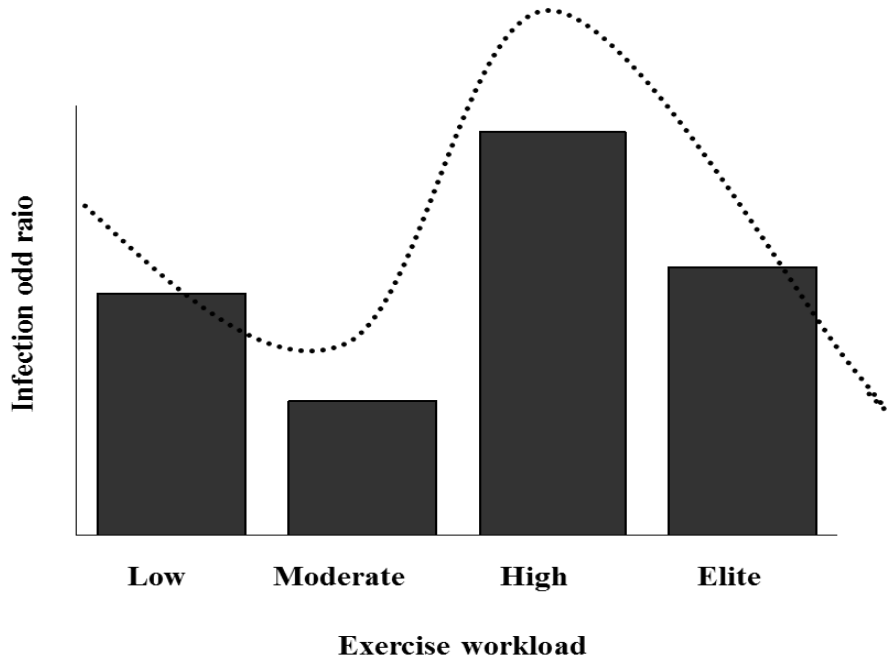


Figure 2.6. The proposed S-shaped curve illustrating the relationship between physical activity and risk of URTI at different workload categories (adapted from Malm, 2006, pp.5).

Inadequate recovery between bouts of high-intensity exercise, especially during times of multiple bouts of exercise such as pre-season training or training camps, may result in impaired immune function causing depression of the immune system resulting in heightened susceptibility to infection (Foster, 1998; Nieman *et al*., 1990). This concept is commonly referred to as the ‘open-

window theory' (see figure 2.7) and was first proposed by Pedersen and Ullum (1994). Repeated bouts of high-intensity exercise on the same day result in an elevated immune response (Ronsen *et al*., 2001), whereas over continuous days immunodepression has been observed (Gleeson *et al*., 2013; Simpson *et al*., 2006).

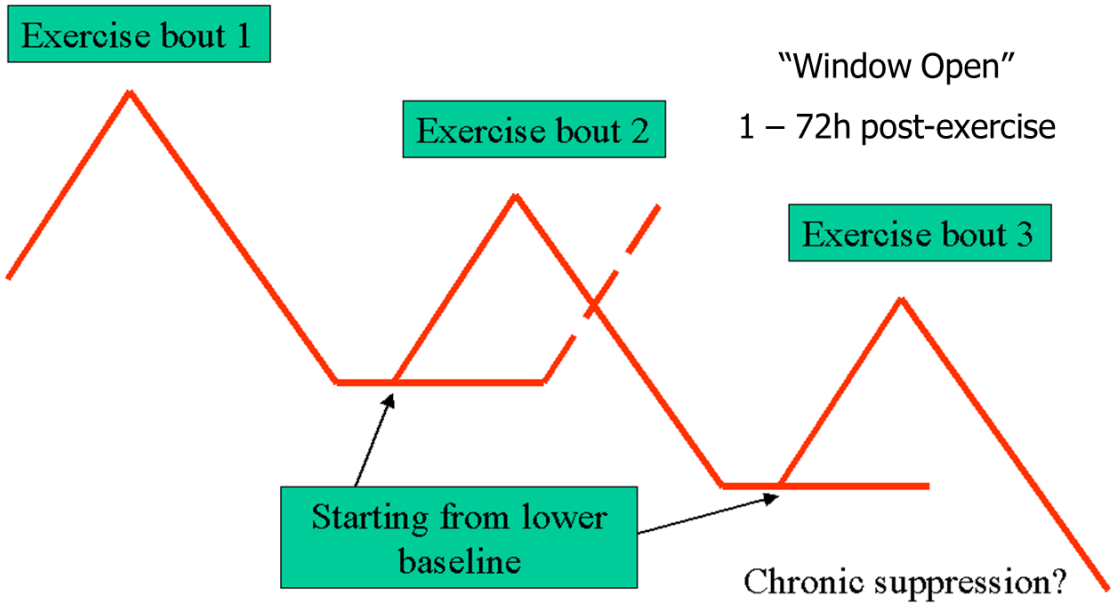


Figure 2.7. The open window theory demonstrating the effects of multiple bouts of exercise on immune cell redeployment (adapted from Pedersen and Ullum, 1994).

2.11 T-Lymphocyte Response to Exercise

Research has demonstrated a biphasic change in the number of circulating lymphocytes as a result of an acute bout of aerobic exercise (Gleeson, 2006). Typically, a near-immediate and transient increase in circulating lymphocytes can be seen in the blood from the onset of exercise (Simpson *et al*., 2006; Shek *et al*., 1995). This initial increase in lymphocytes of the peripheral blood

is termed lymphocytosis and is the most replicated of findings in the discipline of exercise immunology (Gleeson and Bishop, 2005). The lymphocytosis response to exercise has been shown to continue to rise from the onset of exercise for a period of approximately 30 to 60 minutes after the stimulus has ceased, at which point the number of lymphocytes reduces rapidly (Gleeson, 2006; Simpson *et al.*, 2006). This process of a systematic reduction in circulating peripheral blood lymphocyte numbers is called lymphocytopenia (Gleeson, 2006). Indeed, while regular exercise appears to have beneficial effects on the immune system, as outlined in the 'S' shape theory (Malm, 2006), the detrimental effects of frequent bouts of acute high-intensity exercise on the immune system has also been researched (Nieman *et al*., 1997) and will be considered in detail below.

This biphasic response of lymphocytosis and lymphocytopenia has been shown to be relative to the intensity, duration and health status to which the experimental population completes exercise (Ingram *et al.,* 2015; Turner *et al.,* 2010; Gleeson, 2006; Simpson *et al.,* 2006). Although a vast array of research in the field of exercise immunology has been completed, one particular type of leukocyte, namely lymphocytes, has been extensively researched. Lymphocyte populations are divided primarily into B-lymphocytes and T-lymphocytes. Naïve T- and B-lymphocytes enter the blood and migrate to secondary lymphoid organs such as the spleen, lymph nodes and mucosa-associated lymphoid tissue (Appay *et al*., 2009; Romero *et al*., 2007). Within the lymphocyte compartment, a number of subpopulations exist, these include cytotoxic T-lymphocytes (CD8), T-helper (CD4) lymphocytes and gamma delta

(γδ) T-lymphocytes (Gleeson, 2007). Relatively recent reports suggest the phenomena of lymphocytopenia and lymphocytosis are significantly influenced by the effect of circulating stress hormone concentrations induced by physiological or psychological stressors associated with the onset of physical activity (Anane *et al*., 2009; Campbell *et al*., 2009).

2.12 The Relationship between Circadian Rhythm, the Immune and Endocrine Systems

Circulating leukocytes, including lymphocytes, display a distinct circadian rhythm of one sort or another (Esquifino *et al.*, 2004; Haus and Smolensky, 1999; Suzuki *et al*., 1997) with circulating hormone levels a significant influence (Dimitrov *et al*., 2009). Research is unequivocal in determining the influence the two major stress-axes, the SNS and the HPA, exhibit on the immune system (Scheiermann *et al.,* 2013; 2012; Dimitrov *et al*., 2010; Lange *et al*., 2010; Elenkov *et al*., 2000; Suzuki *et al*., 1997; Palm *et al.,* 1996). Both of these stress-axes apply opposing governance of immune defence by their respective conflicting circadian rhythm. The primary hormones affecting circulating immune cell subsets appear to be the stress hormone (catecholamine) adrenaline and the immunosuppressant glucocorticoid cortisol (Dimitrov *et al*., 2009; 2007). For example, circulating catecholamines and cortisol levels display distinct circadian profiles with circulating cortisol levels elevating during the hours of nocturnal sleep peaking upon awakening (awakening response), as illustrated in figure 2.8 (Dimitrov *et al*., 2010; 2009; Lovallo *et al*., 2006). Catecholamines, and especially adrenaline, show a similar awakening response, however diurnal concentrations of the hormone

results in a second near-peak at 20.00 hours (Dimitrov *et al*., 2010; Lange *et al*., 2010; Dimitrov *et al*., 2009).

Catecholamines are synthesized from tyrosine (amino acid) and secreted from chromaffin cells located in the adrenal medulla with adrenaline the most abuntantly-produced hormone (Hinson *et al*., 2010). Catecholamines share a common synthesis precursor; the enzyme tyrosine hydroxylase catalyses tyrosine to dihydroxyyphenylalanine, while regulated by the presence of high concentrations of glucocorticoids phenylethanolamine-N-methyltransferase (PNMT) converts noradrenaline to adrenaline (Hinson *et al*., 2010). The catecholamines adrenaline and noradrenaline play a pivotal role in preparing the body for the rigours of the day and exhibit a clear rhythm with a divergent response between night-time nadir and day-time acrophase (Reilly *et al*., 1997).

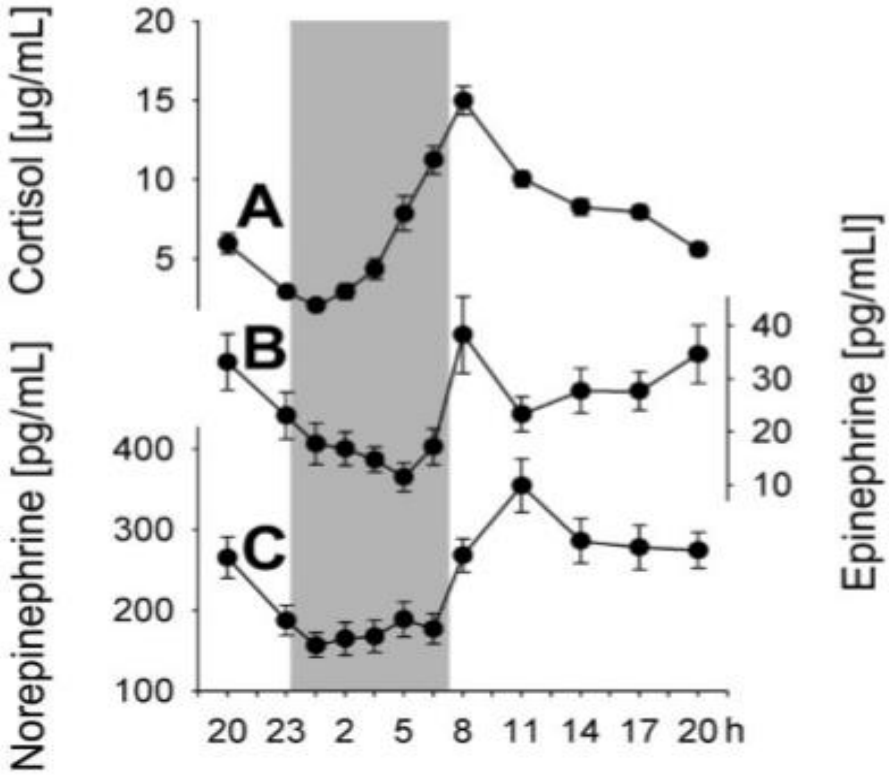


Figure 2.8. Circadian profiles of cortisol (A), epinephrine (adrenaline) (B) and norepinephrine (noradrenaline) (C) (taken from Dimitrov *et al*., 2009, pp.5138).

The release of adrenaline and noradrenaline influences a range of immunological processes including cell differentiation and lymphocyte proliferation, in addition to cytokine production (Meltzer *et al*., 2004). Additionally, T-lymphocytes have been found to respond to catecholamines via adrenoceptors type (α or β) which are expressed by markers on the cell surface (Kruger *et al*., 2008). Moreover, recent publications have highlighted expression of β2-adrenoceptors increases with immune cell activation and differentiation state, i.e. with effector memory phenotypes (Dimitrov *et al*., 2010; 2009; Benschop *et al*., 1996).

Conversely, the HPA derived hormone, cortisol, has been shown to be responsible for the regulation of naïve lymphocytes, in a near antipodal

manner to catecholamines, with acrophase during nocturnal sleep and nadir during the period of heightened daytime adrenaline levels (Dimitrov *et al*., 2009). In addition to heightened nocturnal levels, an awakening response of increased cortisol levels in the hours immediately prior to waking (Lovallo *et al*., 2006) and an increase in response to the onset of high-intensity exercise (Hayes *et al*., 2010) are consistently reported in healthy populations.

The balance between these two opposing stress systems and their respective hormone products appear to be the greatest influence on circulating lymphocytes. For instance, adrenaline levels increase exponentially from the onset of exercise, returning to pre-exercise levels from the cessation of exercise (Ingram *et al*., 2015). Contrary to this, cortisol levels display an initial depression form the onset of exercise-stressor, with a subsequent rise in plasma levels with reduction of exercise intensity or complete cessation (Galbo, 1983). Coinciding with these events, CTLs (cytotoxic CD8+ T-lymphocytes, NK cells) increase in circulatory numbers correlating to adrenaline levels (and indirectly exercise intensity). Furthermore, during the period of heightened cortisol levels, i.e. the cessation of exercise, a greater proportion of naïve lymphocytes replace functionally cytotoxic cell types (Dimitrov *et al*., 2010).

Atanackovic and co-authors (2006) demonstrate this phenomenon with acute stress inducing a decrease in the total number of circulating T-lymphocytes of a naïve and a central memory phenotype, in coincidence with an increase in effector memory and terminally differentiated T-lymphocyte subsets. Accordingly, the relative effects of catecholamines are determined by the type

and quantity of receptors present on target cells, in addition to the local concentration of adrenaline and noradrenaline (Kruger *et al*., 2008). However, with noradrenaline receptors found in almost every cell associated with the immune system their prominence and influence appears vast (Sanders *et al.,* 2001). Importantly, and again, this highlights how malleable the immune system is to inputs from SCN-influenced temporal channels and to exercise-induced physiological responses, as seen with circulating diurnal cortisol levels and adrenaline/noradrenaline response to exercise.

There is a mounting body of literature to date demonstrating that circulating levels of cytotoxic CD8+ lymphocytes exhibit a high adrenergic receptor density which regulates release from vascular endothelium into the blood (Anane *et al*., 2009; Dimitrov *et al*., 2009). Consequently, the subsequent exercise-induced lymphocytopenia is thought to occur as a result of increased secretion of cortisol due to greater HPA activity (Dimitrov *et al*., 2009). Indeed, a key underlying mechanism dictating immune response during lymphocytosis is the propensity to be activated by β2-adrenergic receptor (Sanders and Straub, 2002; Pedersen and Hoffman-Goetz, 2000).

Interestingly, although the vast majority of T-lymphocyte subsets appear to express β2-adrenergic receptors (Sanders and Kavelaars, 2007), exercised-induced lymphocytosis results in an apparent preferential increase in circulating lymphocytes of a cytotoxic nature, for example cytotoxic CD8+, NK cells, and more recently γδ T-lymphocytes have been identified (Anane *et al*., 2009; Campbell *et al*., 2009; Atanackovic *et al*., 2006; Segerstrom and Miller, 2004). Although expressing β2-adrenergic receptors, non-cytotoxic subsets

(CD4+) generally do not display the same magnitude of exercise stress-induced lymphocytosis (Segerstrom and Miller, 2004). It has been postulated that cytotoxic 'attack' subset types show greater mobilisation during periods of greater stress where an enhanced immune defence (Dhabhar and McEwen, 1997), and an increased migration to injured or inflamed tissue, may be beneficial (Viswanathan and Dhabhar, 2005). Moreover as introduced above, naïve and central memory CD8+ T-lymphocytes and cytotoxic T-lymphocytes migratory preference is confined to peripheral tissue (Romero *et al*., 2007; Appay *et al*., 2002; Hamann *et al*., 1997), hence the preferential redistribution of these subset types. Dimitrov *et al*. (2009) (see figure 2.9) investigated the circulatory circadian patterns of CD4+ and CD8+ T-lymphocyte subsets respectively, demonstrating a distinct state of differentiation-specific profiles during diurnal and nocturnal periods with subsets of a naïve (figure 2.9; A and E) , central memory (figure 2.9; B and F) and effector (figure 2.9; C and G) phenotypes display a diurnal acrophase, while greater diurnal variability is present in effector memory phenotypes (figure 2.9; D and H).

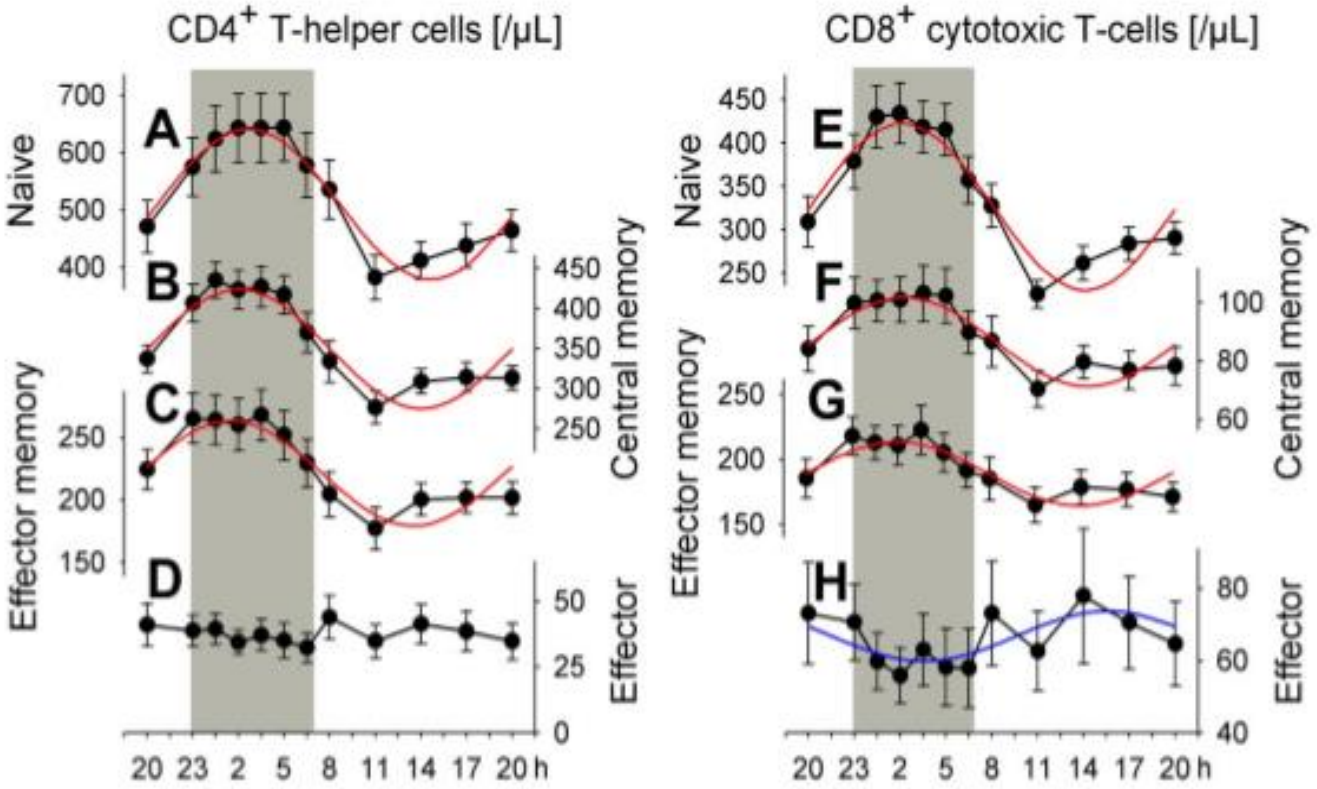


Figure 2.9. Circadian circulatory patterns of CD3+CD4+/CD8+ T-lymphocyte subsets (taken from Dimitrov *et al*., 2009, p. 5137): (A) CD4+ T-helper naïve subset, (B) CD4+ central memory subset, (C) CD4+ effector memory subset, (D) CD4+ memory subsets , (E) CD8+ T-helper naïve subset, (F) CD8+ central memory subset, (G) CD8+ effector memory subset, (H) CD8+ memory subsets.

NK cells show the highest receptor expression and consequently display the greatest mobilisation into the periphery in response to acute stress (Anane *et al*., 2009; Campbell *et al*., 2009). CD8+ T-lymphocytes have intermediate receptor expression levels and exhibit intermediate mobilisation. Whereas, CD4+ cells express substantially less β2 adrenergic receptors and mobilisation is limited in this *pro-rata* manner (Turner *et al*., 2010; Anane *et al*., 2009; Campbell *et al*., 2009; Simpson *et al*., 2007).

Consequently, the effect of adrenaline selectively increasing numbers of effector CD8+ T-lymphocytes is not sufficiently explained by β2 adrenoreceptor expression alone. However, when the redistribution effects of adrenaline are considered together with those effects of cortisol during initial lymphocytosis and subsequent lymphocytopenia, more clarity becomes present. If one uses the analogy of a two-sided coin, one side of the coin may be construed as adrenaline mobilising cytotoxic T-lymphocytes (by way of β2 adrenoreceptors) from endothelial tissue by demarginalisation in a process which takes seconds to occur. The second side may be explained as cortisol depleting naïve and central memory T-lymphocytes from peripheral circulation and being redistributed to the bone marrow by way of high expression of the glucocorticoid sensitive chemokine receptor CXCR4. Importantly, effector CD8+ T-lymphocytes display low expression of this chemokine receptor and are confined to peripheral tissues presumably for immunosurveillance purposes during an active phase.

In addition to lymphocyte cytotoxicity levels and migratory patterns, additional characteristic factors were reported to influence the redistribution and control

of immune defence by Dimitrov *et al*. (2009). For instance, effector or adaptive co-stimulatory functional status, expression of β2-adrenoreceptor, and state of differentiation (i.e. terminally versus naïve) (Campbell *et al*., 2009; Simpson *et al*., 2007; Bosch *et al*., 2005), have all been shown to be synchronising factors linking the immune system to the stress-axes and in turn to confirm biological rhythm within exercise-induced immune response. Campbell *et al*. (2009) defines the characteristics of those lymphocytes that display the most apparent mobilisation into peripheral tissues as a consequence of acute stress-stimulation exhibiting high-tissue migratory potential, high cytotoxicity and low proliferation capabilities. In the most part, these characteristics are distinctive of CD8+ T-lymphocyte subsets and particularly effector-memory subset type, with a rise in circulating effector-memory CD8+ T-lymphocytes by 450% compared to an increase of 84% in naïve CD8+ T-lymphocytes during acute exercise induced stress (Campbell *et al*., 2009). Furthermore, mounting evidence suggests that NK cells show the greatest apparent response to acute stress with increases in excess of 900% reported by two separate studies (Anane *et al.,* 2009; Campbell *et al*., 2009).

The selective mobilisation of lymphocytes of cytotoxic tissue-migrating character in response to acute stress, both within the NK cell and CD8+ T-lymphocyte subset population, may enhance the ability to rapidly remove foreign antigenic challenges and promote immune-surveillance during periods of increased stress (Sallusto *et al*., 2004; 1999). Moreover, the verification of the catecholamines as the driving influence behind this phenomenon has been shown by way of blocking endogenous catecholamines (by stellate ganglion

block) resulting in a decrease of CD8+ cytotoxic T-lymphocyte and NK cell numbers (Dimitrov *et al*., 2010; Yokoyama *et al.,* 2000). It has been demonstrated that the presence of adrenaline selectively increases the numbers of circulating CTLs, with *in vitro* administration of the hormone reducing adhesion to the endothelium (Dimitrov *et al*., 2010). Similarly, Dimitrov and colleagues (2009), to simulate maximal endogenous levels of circulating adrenaline (0.005 μg/kg per minute) and cortisol (8 μg/kg per minute) respectively, intravenously administered the hormones 30 minutes during their respective circadian rhythm nadir with the resulting pattern of circulating CD4+ and CD8+ T-lymphocyte subsets corresponding to acrophase level. In addition to the effect of stress, circadian driven increases in SNS activity mobilise CTLs (such as effector memory (EM) and effector CD8+ T-lymphocytes, NK cells), whereas cells that do not display cytotoxic effector potential (e.g. naïve T-lymphocytes appear considerably less responsive to the effect of catecholamines and are closely influenced by HPA controlled cortisol) (Dimitrov *et al*., 2010).

The mechanism by which the biphasic response is initiated is considered to be two-pronged: by the preferential stimulation of specific cell surface receptors located on cytotoxic lymphocytes; and also as a consequence of increased cardiac output releasing T-lymphocytes from marginal endothelium due to haemodynamic forces (Shephard, 2003). However, it is important to note that these respective physiological processes are not mutually exclusive. CTLs highly express β2-adrenoceptors on their cell surface, but importantly the migratory pattern of CTLs confines them to the peripheral marginal

endothelium where an increase in shear stress detaches them into the blood (Dimitrov *et al*., 2010).

T-lymphocyte differentiation is decisive for the development of T-lymphocyte function. For example, naïve CD4+ T-lymphocytes differentiate preferentially into Th1 cells when they receive T-lymphocyte–receptor stimulation in the presence of the cytokine IL-12 (Seder *et al.,* 1993), while they will differentiate preferentially into Th2 effector cells in the presence of IL-4 (Seder *et al.,* 1992). Hence, any change to the cytokine micro-environment plays a pivotal role in naïve CD4+ T-lymphocyte differentiation, as any catecholamine change may affect the naïve T-lymphocyte differentiation and proliferation (Seder *et al.,* 1992). Cytokines show remarkable diurnal patterns with the cytokines IL-2, IL-6, IL-12, tumour necrosis factor (TNF)-alpha and interferon (IFN)-gamma all displaying maximal acrophase during periods of nocturnal sleep (Lange *et al*., 2010; Meltzer *et al*., 2004).

2.13 Environmental Conditions, Heat, Exercise and Immunity

Environmental conditions play an important role in influencing immunological response. Extremes in temperature, whether hot or cold, result in immunology challenges. For example, exercise in hot conditions results in the body's absorption of heat from the environment, which in addition to endogenous metabolic process raises core body temperature. It is therefore unsurprising that up to 60% of energy derived from metabolism of free fatty acids and glucose is used to heat the body, whereas, the remaining 40% is utilised as fuel in muscle contraction (Peake, 2010). As a consequence of exercise-

induced elevation of core body temperature, cardiac output increases to deliver blood to skin with the aim of acting to cool the body and reduce the rate of core body temperature increase. Catecholamines released from the adrenal medulla, such as adrenaline and noradrenaline, regulate changes in cardiac output and blood flow during exercise (Peake, 2010; French *et al*., 2007; Foster *et al*., 1986). Exercise is also known to stimulate the release of cortisol from the adrenal cortex (Lovallo *et al*., 2006).

Alterations in immune function during exercise in the heat have important implications for athletes. Exercise can increase the incidence of developing upper respiratory tract illness (Cox *et al*., 2008; Nieman *et al.,* 1990) and immune changes during exercise in the heat may contribute to the symptoms of heatstroke (Lim and Mackinnon, 2006). A number of factors may influence the change of circulating immune parameters due to exercise in different environmental conditions, including changes in core body temperature and increased levels of catecholamines and cortisol (Lovallo *et al*., 2006; McCarthey *et al*., 1992; Foster *et al*., 1986).

Catecholamines are released independently of changes in body temperature and result in increased blood flow and leukocyte trafficking (Walsh and Whitham, 2006). These physiological responses to exercise influence the immune system by increasing blood flow and peripheral concentrations of stress hormones to mobilise leukocytes from the bone marrow into peripheral blood compartment and via demarginalisation from the endothelial surface of blood vessels (McCarthey *et al*., 1992). Secondly, by binding to surface receptors on immune cells, stress hormones alter signalling pathways within

these cells that modulate the functional activity of these cells (Dimitrov *et al*., 2009; Miles *et al.*, 1998; Benschop *et al*., 1996). Cytokines increase to a rise in core body temperature may also be considered in influencing circulating leukocyte numbers in an autocrine and paracrine manner (Downing *et al*., 1988; 1987).

Circulating leukocytes appear to be responsive to changes in core body temperature once 38°C is exceeded (Peake, 2010), with a similar response to acute exercise-induced temperature increases (Laing *et al*., 2008; Kappel *et al*., 1991). Cytokines mediate cross-talk between different cells of the immune and endocrine systems as well as the release of immune cells from bone marrow and are an integral component in the inflammatory responses to infection and tissue injury (Gleeson *et al*., 2013; Elenkov and Chrousos, 2002). Passive heating for two hours at 38.5°C to 39.5°C raising core body temperature to ≥38.5°C increases the plasma concentrations of the proinflammatory cytokines IL-1, IL-6, Il-8 and TNF-a, (Laing *et al.*, 2008).

The effect of raised core body temperature on neutrophils is equivocal. Raising core body temperature to 38.5°C reduces neutrophil production of the proteolytic enzyme elastase (Laing *et al*., 2008), whereas two hours of passive heating at 39.5°C had no effect on neutrophil production of reactive oxygen species (Kappel *et al*., 1994). In contrast, others have reported that six hours of passive heating of cancer patients at 41.5°C raises neutrophil bactericidal capacity (Grogan *et al*., 1980). It appears the intensity of an exercise stimulus, and not necessarily the associated rise in body tempretaure *per se*, suggests that exercise-induced leucocytosis is not core body temperature dependent.

Exercise in hot conditions, compared to passive heating via environmental means, increases core body temperature to at least the same extent, but produces a greater magnitude in stress hormone response (Brenner *et al*., 1999). This greater stress hormone response to exercise is due to a greater demand for blood flow and nutrients to contracting skeletal muscles and redistribute heat to the skin for cooling. Several studies have investigated whether exercise in the heat promotes leukocytosis. Circulating leukocyte numbers increase when core body temperature increases by 1°C during exercise (Rhind *et al*., 1999). Moderate intensity exercise or exercise of a shorter duration that does not increase core body by 1°C does not promote any significant leukocytosis (Peake *et al*., 2007; Niess *et al*., 2003; Brenner *et al*., 1999).

The rise in core body temperature and peripheral concentrations of stress hormones accounts for the elevation of circulating leukocyte numbers in response to exercise (Rhind *et al*., 1999). In the hours after exercise, leukocyte cell counts either remain elevated or return to baseline in a similar manner to that after exercise in thermo-neutral conditions (Laing *et al*., 2008; Niess *et al*., 2003; Rhind *et al*., 1999).

Cytokine response to an exercise-induced rise in core body temperature potentially plays an important role as cytokines regulate leukocyte trafficking and function. The cytokines IL-6 and TNF-α (tumor necrosis factor) increase during exercise due to greater body temperature (Peake *et al*., 2007; Starkie *et al*., 2005; Rhind *et al*., 1999), with IL-6 stimulating the production of IL-10 and IL-1ra during the latter stage of exercise (Steensberg *et al*., 2003). IL-8

also increases during exercise due to accumulation of body heat, but it appears to have a limited role in regulating changes in circulating neutrophils (Laing *et al*., 2008; Peake *et al*., 2007).

Research investigating the influence of heat stroke on the incidence of illness reported that lymphocyte activation is depressed in military personnel with prolonged elevation in core body temperature (>40.4°C) compared with peers within core body temperature of normal physiological range (~38.6°C) (DuBose *et al*., 2003). The number of circulating CD8+ lymphocytes and NK cells were higher (DuBose *et al*., 2003), whereas the number of CD4+ T-helper lymphocytes were lower in individuals with heatstroke (~41.4°C) (Hammami *et al*., 1998; Bouchama *et al*., 1992). Moreover, the presence of heatstroke was associated with the number of cytotoxic lymphocytes and correlated with core body temperature (Bouchama *et al*., 1992). A dual pathway model has been proposed to account for the role of cytokines and core body temperature in heatstroke and the redistribution of circulating lymphocytes (Lim and Mackinnon, 2006).

2.14 The Circadian System and Health

The circadian system, as a result of evolution, works optimally when all constituent components work in synergy to the daily cycles controlled by the master pacemaker, the SCN. Dysregulation, due to chronic stress, illness or prolonged poor lifestyle/work patterns disrupts homeostasis with long-term consequences for morbidity and health. Shift-workers have an increased prevalence of hypertension, gastrointestinal disturbance (Sookoian *et al*.,

2007; Knutsson, 2003), developing cancer (Scherhammer *et al*., 2006; 2003) and irregular blood glucose patterns increasing the risk of diabetes (Rudiac *et al*., 2004). Disruption of the endocrine system due to circadian disturbances have been implicated in the development of metabolic conditions and mental health (Chrousos, 2000).

In addition, the neuroendocrine and immune systems are characterised by an integrated communication network of shared signalling proteins and receptors (Blalock *et al*., 1985) which display their own circadian, ultradian and infradian rhythms (Ackerman *et al*., 2012; Dimitrov *et al*., 2009; Wrona, 2006). Cytokines, which can be produced by cells of the immune and neuroendocrine systems, are the most common mediator between these two systems (Chrousos, 1995) but lymphokines (a subset of cytokines) released by lymphocytes can signal to neuroendocrine tissues (Weigent and Blalock, 1987). Moreover, immune cells can synthesize neuroendocrine hormones and many hormones, including cortisol, can interact with the immune system due to the expression of mutual receptors (Dimitrov *et al*., 2009; Blalock *et al*., 1985). The stress hormones, catecholamines and cortisol, appear influential in exercise-induced immune responses and recovery period from exercise (Dimitrov *et al*., 2009; Pedersen *et al*., 1998; 1997). Catecholamines appear to be responsible for acute exercise effects on lymphocyte subpopulations, while cortisol appears to have a delayed effect of immune parameters of approximately two hours (Pedersen *et al*., 1998; McCarthy and Dale, 1988). As a result of this highly integrated communication network research

investigating the effect of diurnal exercise on endocrine and immune responses merits exploration.

2.15 Summary

To date research has demonstrated a distinct circadian rhythm in circulating leukocytes including neutrophils and lymphocytes during everyday living conditions (Lange *et al*., 2010). The fluctuation of circulating virus fighting CD8 T-lymphocytes amongst other immune cells is influenced by the actions of the endocrine system, especially the stress hormone adrenaline and neurotransmitter noradrenaline (Dimitrov *et al*., 2010; 2009). In addition, it has been demonstrated that acute stress can cause an oscillation in circulating T-lymphocyte numbers, with greater exercise intensity resulting in a more marked effect (Turner *et al*., 2010; Anane *et al*., 2009; Simpson *et al*., 2008). However, no research thus far has aimed to explicitly investigate the effect of exercise-induced immune response at differing times of day (morning versus evening). It is therefore proposed that an investigation assessing exercise-induced immune response at conflicting diurnal time-points is warranted. Indeed, it is anticipated that such a study may reveal a divergent immune response in circulating lymphocytes due, in part, to the influence of circadian phase (time-of-day). Thus far no such research has examined such a hypothesis.

2.16 Study Aims

The predominant aim of this thesis was to investigate the effect of high-intensity self-paced exercise in highly trained and recreationally active male runners at two different times of the day that this activity is commonly undertaken. In particular, physiology, immune and hormone parameters were examined to determine whether a diurnal phase response was exhibited.

The specific aims of each data chapter and the studies conducted for this thesis are as follows:

- **Chapter 4:**
    - ***Aim 1:*** To examine if the diurnal phase exercise is undertaken at influences macro physiology parameters, including rate of perceived exertion (RPE), heart rate (HR), core body temperature (CBT) and exercise performance in trained male distance runners.

    Hypothesis: Parameters of physiology display circadian variation with a divergent diurnal phase response to morning and evening exercise.

    - ***Aim 2:*** To determine whether a relationship between diurnal phase and chronotype classification may effect physiological responses.

    Hypothesis: Chronotype score and categorisation (based on the Morningness-Eveningness questionnaire scoring) would significantly impact exercise performance and physiology.

    - ***Aim 3:*** To assess lung function in response to an acute bout of self-paced high-intensity exercise in a cold environment (6°C) at morning and evening time-points.

Hypothesis: Lung function would be negatively affected by an acute bout of self-paced high-intensity exercise in a cold environment (6°C) and diurnal variation.

- ***Aim 4:*** To investigate immune (leukocytes and lymphocyte subsets) and endocrine (catecholamines and cortisol) parameters in response to high-intensity exercise and diurnal phase.

Hypothesis: Leukocytes, lymphocyte/lymphocyte subsets and catecholamines and cortisol would be effected by high-intensity exercise and diurnal phase.

- **Chapter 5:**
  - ***Aim 5:*** To investigate training status and diurnal phase on exercise response and physiological parameters.

Hypothesis: The diurnal phase exercise is undertaken at would result in a differential physiological response in recreational and experienced distance runners.

- ***Aim 6:*** To investigate neutrophil and lymphocyte responses to exercise at diurnally different time-points in recreational and experienced distance runners.

Hypothesis: A significant diurnal response to exercise would be observed in recreational and experienced runners.

- ***Aim 7:*** To examine the effect of exercise and diurnal phase on CD8+ T-lymphocyte subsets of early, intermediate and late phenotypes in experienced and recreational runners.

Hypothesis: Late differentiated CD8+ subsets would be preferentially mobilised in response to evening exercise and training status would have a significant effect on this response.

- ***Aim 8:*** To investigate the effect of exercise and diurnal phase on lung function ability in recreational and experienced runners in cold environmental conditions.

Hypothesis: Lung function in response to exercise would be subject to circadian rhythms with higher capabilities in the evening and recreational runners would report significant impairment of lung function and diurnal phase.

- ***Aim 9:*** To assess whether cortisol response to exercise trial would be influenced by training status.

Hypothesis: Cortisol concentrations in peripheral blood displays a circadian pattern with higher values in the morning and cortisol concentrations would respond differently according to training status.

- **Chapter 6:**
  - ***Aim 10:*** To investigate the association between Morningness-Eveningness Questionnaire (MEQ) score and parameters of physiology at rest and in response to exercise at morning and evening time-points.
  - ***Aim 11:*** To investigate the association between MEQ score and parameters of the immune system (leukocytes, lymphocytes, CD8+ T-lymphocyte subsets and neutrophils).

Hypothesis: MEQ score, due to known advancement of circadian phase, would significantly influence physiological and immunological parameters at rest and in response to exercise.

Chapter 3: General Methodology

3.1 Introduction

Chapter 3 states the general methodologies used, unless otherwise stated, for all studies undertaken for this thesis, as approved by the ethics committee at Edinburgh Napier University.

3.2 Participants

Entry onto both studies included in this thesis was subject to strict requirements. Participants, all male, completed a medical screening form (PAR-Q) (Appendix 5) and a study consent form (Appendix 2). To preserve the integrity of the population cohort, all participants were required to be free of symptoms of illness or injury four weeks prior, did not report sleep or gastrointestinal conditions, were not currently using ergogenic/sports supplements and adhered to the same nutrional intake and time-of-day pattern prior to both experimental trials. In addition, due to concerns regarding lung function ability and premature aging of immune cells (Chaudhuri *et al*., 2006), all participants were strictly non-smokers, refrained from exercise and alcohol 24-hours prior to exercise trials and caffeine six hours pre-exercise. Participants who were taking medication affecting the immune system were excluded from the study. To maintain study validity, simulated laboratory-based performance experimental procedures, as described by Reilly and Waterhouse (2009), were employed with participants adhering to regular sleep-wake, activity and eating schedules between and on trial days, in a counter-balanced manner. Each participant provided written informed consent

(Appendix 2) and were aware they were free to withdraw from the study at any time.

3.3 Participant Familiarisation

Participants not familiar with running on a treadmill (Woodway, ergo ELG 55, Germany), were required to complete one familiarisation trial before being recruited onto the study.

3.4 Pre-Trial Assessment of Aerobic Fitness

Participants attended the Human Performance Centre at Edinburgh Napier University having refrained from ingesting caffeine-containing products from the night before, as well as refraining from any alcohol intake 24-hours prior to participation. After 10 minutes rest in a supine position and prior to commencing the trial, blood pressure was measured (Nonin Puresat Avant 2120, Nonin medical Inc, Minnesota, USA; Ultra-Check® Blood Pressure Adult Cuff, Statcorp Medical, Florida, USA) on the non-dominant arm.

To assess suitability for entry into the study cohort, an assessment of aerobic fitness was first completed outwith experimental testing times between the hours of 11.00hrs and 15.00hrs during UK summertime on separate occasions. An incremental exercise test on a motorized treadmill (Woodway) using online gas analysis (CPX MedGraphics, Oldham, UK), commenced at an initial speed of 10km·h^{-1} with 0% gradient. Every three minutes the treadmill speed was increased by 3km·h^{-1} until achieving a maximum speed of 16km·h^{-1}. At this stage, and after running duration of three minutes, at 16km·h^{-1} the

treadmill gradient increased by 2.5% every minute until the runner reached maximum fatigue (Simpson *et al*., 2006). Runners were deemed to have reached their $\dot{V}O_2max$ when heart rate (HR) was 220 beats·min^{-1} minus the runner's age; where the respiratory exchange ratio (RER) value was greater than 1.10; and $\dot{V}O_2$ reached a plateau. At the latter stages of the test, the participants were encouraged verbally.

3.5 Experimental Design

Participants were required to complete a PAR-Q questionnaire, a Morningness-Eveningness Questionnaire (MEQ) (Horne and Ostberg, 1976) (Appendix 3) and an Arousal and Alertness Questionnaire (Appendix 7) on trial days. Participants who presented with cold or flu symptoms, injury or contraindications to exercise were asked to return after they recovered fully. The trial consisted of a 10 kilometer (km) time-trial run on a motorised treadmill (Woodway, ergo ELG55, Germany) at two different times of the day (09.00hrs and 16.00hrs) on two separate occasions. The trials were performed in a thermal-neutral controlled environmental chamber (Weis-Gallenkamp, UK) set at a standardised 6°C to simulate mean Scottish winter temperatures. This temperature was set to conform to the optimum temperature to assess core body temperature response to time-trial performance (Boukelia, 2015). A minimum of seven days was required between the first and second trial and to ensure circadian parameters such as activity, rest and nutritional status were comparable. Written informed consent was obtained from all participants prior to commencement of each trial.

3.6 Experimental Procedures

On experimental trial days, all participants were required to report to the Human Performance Laboratory at Edinburgh Napier University a minimum of ninety minutes prior to trial commencement. The following questionnaires and paperwork were completed by each participant:

- Research Participant Information Sheet (Appendix 1)
- Study Consent Form (Appendix 2)
- Morningness-Eveningness Questionnaire (MEQ) (Appendix 3)
- Venepuncture Blood Donation Declaration Form (Appendix 4)
- Physical Activity Readiness Questionnaire (PAR-Q) (Appendix 5)
- Caffeine Consumption Diary (CCD) (Appendix 6)
- Arousal and Alertness Questionnaire (Appendix 7)

On completion of all relevant prerequisites, and without any contraindications being revealed, the experimental trial began at this point. The trial consisted of a self-determined (self-paced) 10km time-trial, with speed of the treadmill under the control of the participating athlete. In addition, the athlete was unaware as to the distance covered or time expired while exercising, to conform to normal time-trial procedures. However, a 1km remaining notification was disclosed to the athlete. The experimental trials were completed at two differing diurnal time-points (study 1, chapter 4: 09.00hrs and 16.00hrs; study 2, chapters 5 and 6: 09.00hrs and 17.00hrs) on separate days, with a standardised minimum of seven days between trials. All trials were completed on weekdays. During the trial, subjective Ratings of Perceived

Exertion (RPE) (Appendix 8) on a scale of 6-20 (Borg, 1970) and HR (Polar Electro, Finland) were recorded at 1km stages throughout.

3.7 Environmental and Core Body Temperature Assessment

Environmental temperature has been demonstrated to advance or influence lung function capabilities (Kippelen *et al*., 2012) and circadian phase (Moul *et al*., 2002). Temperatures greater than 35°C have demonstrated eliciting an immunological response (Laing *et al*., 2008; Starkie *et al*., 2005). Therefore, all trials were performed in a temperature controlled environmental chamber set at a standardised, thermal-neutral 6°C (Scottish mean winter temperature). Similarly, particpants were exposed to standardised laboratory lighting of 500 lux (for a minimum of 90 minutes) prior to both experimental trials. These methodological controls were designed to preserve internal validity from exogenous factors that may mask endogenous circadian values and are commonplace in the study of circadian rhythms; they were not designed to increase difficulty or cause complications to the exercising athlete.

Core body temperature (CBT) was measured throughout all trials using a core temperature (CoreTemp) monitoring pill (Core Body Temperature Sensors, 262K, HT 150002, Florida, U.S.A.) and CoreTemp data recorder (HQInc, Florida, U.S.A) (in accordance with Byrne and Lim, 2007). Findings from our laboratory found the CoreTemp pill, compared to tympanic and aural assessment, to be the most accurate method to record core body temperature when administered a minimum of 90 minutes prior to trial commencement (Boukelia, 2015). The assessment of CBT, by way of pilot data, was validated

over a full circadian cycle (24-hours) at rest (figure 4.3, chapter 4) and in an exercise condition (figure 4.4, chapter 4).

3.8 Lung Function Tests

Before exercise (pre), immediately after exercise (post) and one hour into recovery (1hr post), the participants performed a spirometry test, with values of forced vital capacity (FVC), forced expiratory volume in 1 second ($FEV_{1)}$, forced expiratory flow at 25-75% of the pulmonary volume (FEF_{25-75}) and peak expiratory flow (PEF) measured (Gomes *et al*., 2010). Briefly, in an upright standing position the participants inhaled filling their lungs maximally, pausing briefly before they exhaled into the mouthpiece of the spirometer (Compact II: Type C, Vitalograph Ltd., UK) until all air was expired. The participants performed the spirometry test three times with the highest respective values recorded in accordance with trial-retrial standards set by Miller *et al*. (2005). Familiarisation trials were completed until all participants were comfortable with the procedure.

3.9 Biochemistry

Blood samples were drawn from the participants while in a supine position pre, immediately-post and 1hr post-exercise; pre-exercise and 1hr post exercise respective blood samples were drawn after 10 minutes of supine rest, post exercise samples were collected immediately upon trial ceasing. Peripheral blood was collected in 6 millilitre (ml) (BD Biosciences, UK) vials, coated in ethylenediaminetetraacetic acid (EDTA) (Becton-Dickinson, Oxford, UK) using

¾ BD vacutainer safety-lok 25G needles (Becton-Dickinson, Oxford, UK). The samples were collected by venepuncture from the antecubital vein by a qualified technician. Total blood leukocyte counts were determined using an automated haematology analyser (Sysmex, XS 1000i, UK), with each sample analysed in duplicate.

Four 6ml EDTA tubes and one 4ml EDTA tube (BD Biosciences, USA) were used for determination of lymphocyte subpopulations and plasma stress hormones (cortisol, adrenaline and noradrenaline) for chapter 4. EDTA vacutainers containing whole blood were centrifuged at 3000rpm for 10mins at 22ºC. Once finished, the band of plasma was pipetted from the separated whole blood and into 3ml eppendorfs, labelled and immediately stored at -80ºC for future ELISA analysis (chapter 4).

For chapter 5, two 6ml EDTA tubes, one 4mL EDTA tube and one 5ml serum tube (BD Biosciences, USA) were used for determination of lymphocyte subpopulations and (serum) cortisol.

3.10 ELISA Analysis

Serum cortisol (R&D Systems, UK) and plasma catecholamine (adrenaline and noradrenaline) (CatCombi, IBL, Germany) levels were measured using respective enzyme linked immunoabsorbent assay (ELISA) kits. Plates were read on a spectrophotometerat a wavelength of 450nm (Labtech LW5000, UK).

Plasma Catecholamine

3.11 Lymphocyte Isolation Procedure

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood using density gradient centrifugation (Lymphoprep®, Axis-Shield, Oslo, Norway). Plasma was extracted for ELISA analysis (chapter 4) and equal 0.9% NaCl added to replace. The 6ml sample was then diluted in equal measure (50:50) with 0.9% NaCl. The 6ml of the diluted blood solution was carefully layered on top of 3ml of Lymphoprep solution (Axis-Shield, Oslo, Norway). This solution was centrifuged at 800G, 22ºC for 30 minutes. The band of lymphocytes which formed was removed and pipetted into a centrifuge tube where 0.9% NaCl (Baxter, UK) was added in order to make up a 40ml cell suspension. Cells were then washed for 10mins at 250G at 22ºC, the peripheral blood mononuclear cells (PMBCs) then adhered to the bottom of the centrifuge tube in the form of a clear pellet and excess supernatant was removed. PMBCs were once again resuspended in 40ml of PBS-BSA (phosphate buffer saline 2% bovine serum albumin and 0.02% sodium azide (Sigma-Aldrich, Irvine, Scotland)), and washed again at 250G for 10mins at 22ºC. Once more the supernatant was removed and PMBCs were re-suspended in 1ml PBS-BSA. Finally, trypan blue staining method was used to obtain the required number of cells (500,000) for flow cytometry analysis.

3.12 Cell Analysis and Cell Surface Phenotyping

Isolated lymphocytes (0.5×10^6) were incubated for 45mins at room temperature with 100 microliter (µl) (working dilution) of each monoclonal antibody (MAb): CD3+CD8+ T-lymphocytes subsets (were left to incubate for

45mins at room temperature. All samples were incubated with an APC-conjugated anti-CD3 monoclonal antibody (table 3.1) (BD Biosciences, San Jose, CA).

Table 3.1. Antibody panel for CD8+ T-lymphocyte surface markers.

Antibody	Filter	Cat no.	Company
CD3	APC (SK7)	345767	BD
CD4	PE-CY5	555348	BD
CD8	PE-CY5	555636	BD
CD8	PE	345774	BD
CD27	FITC	555440	BD
CD27	PE	555441	BD
CD28	PE	555729	BD
CD45RA	PE	555489	BD
CD45RA	FITC	335039	BD

Combinations of one FITC, PE-CY7, PE-CY5, or PE-conjugated monoclonal antibody with specificity for CD45RA / CD8 / CD27, (table 3.2 below). All MAbs used were previously titrated to determine optimal conditions for analysis by flow cytometry.

Table 3.2. CD8+ T-lymphocyte surface markers and state of differentiation.

Cell Description	T-Lymphocyte Marker	Identification	Reference
Naïve	CD3+CD8+	CD45RA+CD27+	Turner *et al*., 2010
Central-memory (CM)	CD3+CD8+	CD45RA-CD27+	Turner *et al*., 2010
Effector-memory (EM)	CD3+CD8+	CD45RA-CD27-	Turner *et al*., 2010
CD45RA+effector memory (RAEM)	CD3+CD8+	CD45RA+CD27-	Turner *et al*., 2010

3.13 Flow Cytometry

Cell phenotype data was attained using CELLQuest Pro software (BD Biosciences, San Jose, CA, USA) on a FACSCalibur flow cytometer equipped with a 15mW argon ion laser emitting light at a fixed wave length of 488nm. An electronic gate was placed around the lymphocyte population in the flow cytometry forward and side scatter mode. Forward scatter against Allophycocyanin (APC) fluorescence was then used to identify and individually gate CD3+ and CD3- cell populations (illustrated in figure 3.1), CD8 expression was determined using the CD3+ gate, with 10000 gated events required for analysis. Lymphocyte subset numbers were calculated by multiplying the percentage values obtained from the flow cytometer by total lymphocyte count as reported by the haematology analyser.

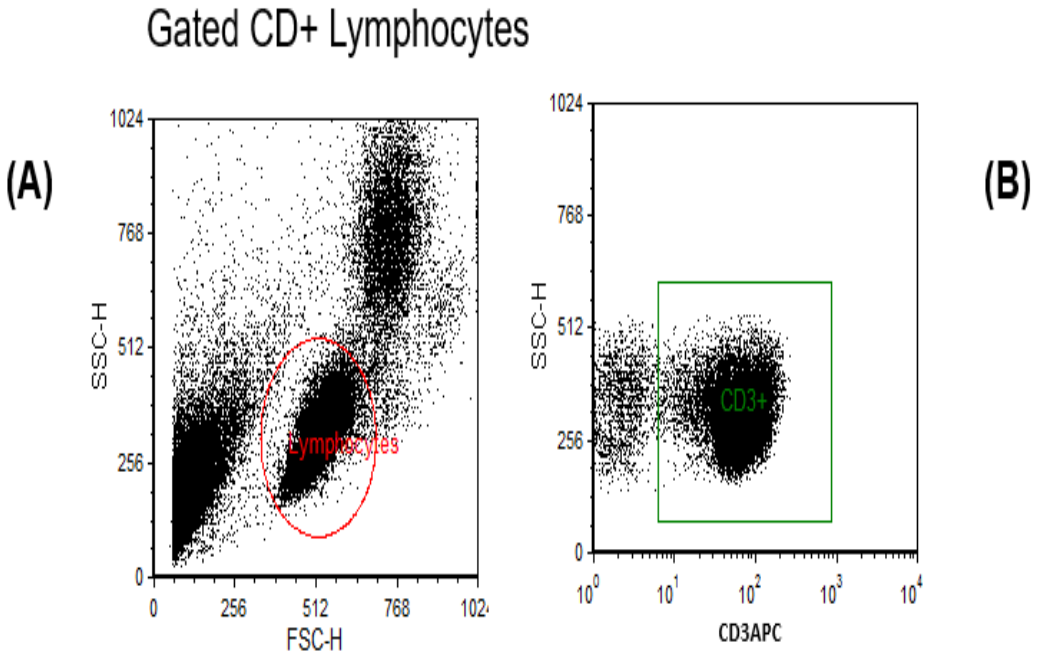


Figure 3.1. CD3+ T-lymphocytes determined by forward scatter (FSC-H) and side scatter (SSC-H) on a flow cytometer. Red circle shows gated (A) lymphocyte population, and green highlighted area shows CD3+ T-lymphocytes gated from lymphocyte population (B).

3.14 Statistical Analysis

SPSS software version 20 (Chicago, IL) was used to complete statistical analyse; normal distribution using the Kolmogrov Smirnov non-parametric test. The effect of time (i.e. pre, post and 1hr post) and diurnal time-points (morning - AM and evening - pm) were analysed using repeated measures analyses of variance (ANOVA). Only significant interactions (time x diurnal time-point) are presented unless otherwise stated. Bonferroni corrected paired T-tests were utilised when significance was found ($p < 0.01$). Assumptions of sphericity in data were checked for each ANOVA, and, where appropriate, adjustments were made to the degrees of freedom. Paired sample T-tests were used to determine fitness status (study 2) and physiology parameters for pre and post -exercise and for diurnal phase (AM pre vs PM pre). Linear regression was used to examine correlation with r value reported. Statistical significance was recorded as $p<0.05$. All results are presented as mean ± SE unless otherwise stated.

Chapter 4: Diurnal Variation in Physiology and Immune Responses to High-Intensity Aerobic Exercise in Men

4.1 Introduction

Circadian rhythms are integral to the normal functioning of physiological processes (Adan *et al*., 2012; Kantermann *et al*., 2007; Ko and Takahashi, 2006; Freedman *et al*., 1999). Diurnal variations in physiological performance and the physiological responses to exercise have been investigated extensively (Reilly and Edwards, 2007; Waterhouse *et al*., 2004; Reilly and Bambaeichi, 2003; Atkinson and Reilly, 1996). Data collected from equally distributed time-points over a 24-hour circadian period may be used to locate peak (acrophase) and low point (bathyphase) of a cycle using a cosine mathematical model (Reilly *et al*., 2007; Refineti, 2006; Forsyth and Reilly, 2004).

Many physiological parameters associated with exercise performance display distinct diurnal rhythm with optimal values consistently reported in the hours of early evening (around 16.00 hours (hrs) to 20.00hrs) (Edwards *et al*., 2013; Reilly and Waterhouse, 2009). For example, blood pressure (Guo and Stein, 2003; Uzu and Kimura, 1999; Uzu *et al*., 1997), muscular strength and peak force generating ability (Guette *et al*., 2005; Souissi *et al*., 2003), broad jump performance (Reilly and Down, 1992), anaerobic power (Souissi *et al*., 2007) and distance running (Drust *et al*., 2005; Atkinson and Reilly, 1996; Winget *et al.*, 1985) peak from 15.30hrs to 20.30hrs with amplitudes ranging from 2% to 11% of mean daily cosine values (Reilly and Waterhouse, 2009; Reilly *et al*, 2007; Reilly and Edwards, 2007). Similary, and as a consequence in many regards, linear improvements in sporting performance from morning to evening have been reported in swimming (Kline *et al*., 2007; Martin *et al*.,

2007; Deschodt and Arsac, 2004; Arnett, 2001; Baxter and Reilly, 1983), cycling (Atkinson *et al*., 2005; Deschodt and Arsac, 2004; Reilly and Baxter, 1983), racket sports (Edwards *et al*., 2005; Atkinson and Speirs, 1998), resistance training (Teo, 2011), in intermittent high-intensity aerobic sports such as soccer (Chtourou *et al*., 2012; Reilly *et al*., 1997) and rowing (Forsyth and Reilly, 2004).

Although an abundance of research reports circadian variation in physiological parameters, contradictory observations have been presented (Racinais *et al*., 2005; O'Connor *et al*., 2004; Youngstedt and O'Connor, 1999; Deschenes *et al*., 1998; Dalton *et al*., 1997). A series of important limiting factors have been observed including fatigue, inter-individual variation, habitual time-of-day training, unfamiliarity with exercise mode or intensity, nutritional status and chronotype (Prasai *et al*., 2013; Racinais *et al*., 2005; Youngstedt and O'Connor, 1999; Deschenes *et al,*. 1998; Dalton *et al*., 1997) and not controlling for biological rhythms (masking effects) (Okusaga and Postolache, 2013).

Chronotype refers to the innate circadian phenotype for preferred activity, and indeed inactivity (rest), that is specific to an individual (Taillard *et al*., 2005; Roenneberg *et al*., 2003; Horne and Ostberg, 1976). Using Horne and Ostberg's (1976) Morningness-Eveningness questionnaire (MEQ), there are five main categories of chronotype: definitely morning-type, moderately morning-type, intermediate (or neither-types), evening-type and definitely evening-type with the distribution of chronotypes within any given population 'bell-shaped' (Horne and Ostberg, 1976). The vast majority of the global

population are classified as intermediate-types (moderately morning or moderately evening-type) with approximately 10% of the population evenly distributed between morning or evening phenotypes, respectively (Henst *et al.*, 2015; Kabrita *et al*., 2014; Kudielka *et al*., 2006; Zavada *et al*., 2005; Smith *et al*., 2002). As their name would suggest, morning chronotypes inherently prefer being active and alert earlier in the first third of the diurnal day whereas evening-types display greater activity in the latter third of the day, with a divergent pattern of sleep onset/rising between chronotypes (Taillard *et al*., 2005; Smith *et al*., 2002). Additionally, core body temperature (CBT) as a surrogate for circadian phase, displays a two hour phase advancement in morning chronotypes compared to evening phenotypes, when assessed in the hours of the morning in a cohort of morning only chronotypes (Henst *et al*., 2015). A conflicting response in CBT is noticed between evening and morning chronotypes when observed at evening time-points (Reilly *et al*., 2007; Taillard *et al*., 2005; Roenneberg *et al*., 2003), thus circadian phase and exercise/sports performance can be seen to be influenced by chronotype (Novakova *et al*., 2013).

Research investigating circadian, or diurnal, rhythms in physiological parameters are presented with methodological issues around controlling for external factors that entrain or synchronise the circadian cycle to the environment (Wright *et al*., 2013; Reilly and Waterhouse, 2009). External cues or zeitgebers, for example the light-dark cycle, provide an input that fine tunes and entrains circadian phase (Appleman *et al*., 2013; Wright *et al*., 2013; Chang *et al*., 2012). Difficulties in how to control or approach these ‘masking

effects' (of zeitgebers) are a perennial issue in biologic rhythm research, with methodologies that impose prescribed zeitgebers (activity, feeding times, defined light-dark and sleep) over a 24-hour period to standardise and purify rhythms (Roenneberg *et al*., 2003), such as 'free-running protocol', 'constant routine protocol' and 'forced-desynchronisation protocol' commonly used (Minors and Waterhouse, 2013; Reilly and Waterhouse, 2009; Minors and Waterhouse, 1986). Protocols such as these are essential for laboratory research, however, 'real world' research pertaining to, for example, field sports or competitive race events, commonly use a cross-over model as an experimental control.

Some parameters of lung function have been reported to display a diurnal rhythm with greater performance in early evening (Goel *et al*., 2015; Medarov *et al*., 2008), while many elite and highly-trained athletes have been reported to suffer from exercise-induced impairment to lung function (Fitch, 2012). Airway hyper-responsiveness (AHR) is the most commonly reported medical condition affecting athletes (Fitch, 2012; Fitch and Andersen, 2012; Kippelen *et al.,* 2012). AHR is injury to the epithelium lining of the airways (Couto *et al*., 2013) which occurs during or as a result of high-intensity aerobic exercise (Rundell, 2012). High rates of ventilation (commonly >200L/min) in cold, polluted or chlorinated water and an accumulated effect of repeated bouts of exercise in these conditions are understood to be the main factors causing AHR (Fitch, 2012; Rundell, 2012; Bougault *et al*., 2009; Chimenti *et al*., 2009). Additionally, the continual regeneration and repair of the airway epithelium lining is thought to result in structural alterations and further development of

AHR (Kippelen *et al.*, 2012; Chimenti *et al*., 2010). The phenomena of AHR commonly manifests as an increase in a type of inflammatory leukocyte, neutrophils (Belda *et al.,* 2008; Morice *et al.,* 2004).

In addition to physiology, many biologic parameters exhibit robust circadian rhythms. For example, haematology (Ackermann *et al*., 2012), immune (Labrecque and Cermakian, 2015; Lange and Born, 2011; Bollinger *et al*., 2009; Dimitrov *et al*., 2009; Benedict *et al*., 2007; Dimitrov *et al*., 2007; Dimitrov *et al*., 2004; Born *et al*., 1997; Suzuki *et al*., 1997; Palm *et al*., 1996) and endocrine parameters (Dimitrov *et al*., 2009; Redwine *et al*., 2000) peak at distinct diurnal or nocturnal cycles respectively. Importantly, under healthy, regular sleep-wake conditions these rhythms display a robust circadian cycle synchronised by intrinsic (endogenous) and extrinsic (exogenous) factors (Besedovsky *et al*., 2012; Born *et al*., 1997). However, through environment conditions such as nightshift work or chronic illness, rhythmicity may be lost (Adan *et al*., 2012).

Under normal sleep-wake and lighting conditions, circadian rhythms have been identified in a range of leukocytes, with circulating lymphocyte subsets displaying distinct diurnal or nocturnal levels (Labrecque and Cermakian, 2015; reviewed by Lange *et al*., 2010; Dimitrov *et al*., 2009). Inconsistencies in circadian levels of the leukocyte neutrophils have been found with either diurnal or nocturnal values (Ackermann *et al*., 2012; Lange and Born, 2011; Dimitrov *et al*., 2007; Born *et al*., 1997). T-lymphocyte subsets show the greatest variance of diurnal or nocturnal heightened levels (Ackermann *et al*., 2012; Dimitrov *et al*., 2009). Mature T-lymphocytes express CD3 and either

CD4 or CD8 cell surface molecules, hence allowing for identification of CD4+ T-helper (Th) cells and CD8+ cytotoxic T-lymphocytes (CTLs). Using cell surface markers (CD27 and CD45RA), T-lymphocytes can be further divided into four groups of subsets: naïve (NA), central memory (CM), effector memory (EM), and CD45RA-effector memory (RAEM), each of which signify a distinct differentiation or activation status and tissue migratory patterns (Appay *et al*., 2009; 2008; Romero *et al*., 2007). An additional cell surface marker, co-stimulatory receptor CD28, has been used to detect distinction populations of CD8+ T-lymphocyte subsets and differentiation status. However, a great deal of overlap exists between differentiation status and markers of naïvety/senescence (CD45RA) (Romero *et al*., 2007; Appay *et al*., 2002).

Current consensus suggests the leukocyte cell surface marker CD45RA, which is one of the leukocyte CD45 surface protein isoform, as being an accurate method to distinguish naïve from experienced subsets due to the RA isoform being upregulated and replaced by a RO isoform after antigenic recognition allowing for greater precision in identifying subset types (Appay *et al*., 2008; Romero *et al*., 2007; Appay *et al*., 2002). Importantly, solely using a CD45RA cell surface marker to identify naïve subsets is inadequate as terminally-differentiated revertant memory subsets re-express the CD45RA surface-marker, hence the necessity for phenotype identification with co-expressed markers for naïvity, for example co-stimulatory molecules CD27 and CD28 profiles (Appay *et al*., 2008; Hamann *et al*., 1997).

Under regular sleep-wake conditions, naïve CD4+ and CD8+ T-lymphocyte subsets show an acrophase during nocturnal sleep whereas effector,

experienced-subsets, EM and RA-effector memory (RAEM), show a diurnal acrophase (Dimitrov *et al*., 2009). This circadian rhythm in T-lymphocyte subsets is controlled by the two major stress-hormone systems, by way of release of glucocorticoids, such as cortisol via the hypothalamus pituitary adrenal (HPA) system, and release ofcatecholamines via the sympathetic nervous system (SNS). Naïve phenotypes are governed by cortisol and EM/RAEM phenotype CD8+ subsets which are influenced by catecholamines (Dimitrov *et al*., 2009) by way of binding to cell surface β_2 adrenergic receptors (Anane *et al*., 2009; Atanackovic *et al*., 2006). Verifying the effect of catecholamine and cortisol on lymphocyte subsets, the infusion of these hormones and blocking their receptors (β-blockers) outside their normal circadian patterns, have evoked changes in circulating T-lymphocyte subsets that mimick endogenous circadian patterns (Dimitrov *et al*., 2009).

The aim of this study was to investigate the relationship between time of the day (diurnal) and exercise responses in physiological, immunological and endocrinological parameters.

It was hypothesised that:

1. Parameters of physiology display circadian variation with a divergent diurnal phase response to morning and evening exercise.
2. Chronotype score and categorisation (based on the Morningness-Eveningness questionnaire scoring) would significantly impact exercise performance and physiology.
3. Lung function would be negatively affected by an acute bout of self-paced high-intensity exercise at 6°C due to an interaction between

diurnal variation in physiology, training status of participants, high-intensity of exercise and inflammatory factors.

4. Leukocytes, lymphocyte subsets, catecholamines and cortisol would be effected by high-intensity exercise and diurnal phase.

4.2 Methodology

Chapter 3 details the comprehensive methods used for this study. Methods specifically used in this study only are described below.

4.2.1 Participants

Sixteen trained and experienced, male distance runners (mean ± SD: age: 30 ± 4.5 years, body mass: 72 ± 4.7kg, height 177 ± 5.9cm, $\dot{V}O_2max$ 61.2 ± 3.7 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$) were recruited. All participants were healthy, non-smokers and were not taking any medication in the previous weeks. Each participant completed all prerequisite paperwork and questionnaires as described in detail in chapter 3. Ethical approval was granted by the ethics committee at Edinburgh Napier University and all participants provided written informed consent.

4.2.2 Experimental Procedures

The experimental procedures are described in detail in chapter 3. Figure 4.1 (below) briefly outlines experimental trial sequence.

Experimental days consisted of participants arriving at the Human Performance Laboratory at Edinburgh Napier University a minimum of 90 minutes prior to trial commencement (07.30hrs for the morning (AM) trial and

14.30hrs for the evening (PM) trial. The trial consisted of a 10 kilometer (km) self-paced blinded (no input as to running speed) time-trial run on a motorised treadmill (Woodway, ergo ELG55, Germany) at two different times of the day (09.00hrs and 16.00hrs) on two separate occasions, with a minimum of seven days between trials. The trials were completed in a randomised counter-balanced manner. All participants were experienced 10km runners. The trials were performed in an environmental chamber (Weis-Gallenkamp, UK) with the temperature set for a standardised 6°C (mean Scottish winter temperature) for the duration of the exercise protocol.

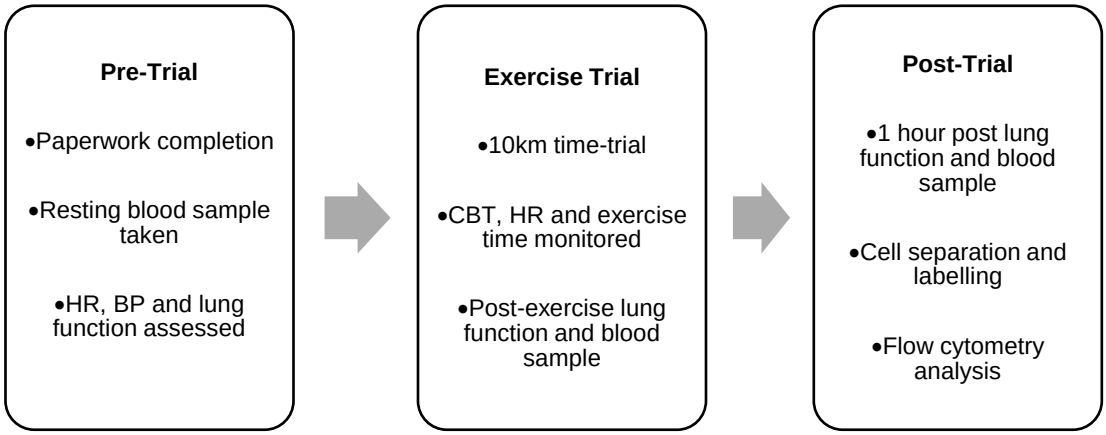


Figure 4.1. Diagram of testing sequence.

Core body temperature (CBT) was measured, to two decimal places, during the periods pre, during and post experimental trial using a core temperature (CoreTemp) monitoring pill (Core Body temperature Sensors, 262K, HT 150002, Florida, U.S.A.) and CoreTemp data recorder (HQInc, Florida, U.S.A). The core temperature pill was administered at least 90 minutes prior to trial commencement. The assessment of CBT was validated over a full circadian cycle (24-hours) at rest (figure 4.3) and with exercise (figure 4.4) as per

previous laboratory findings (Boukelia, 2015). Lung function and blood samples were obtained pre, post and 1 hour (hr) post-exercise, as described in chapter 3.

4.2.3 Lymphocyte Isolation, Labelling and Analysis

Briefly, whole blood was separated, labelled with monoclonal antibodies (MAb) and analysed by FACSCalibur flow cytometry using CELLQuest Pro software (BD Biosciences, San Jose, CA, USA). Plasma at each sampling point was immediately frozen for *post hoc* analysis, as described in detail in chapter 3

4.2.4 Statistical Analysis

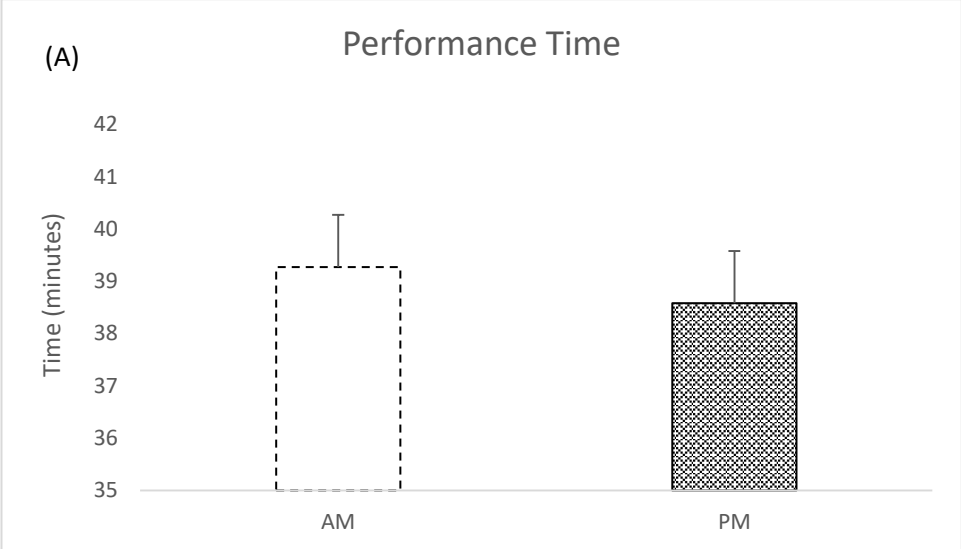
SPSS software version 20 (Chicago, IL) was used to complete statistical analysis. Normal distribution was assessed using the Kolmogrov Smirnov or Shapiro-Wilk test (Newell *et al*., 2010). The effect time (i.e. pre, post and 1hr post-exercise) and diurnal time-point (AM and PM) were analysed using repeated measures analyses of variance (ANOVA). Only significant interactions (time x diurnal time-point) are presented unless otherwise stated. Bonferroni corrected paired T-tests were utilised when significance was found ($p < 0.01$). Assumptions of sphericity in data were checked for each ANOVA, and, where appropriate, adjustments were made to the degrees of freedom. Paired sample T-tests were used to determine parameters for pre and post-exercise and for diurnal phase (AM pre vs PM pre). Pearson's correlation was used to measure the linear correlation between two variables. Spearman's rank order correlation was utilised for non-normally distributed data. Statistical

significance was recorded as $p<0.05$. All results are presented as mean ± SE unless otherwise stated.

4.3 Results

4.3.1 Results Part 1: The effect of morning and evening time-trials on parameters of physiology

All participants completed the exercise trials (09.00hrs/AM, 39.45 ± 4.1min and 16.00hrs/PM, 38.97 ± 4.1min) successfully. Figure 4.2 illustrates that no significant main effect of time-of-day (paired-sample T-test; $P > 0.05$) was found, however PM time-trial performance was quicker overall by 0.48min (29 seconds).



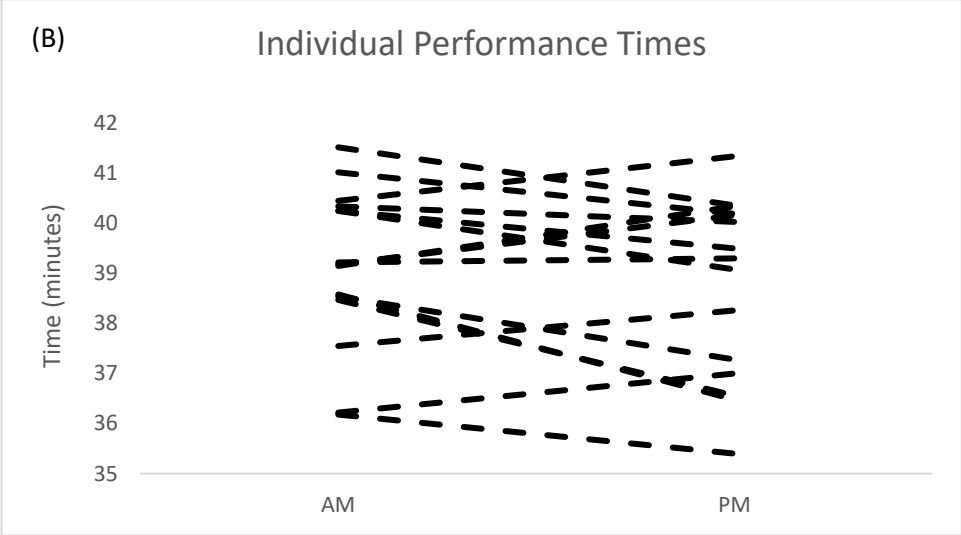


Figure 4.2. (A) Group performance (time in minutes to complete), and (B) individual performance times at 09.00hrs (AM) and 16.00hrs (PM). Values are mean ±SD, coefficient of variation percentage (CV%) AM = 10.39% (min = 6.1%, max = 13.0), PM = 10.52% (min = 5.3, max = 13.2).

Core body temperature recordings of one participant over two complete days are shown in figures 4.3 and 4.4. The increase in body temperature (masking effect) can clearly be seen in figure 4.4. with a temporal change in the variable, caused by an exogenous influence, in this case the exercise, with a realignment to diurnal CBT following recovery.

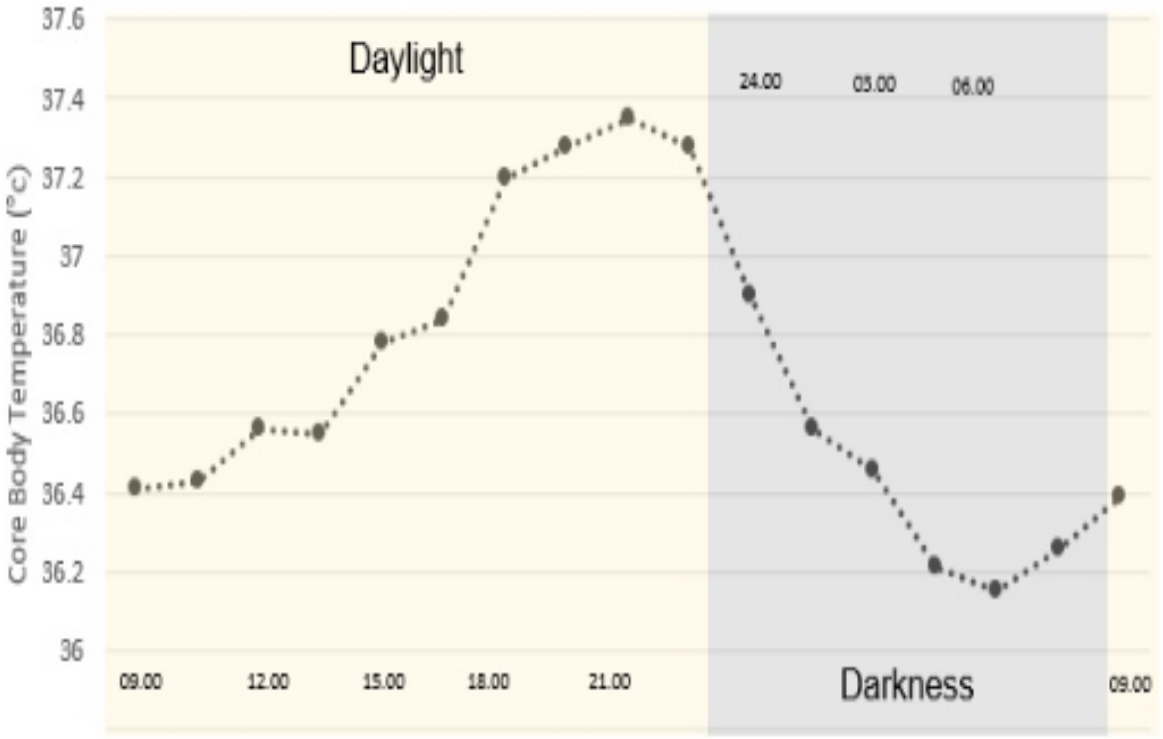


Figure 4.3. CBT (in degrees Celsius) recorded every 10 seconds (displayed in hourly mean form) from 09.00hrs to 0.900hrs over a 24-hour light-dark cycle at rest (n=1, male).

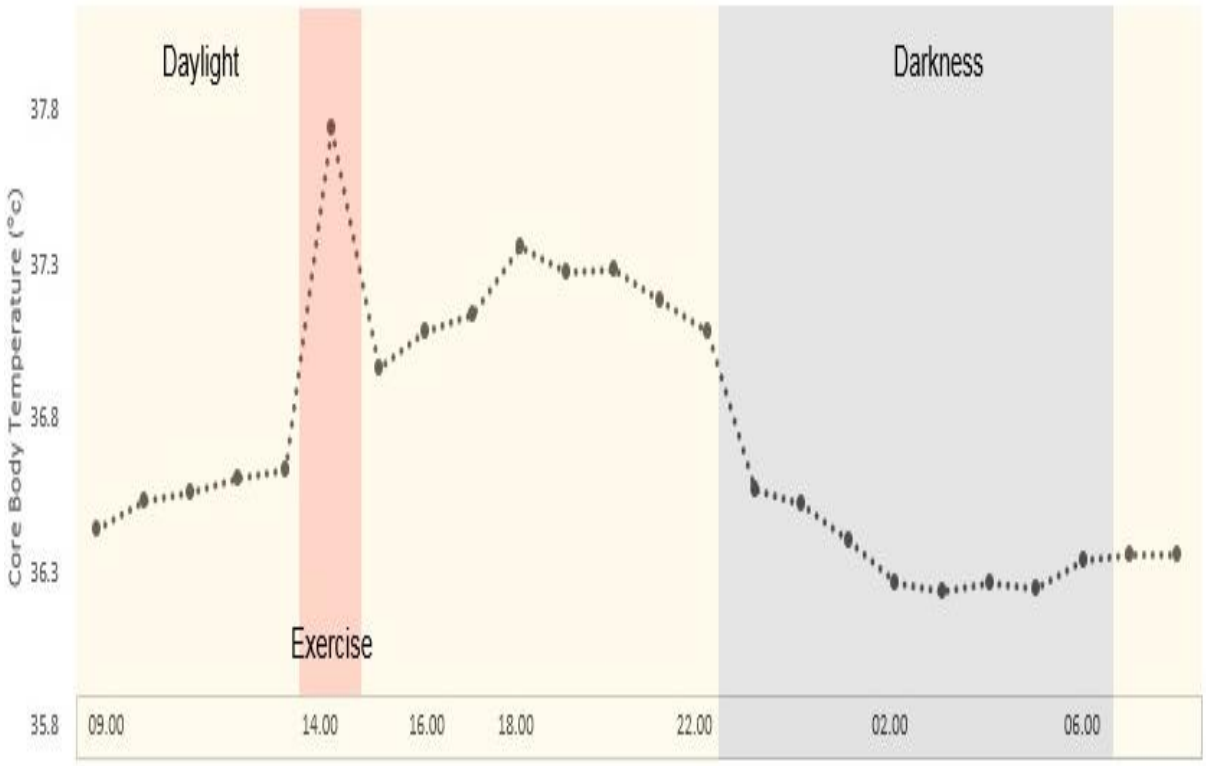


Figure 4.4. CBT (in degrees Celsius) recorded every 10 seconds (displayed in hourly mean form) from 09.00hrs to 0.900hrs over a 24-hour light-dark cycle with afternoon exercise bout (aerobic jog ~70% maximum heart rate, 55 minute duration) (n=1, male).

Sampling issues resulted in an incomplete CBT data set (n=7). An exercise effect on CBT was found in pre and post-exercise for both 09.00hrs (AM) and 16.00hrs (PM) trials (paired sample T-test, $P < 0.05$) as shown in figure 4.5. Diurnal variation in resting (pre-exercise basal levels) CBT was observed with higher values in the PM (m=37.12°C, SD=0.37) compared to AM (m=36.48°C, SD =0.5) trial conditions ($t(15) = 6.36$, $p=0.001$). Statistical difference between AM and PM trials was observed at the 1km stage (paired sample T-test $P < 0.05$) but at no other stage.

A significant difference in CBT from pre (m=36.48°C, SD=0.5) to post-exercise trial (m=37.82°C, SD=0.68) (on finishing the 10km time-trial) was found in the

AM condition (t(15)=8.11, p=0.001). A similar response in the PM condition was also revealed with pre (m=37.12°C, SD=0.37) significantly lower (t(15), p=0.001) than post-exercise (m=37.92°C, SD=0.59). This trend in CBT increasing equates to an increase from pre to post-exercise of 3.67% and 2.15% in the AM and PM trials respectively. A significant circadian difference in resting CBT was observed between AM and PM trials (09.00hrs and 16.00hrs) (t (15), 6.36, p=0.000).

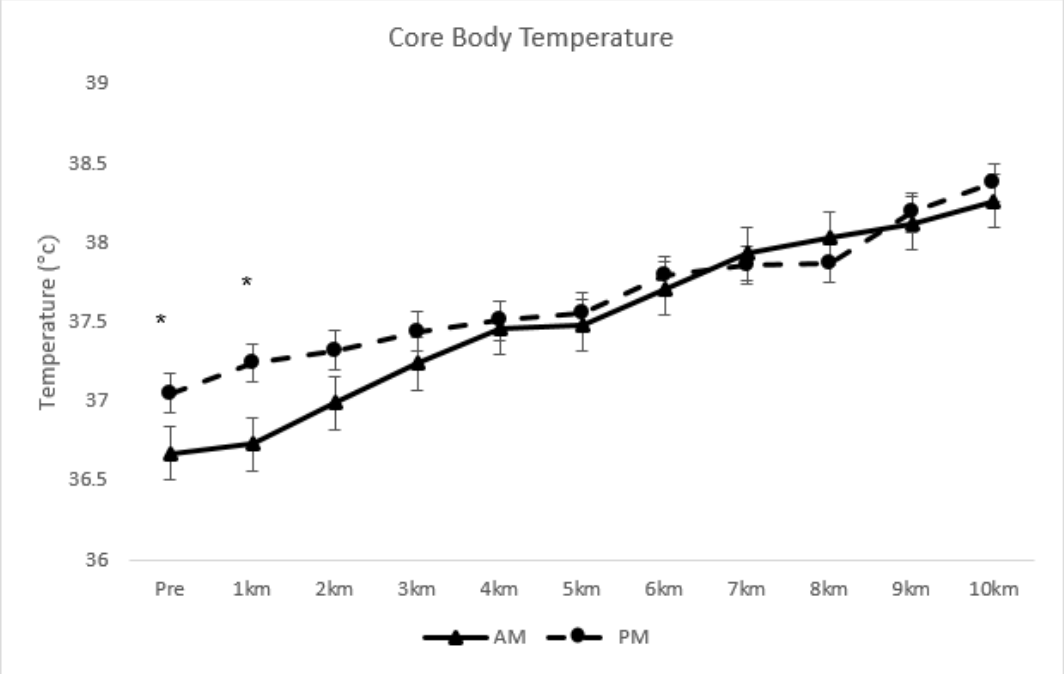


Figure 4.5. CBT recorded at 1km intervals throughout a 10km time-trial at 09.00hrs (AM) and 16.00hrs (PM). Values are mean ±SD, statistical significance is set at P < 0.05 (n=7).

Figure 4.6 demonstrates no diurnal difference for heart rate (HR) (t(15)=0.81, p=0.43) (beat per minute) observed between resting AM (m=61bpm, SD=12) and PM trials (m=62bpm, SD=11.5). However, an exercise response was found in both AM and PM trials from pre to post-exercise (P < 0.05), respectively.

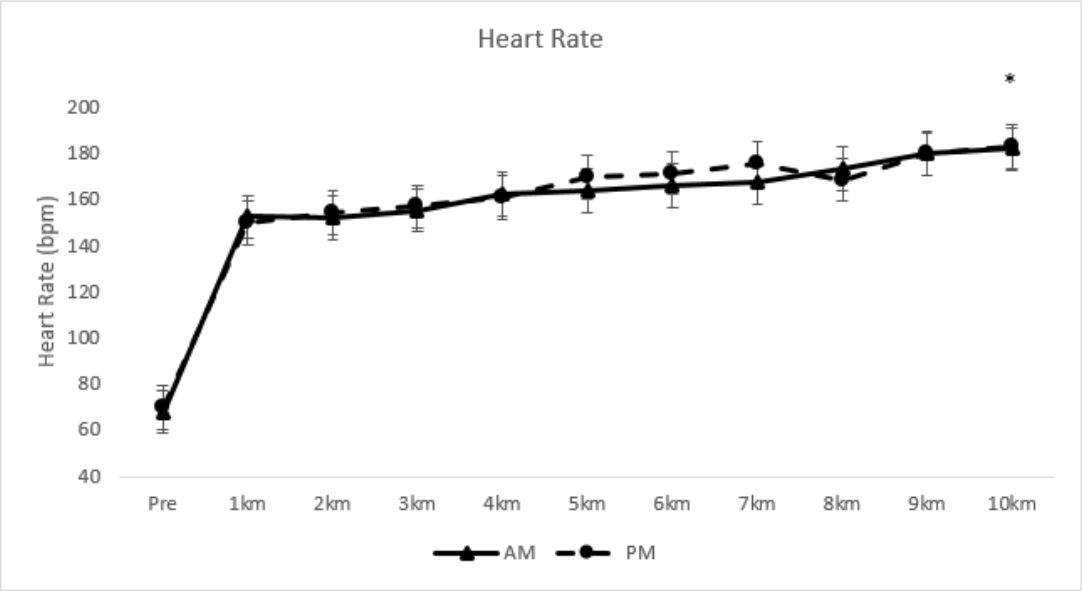


Figure 4.6. HR response recorded at 1km intervals throughout a 10km time-trial (n=16). Values are mean ±SD, statistical significance is set at P < 0.05 (*).

Figure 4.7 illustrates a strong, positive correlation between distance completed in the AM (r^2=.95) and PM (r^2=.92) and rate of perceived exertion (RPE) (Borg, 1970), respectively. No significant difference was observed between AM and PM at any stage (p= >0.05).

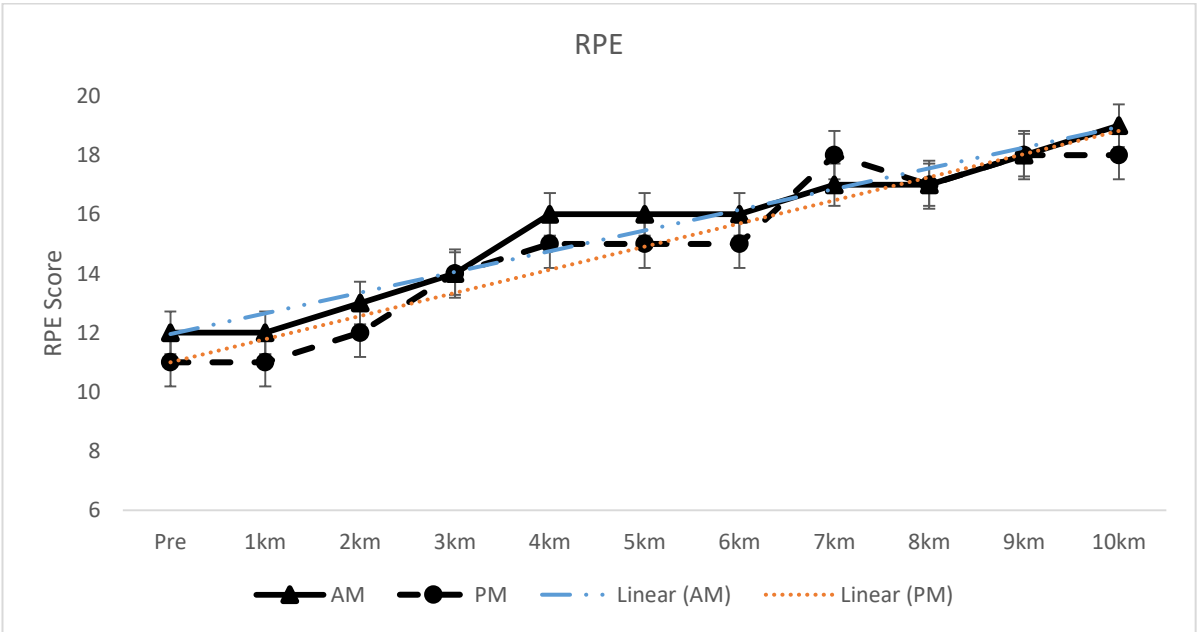
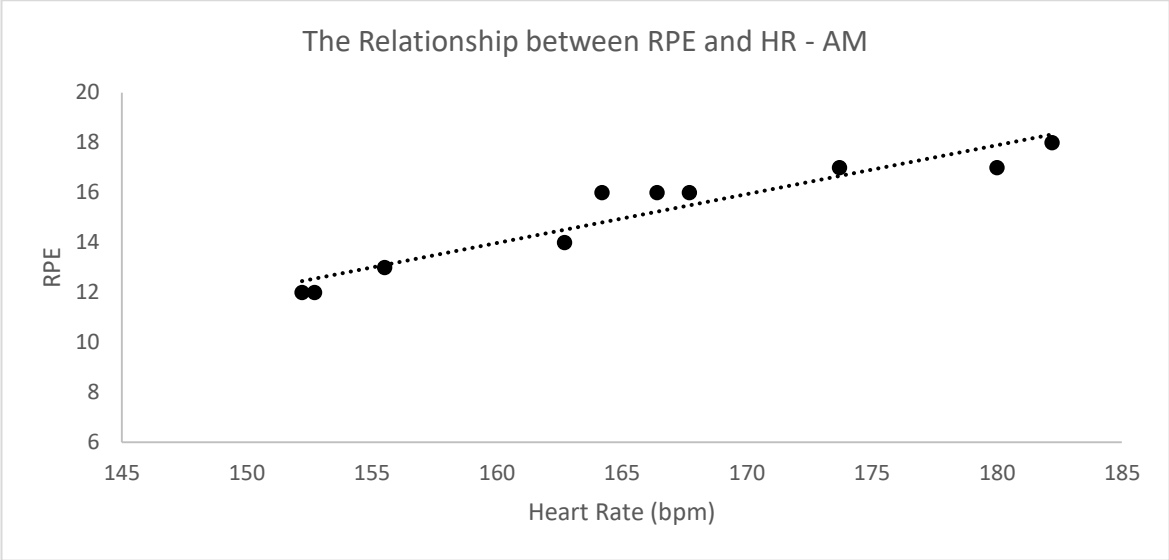


Figure 4.7. RPE recorded at 1km intervals throughout a 10km time-trial at 09.00hrs (AM) and 16.00hrs (PM). Values are ±SD, statistical significance is set at P < 0.05.

HR and RPE displayed a positive correlation in both AM (r^2=.90) (figure 4.8A) and PM (r^2=.80) (figure 4.8B) trials, with greater variability in the PM trial.

(A)



(B)

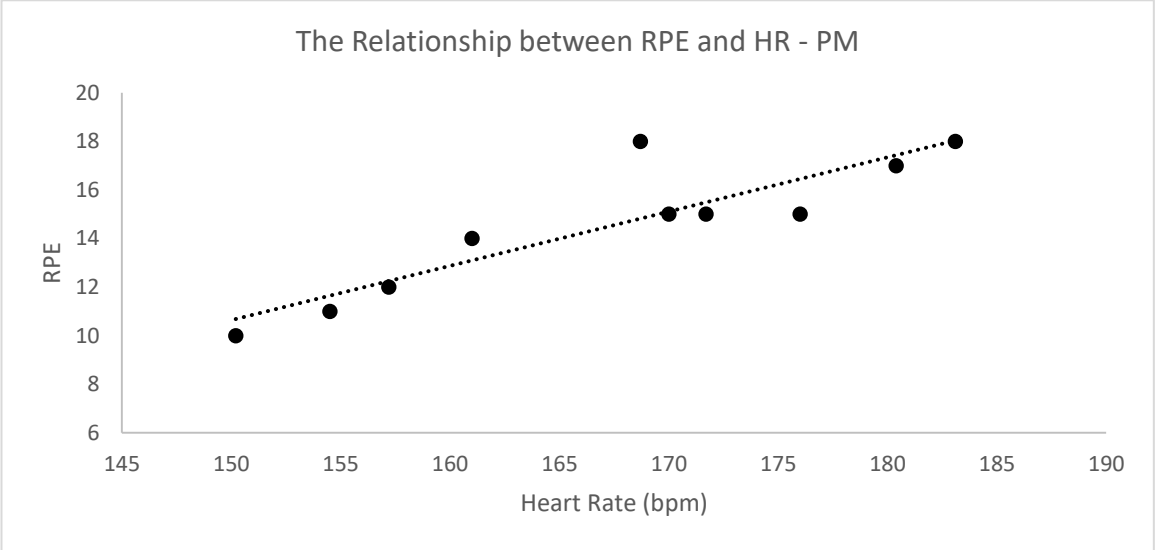
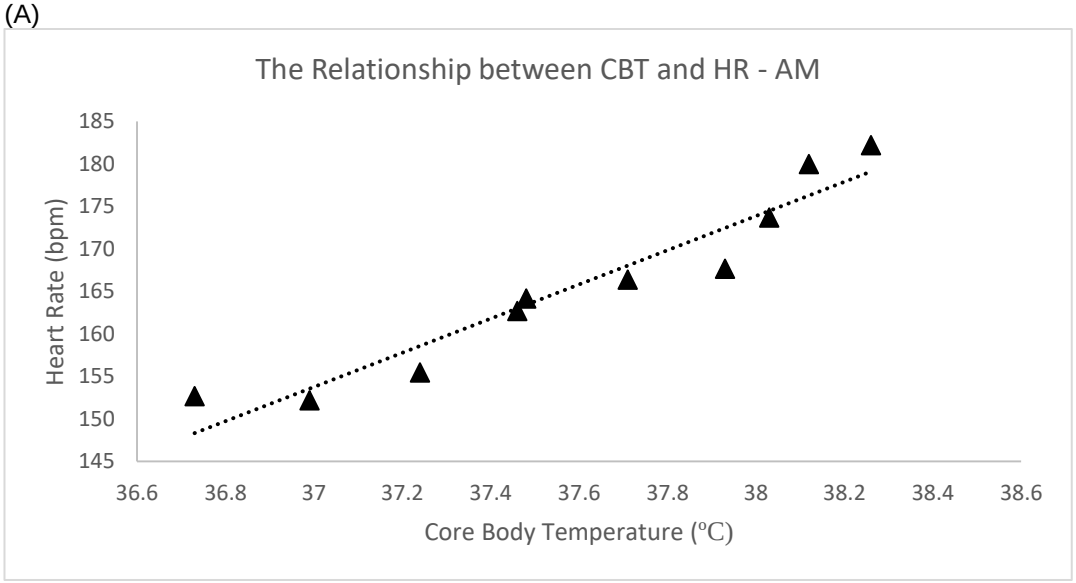


Figure 4.8. RPE and HR recorded at 1km intervals throughout a 10km time-trial at (A) 09.00hrs (^{2}r=.90) and (B) 16.00hrs (r^2=.79), statistical significance is set at P < 0.05.

A positive relationship between increasing CBT and HR is illustrated in figure 4.9A (AM trials) and figure 4.9B (PM trials).

(A)



(B)

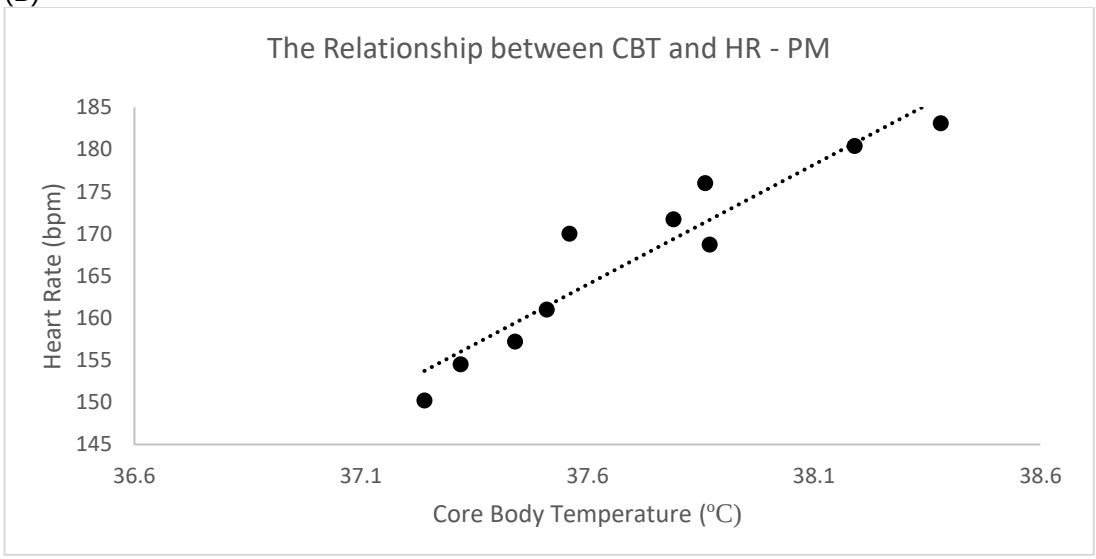
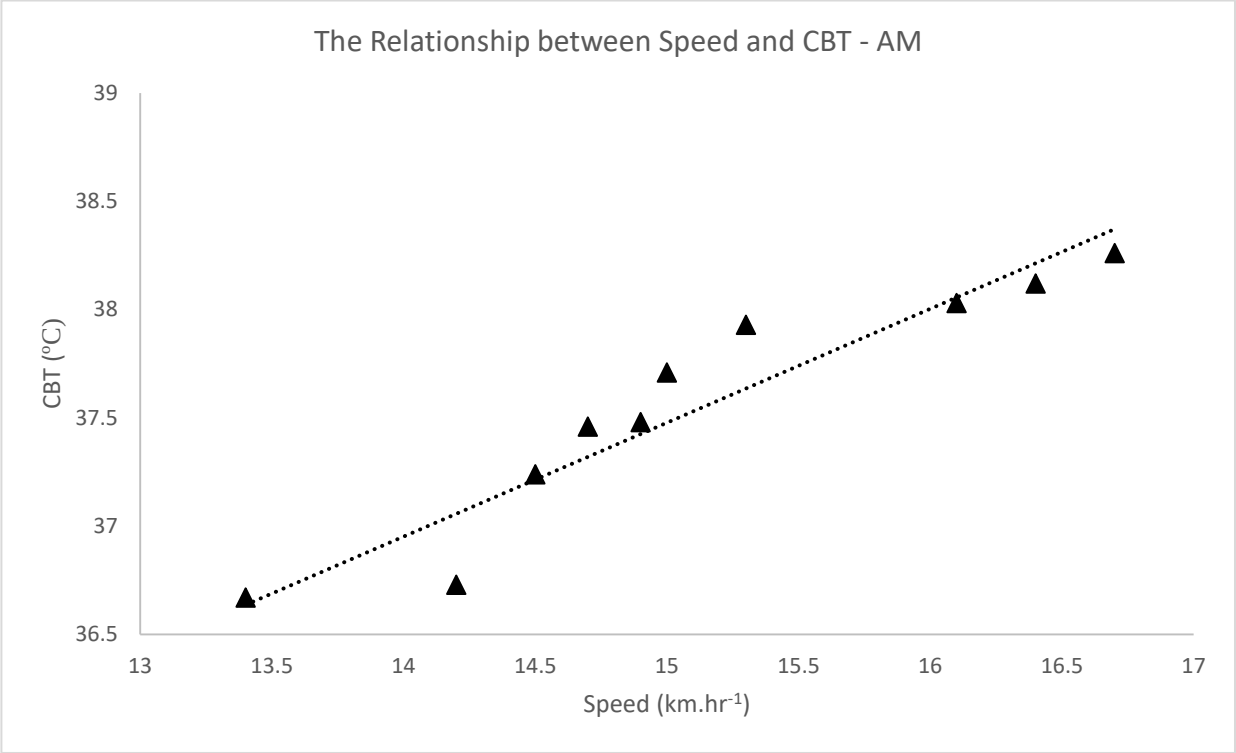


Figure 4.9. Relationship between CBT and HR recorded at 1km intervals throughout a 10km time-trial at (A) 09.00hrs (r^2=.92) and (B) 16.00hrs (r^2=.89), statistical significance is set at $P < 0.05$. Due to technical issues resulting in erroneous and incomplete core body temperature data, n=7.

As reported in figure 4.10A (AM trials) and figure 4.10B (PM trials), CBT showed an increase in line with higher self-paced treadmill speeds.

(A)



(B)

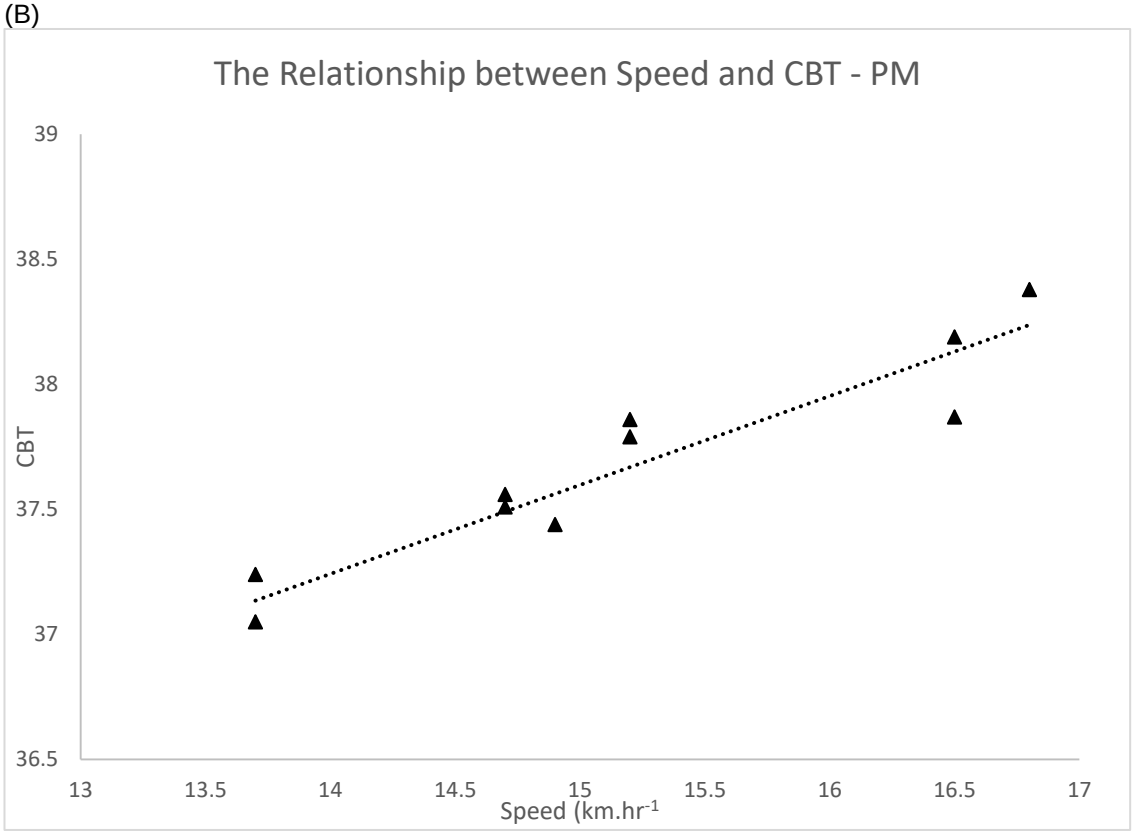


Figure 4.10. Relationship between treadmill speed and CBT at 1km intervals during a 10km time-trial at (A) 09.00hrs (r^2=.88) and (B) 16.00hrs (r^2=.85), statistical significance is set at P < 0.05.

Lung function ability was compared pre, post and 1hr post time-trials in the AM and PM. No significant differences were discovered for the four lung function tests completed (Forced Vital Capacity (FVC), Forced Expiratory Volume in 1 second (FEV_1), Peak Expiratory Flow (PEF) and Forced Expiratory Mid-point Flow (FEF_{25-75})) (time x exercise interaction) (table 4.1). Lung function appeared to return to baseline levels one hour after the completion of the treadmill test, irrespective if conducted morning or late afternoon.

Table 4.1. 09.00hrs (AM) and 16.00hrs (PM) lung function pre, post and 1hr post 10km self-paced time-trial at 6°C. Lung function parameters include: FVC, FEV_1 both measured in litres (L), PEF ($L \cdot min^{-1}$) and $FEF_{25-75,}$ values are ±SD, statistical significance is set at $P < 0.05$.values are ±SD, statistical significance is set at $P < 0.05$.

Lung Function Test	Trial	Pre	Post	1hr Post	Main Effects of Time	Main Effects of Exercise	Time x Exercise Interaction
FVC	AM	5.64± 0.91	5.55± 0.93	5.63±0.9	$F_{(1,15)} = .033$; p = **NS**	$F_{(2,30)} = 3.114$; p = **NS** (.059)	$F_{(2,30)} = 1.816$; p = **NS**
	PM	5.67± 0.9	5.42± 0.88	5.49± 0.9			
FEV1	AM	4.5± 0.64	4.38± 0.66	4.6±0.71	$F_{(1,15)} = .08$; p = **NS**	$F_{(2,30)} = 2.526$; p = **NS**	$F_{(2,30)} = .756$; p = **NS**
	PM	4.49± 0.65	4.37± 0.64	4.43±0.74			
PEF	AM	728± 72	697±89	713±70	$F_{(1,15)} = 1.542$; p = **NS**	$F_{(2,30)} = 2.754$; p = **NS**	$F_{(2,30)} = .485$; p = **NS**
	PM	699±75	678±67	672±69			
FEF_{25-75}	AM	4.23±1.1	4.3±1	4.28±.96	$F_{(1,15)} = .045$; p = **NS**	$F_{(2,30)} = .458$; p = **NS**	$F_{(2,30)} = .852$; p = **NS**
	PM	4.22±1.	4.31±1	4.37±.9			

The FVC lung function test, as marked in figure 4.11, showed a trend of non-significant lower values post and 1hr post-exercise at PM trials.

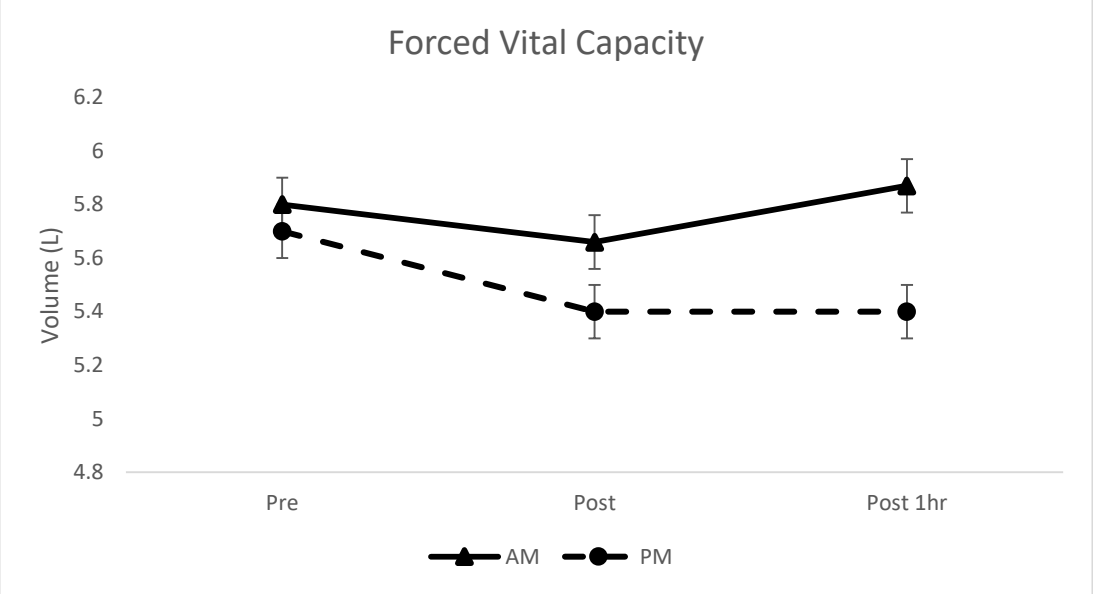


Figure 4.11. Morning and evening variation in FVC (L) pre, post and 1hr post 10km self-paced time-trial. Values are mean ±SD, statistical significance is set at $P < 0.05$.

No significant statistical difference (by repeated measure ANOVA) was observed in self-reported respiratory symptoms via the completion of the questionnaire (data not displayed).

Paired sample T-test found perceived alertness (figure 4.12) was significantly improved prior to PM (m=5.19, SD=1.6) compared to AM (m=4.19, SD= 1.56) trial ($t(15) = 2.284$, $p = 0.037$). No significant difference was observed between AM (m=3.44, SD= 1.32) and PM (m=2.44, SD= 1.5) trials ($t(15) = 1.879$, $p>0.05$) in self-reported arousal.

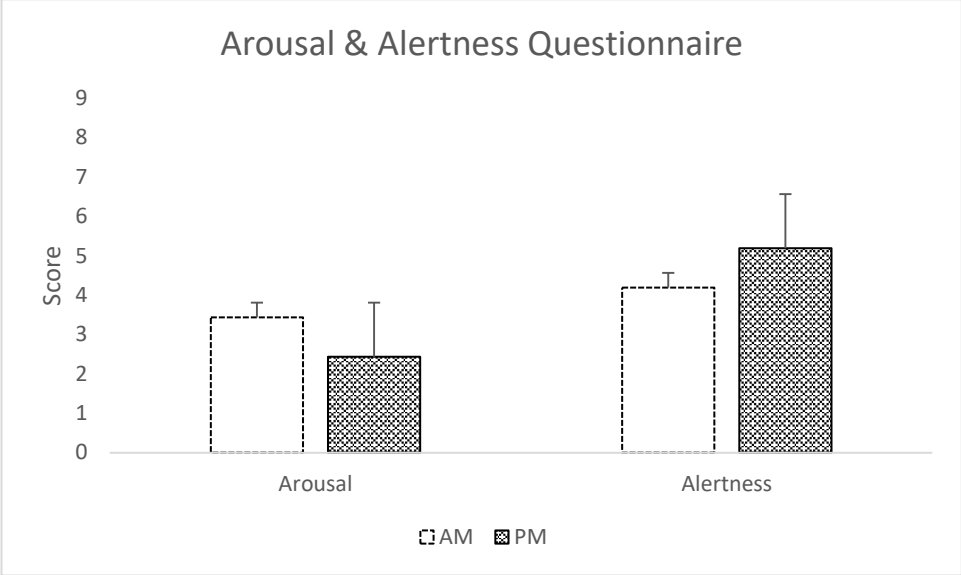


Figure 4.12. Aroual and alertness score. Values are ±SD, statistical significance is set at P < 0.05.

4.3.2 Chronotype Distribution and Performance

The distribution of chronotypes and 10km time-trials performance times (AM and PM) are displayed on figure 4.13. No extreme chronotypes (definitely morning or definitely evening) were found in the study cohort.

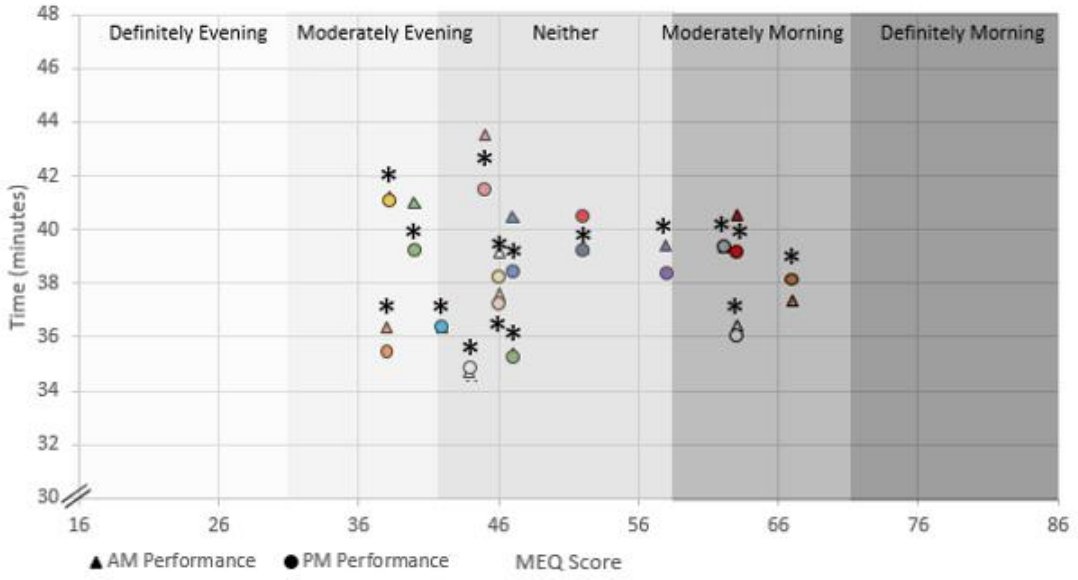


Figure 4.13. Chronotype distribution within the study cohort using MEQ (plotted score) and time-trial performance (time to complete) (y-axis) at 09.00hrs (AM) and 16.00hrs (PM). Individual performance times (AM and PM) are plotted using unique colour coding against respective MEQ score (x-axis), (*) signifies individual participants AM and PM trials – note, some completion times may overlap.

Figure 4.14 displays mean performance time (time to complete) at 09.00hrs and 16.00hrs with chronotype scores (MEQ) divided according to 50th percentile; MEQ_{low} and MEQ_{high}. MEQ_{low} values range from MEQ scores 16-50 and represent evening and evening orientated neither chronotypes while MEQ_{high} values range from MEQ score 51-86 and represents morning and morning leaning-neither types.

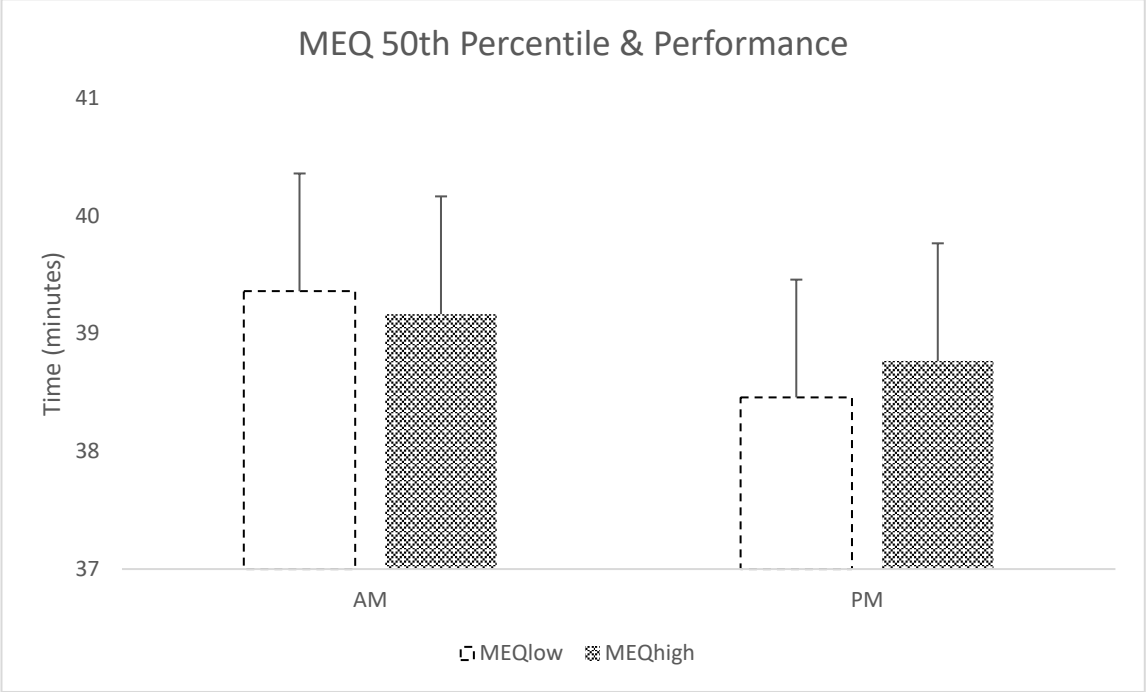
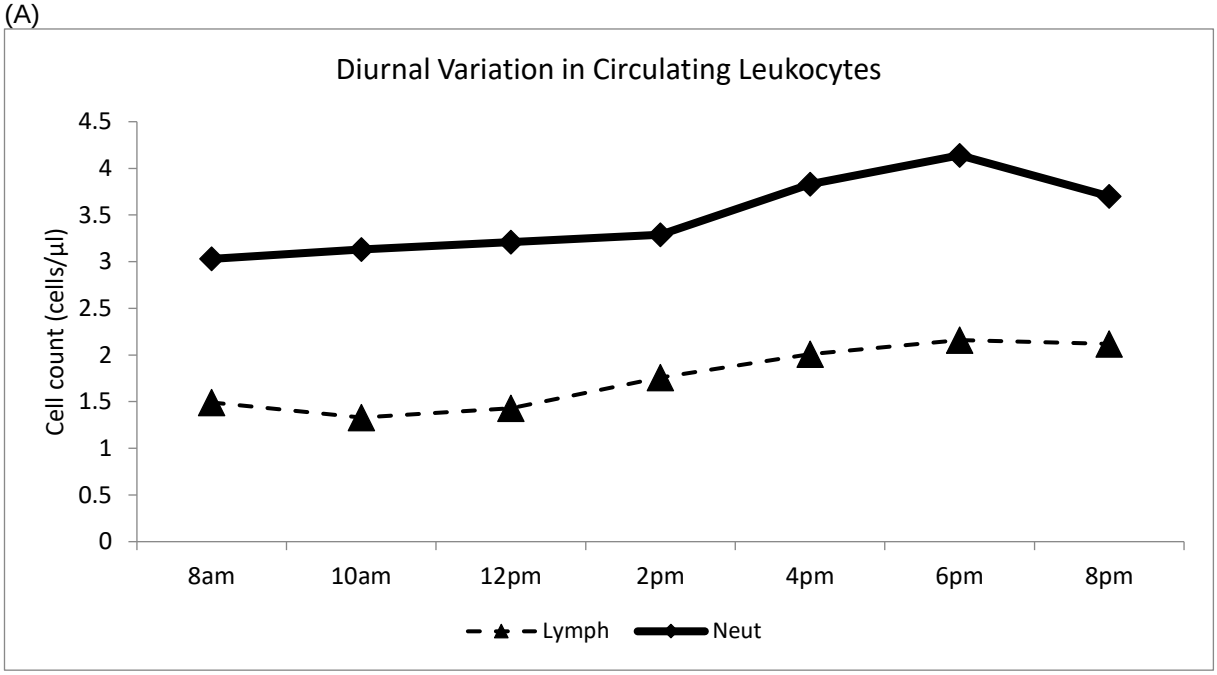


Figure 4.14. Mean performance time (time to complete) at 09.00hrs and 16.00hrs in MEQ_{low} and MEQ_{high}. Chronotype separated into MEQ categories according to MEQ score; 16-50 evening-intermediate types (MEQ_{low}) (m=59.5, SD=5.2, n=7), and 51-86 morning-intermediate types (MEQ_{high}) (m=42.5, SD=4.4, n=9). Values are mean ±SD, statistical significance is set at $P < 0.05$.

4.3.3 Results Part 2: The effect of morning and evening time-trials on immune parameters

Figure 4.15 displays the diurnal variation in lymphocyte and neutrophil cell counts monitored from 08.00hrs to 20.00hrs (A) (sampled every 2 hours) and the percentage of lymphocytes and neutrophils of leukocytes (B) for one participant, illustrating a diurnal influence in circulating numbers (lymphocytes increased by 42.3% from 08.00hrs to 20.00hrs, concomitantly neutrophils increased by 22.1%. Neutrophils displayed a peak increase at 18.00hrs with a 36.6% change from 08.00hrs).

(A)



(B)

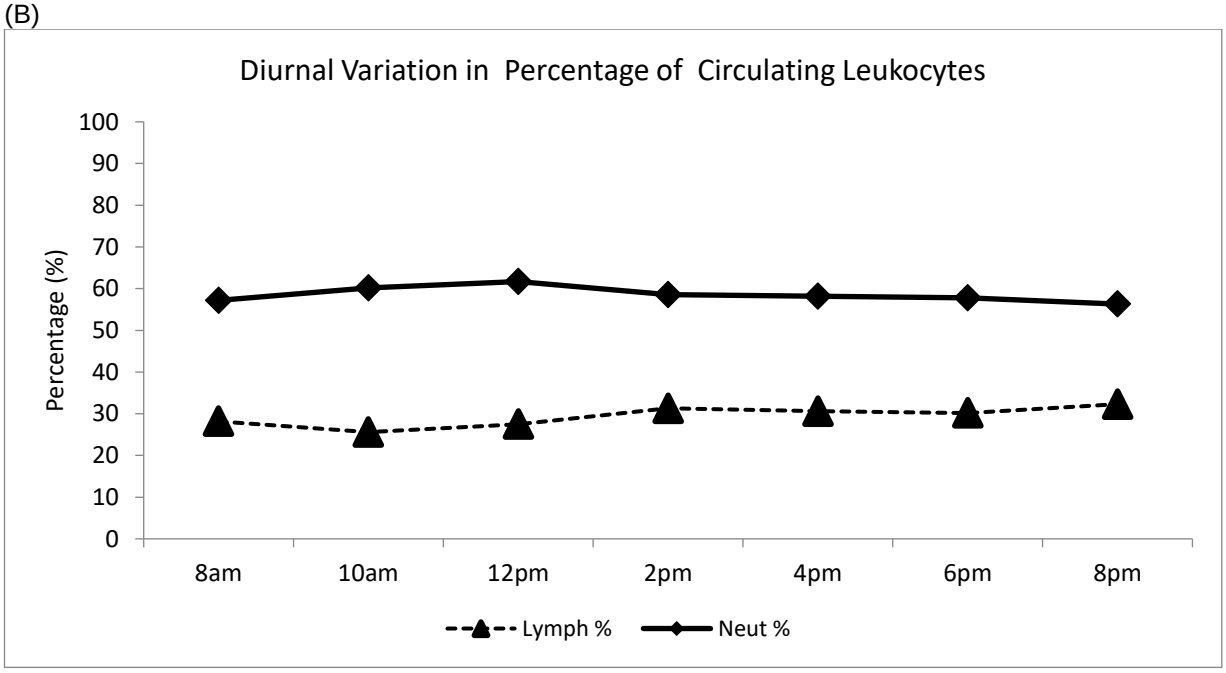
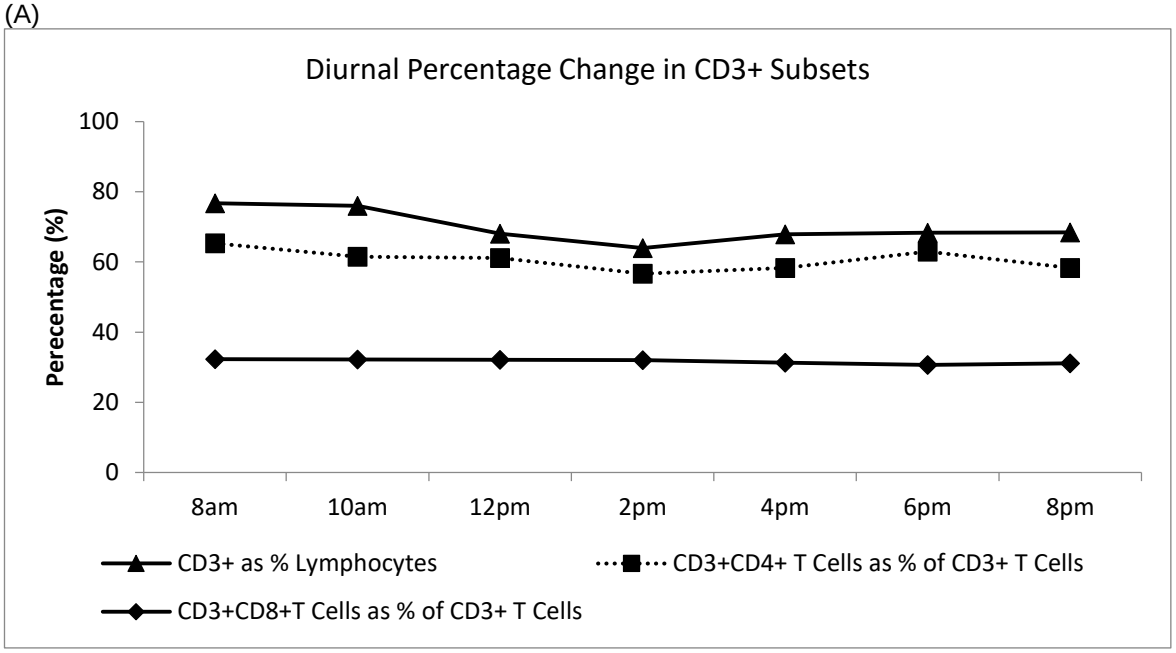


Figure 4.15. Diurnal variation in (A) neutrophil and lymphocyte cell counts and (B) percentage of neutrophil and lymphocytes, across the solar day from 08.00hrs until 08.00hrs. Pilot data, n=1.

Figure 4.16 illustrates the diurnal variation in percentage of CD3+, CD4+ and CD8+ (A) and cell count of CD3+, CD4+ and CD8+ (B) monitored from 08.00hrs to 20.00hrs (n=1).

(A)



(B)

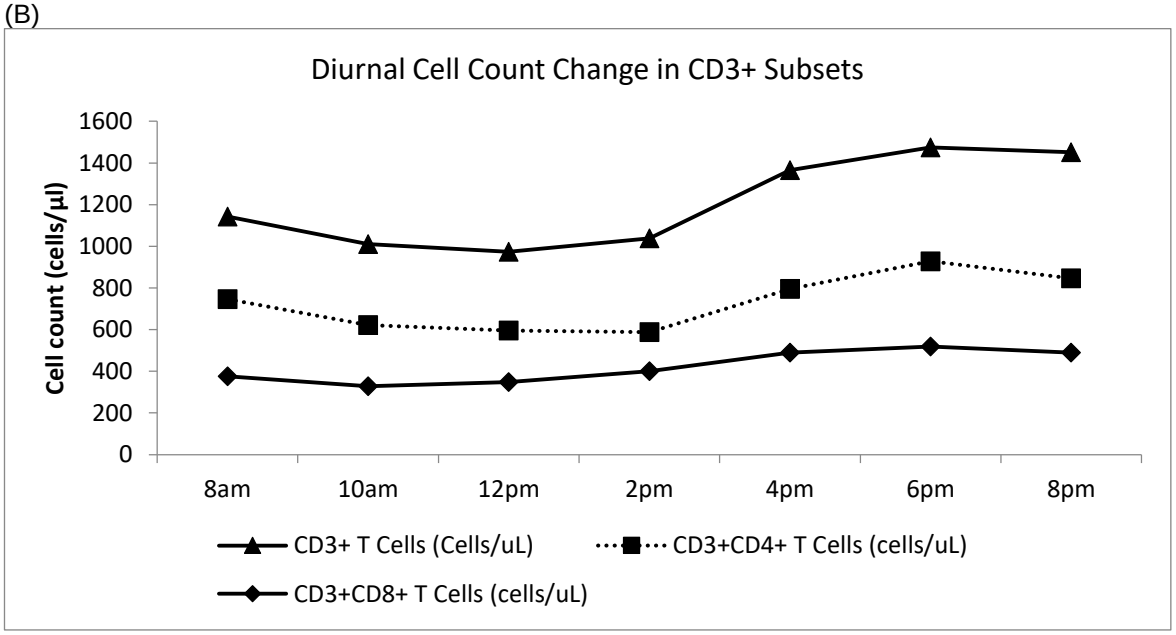


Figure 4.16. Diurnal variation in (A) percentage of CD3+,CD4+,CD8+ T-lymphocyte and (B) CD3+,CD4+,CD8+ T-lymphocyte subset cell counts. Samples were collected from 08.00hrs until 08.00hrs.

A significant trial (AM/PM) effect on circulating peripheral lymphocytes was observed after 10km time-trials by repeated measures ANOVA (time of trial (AM/PM) x time-point (pre, post, post 1hr) (<0.05) as shown in table 4.2.

Table 4.2. Circulating leukocyte, neutrophil and lymphocyte cell counts pre, post and 1hr post 10km time-trial at 6°C, values are ±SD, statistical significance is set at P < 0.05.

Cell Type	Trial	Pre	Post	1hr Post	Main effects of Time	Main effects of Exercise	Time x Exercise Interaction
Leukocytes	AM	4300 ± 164	6971 ± 229	4767 ± 471	$F_{(1/15)} = 7.686$; p = .001	$F_{(2/30)} = 6.53$; p = .001	$F_{(2/30)} = 19.64$; p = .001
	PM	4860 ± 195	8284 ± 547	7015± 974			
Neutrophils	AM	2111 ± 240	3248 ± 381	3176 ± 389	$F_{(1/15)} = 13.624$; p = .002	$F_{(2/30)} = 45.509$; p = .000	$F_{(2/30)} = 9.755$; p = .001
	PM	2446 ± 280	3837 ± 375	4843 ±432			
Lymphocytes	AM	1534 ± 164	2930 ± 328	1063 ± 114	$F_{(1/15)} = 7.296$; p = .016	$F_{(2/30)} = 10.7$; p = .000	$F_{(2/30)} = .531$; p = NS
	PM	1765 ± 171	3523 ±417	1458 ± 154			

Peripheral lymphocytes displayed a biphasic response to AM and PM exercise (figure 4.17) with a significant pre to post-exercise effect observed.

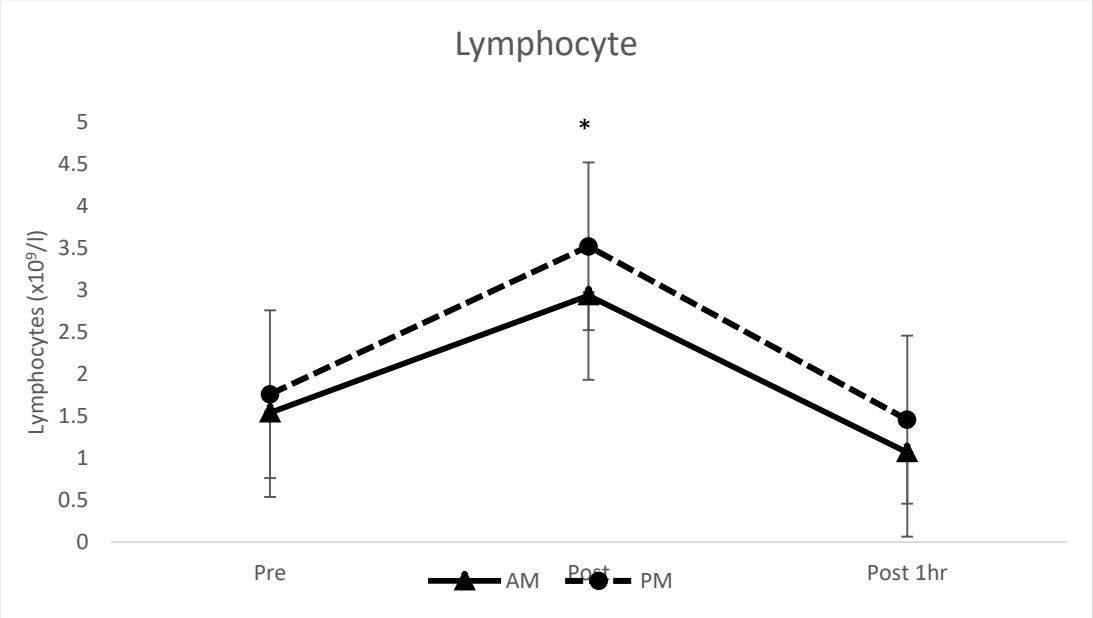


Figure 4.17. Peripheral lymphocyte cell counts in response to a 10km self-paced time-trial at AM (09.00 hrs) and PM (16.00 hrs) time-points at 6°C. No significant main effect of trial x time interaction ($F_{(2/30)} = .531$; $p > 0.05$), however a significant exercise effect (*) was discovered ($F_{(2/30)} = 10.7$; $p < 0.001$), values are ±SD, statistical significance is set at $P < 0.05$.

Neutrophils displayed greater mobilisation in response to PM exercise with a significant effect of time (AM/PM), exercise response and interaction between the two observed (figure 4.18).

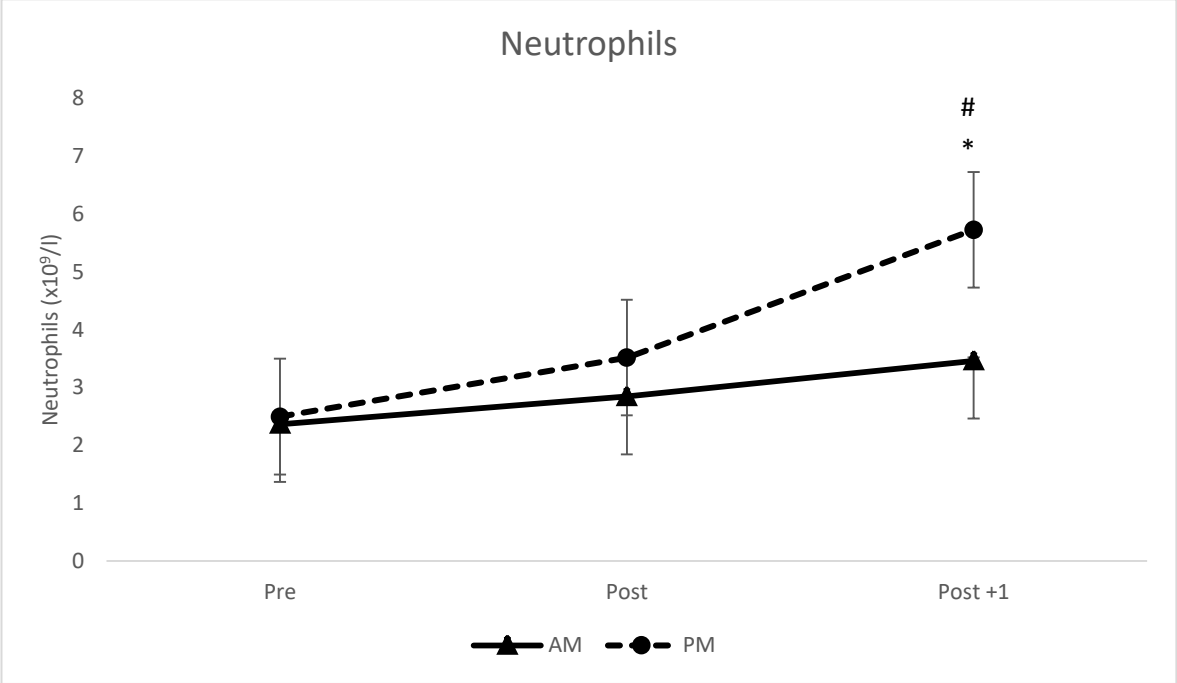


Figure 4.18. Peripheral neutrophil cell counts in response to a 10km self-paced time-trial at AM (09.00hrs) and PM (16.00hrs) time-points at 6°C. A significant main effect of trial x time interaction ($F_{(2/30)} = 9.755$; $p = <0.001$) and main effect of exercise (*) ($F_{(2/30)} = 45.509$; $p = <0.001$) was observed in neutrophils. Paired sample T-test revealed a significant time of day (diurnal) increase 1hr post-exercise in the PM time-trial condition (#) (P<.05), values are ±SD, statistical significance is set at $P < 0.05$.

Table 4.3 displays the percentage of circulating peripheral leukocytes for neutrophils and lymphocytes pre, post and 1hr post high-intensity exercise. Unreported cell types for monocyte, basophil and eosinophil equate to 100% of leukocytes.

Table 4.3. Proportion of neutrophil and lymphocyte cell types displayed as a percent (%) of peripheral blood (monocyte, eosinophils and basophils are not displayed).

Cell Type (%)	AM			PM		
	Pre	**Post**	**1hr Post**	**Pre**	**Post**	**1hr Post**
Neutrophils	49.15	46.5	66.7	50.2	46.3	69
Lymphocytes	35.8	42.1	22.4	36.2	42.5	20.8

Table 4.4 shows circulating peripheral cell counts (cells/µl) of lymphocyte and T-lymphocyte subsets; T-helper CD4+, cytotoxic CD8+ pre, post and 1hr post 10km time-trial completed at 09.00hrs and 16.00hrs at 6°C. Main effects of time, exercise and main effect of time x exercise interaction are displayed, (p=<.05, NS= p>0.05)

Table 4.4. Cell counts for lymphocyte subsets pre, post and 1hr post AM and PM exercise trials, values are ±SD, statistical significance is set at P < 0.05.

Cell Type	Trial	Pre	Post	1hr Post	Main effects of Time	Main effects of Exercise	Time x Exercise Interaction
Lymphocytes	AM	1534 ± 164	2930 ± 328	1063 ± 114	$F_{(2/30)}$ = 7.296; p = **.016**	$F_{(2/30)}$ = 10.7; p = **.000**	$F_{(2/30)}$ = .531; p = **NS**
	PM	1765 ± 171	3523 ±417	1458 ± 154			
CD4+ T-lymphocytes	AM	581 ± 102	684 ± 96	598 ± 97	$F_{(2/30)}$ = 1.798; p = NS	$F_{(2/30)}$ = 5.89; p = 0.024	$F_{(2/30)}$ = 2.474; p = NS
	PM	629 ± 84	698 ± 87	614 ± 75			
CD8+ T-lymphocytes	AM	423 ± 87	511 ± 104	376 ± 68	$F_{(2/30)}$ = 2.86; p = NS	$F_{(2/30)}$ = 6.452; p = 0.04	$F_{(2/30)}$ = 2.05; p = **NS**
	PM	428 ± 76	507 ± 97	363 ± 62			

The response of $CD8^+$ T-lymphocyte subsets characterised by the expression of cell surface markers CD27+ $CD45RA^+$ (NA); CD27+ CD45RA- (CM); CD27- $CD45RA^-$ (EM) and CD27- CD45RA+ (RAEM) are displayed in figure 4.19. No significant time x exercise interaction was found; NA subset, F(2,30) = 3.02; p>.05, CM subset, F(2,30) = 4.455, p>.05, EM subset, F(2,30) = 6.307; P >.05, or RAEM subset, F(2,30) = 7.432; p >.05.

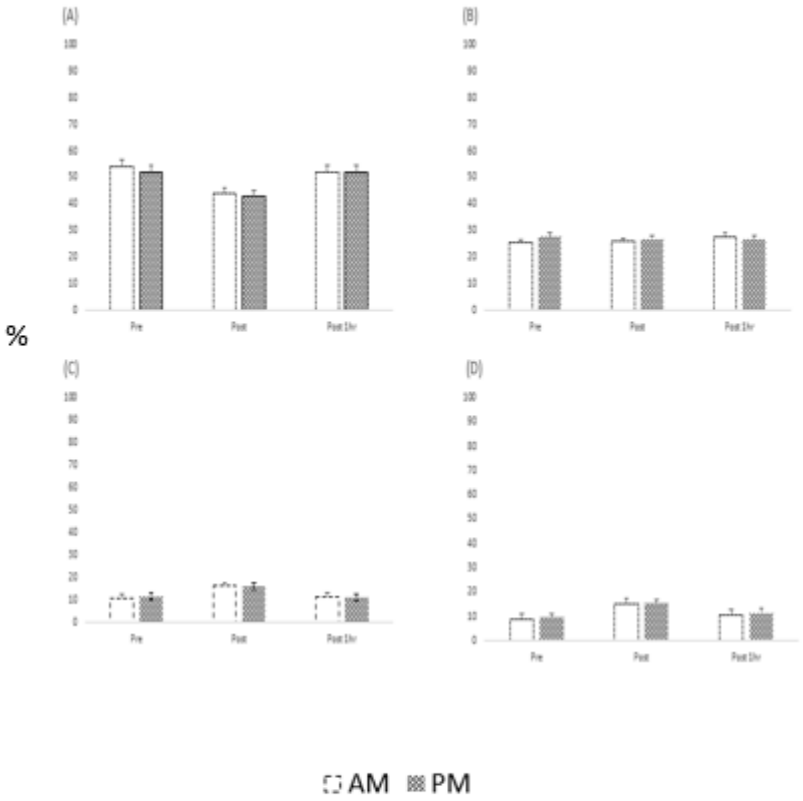


Figure 4.19. Percentage of circulating (A) NA (CD3+CD8+CD45RA+CD27+), (B) CM (CD3+CD8+CD45RA-CD27+), (C) EM (CD3+CD8+CD45RA-CD27-) and (D) RAEM (CD3+CD8+CD45RA+CD27-) pre, post and 1hr post 10km time-trial completed at 09.00hrs and 16.00hrs at 6°C, values are ±SD, statistical significance is set at P < 0.05.

Table 4.5. illustrates the stress hormone response to exercise, time (AM/PM trials) and time x exercise main effect interaction. No exercise x time effect was found for adrenaline ($F(2,10) = .506$; $p>.05$) or cortisol ($F(2,10) = .256$; $p>.05$).

Table 4.5. Adrenaline and cortisol response to a 10km time-trial at differing diurnal time points, values are ±SD, statistical significance is set at $P < 0.05$.

Hormone	Trial	Pre	Post	1hr Post	Main Effects of Time	Main Effects of Exercise	Time x Exercise Interaction
Adrenaline (pg/dl)	AM	22.8± 12.0	85± 25.94	32.16±16.32	$F_{(1,5)} = .667$; p = NS	$F_{(2,10)} = 15.98$ p = .001	$F_{(2,10)} = .506$; p = NS
	PM	32.33± 22.2	96.1± 41.0	30.13±20.2			
Cortisol (µg/dl)	AM	16.08± 3.25	16.55± 1.88	16.3±2.32	$F_{(1,5)} = 6.812$; p = .048	$F_{(2,10)} = 4.57$; p = .039	$F_{(2,10)} = .256$; p = NS
	PM	15.05± 2.49	16.01± 2.38	12.3± 3.28			

Cortisol response to 10km time-trial exercise at differing diurnal time points displayed no time by exercise interaction (repeated measure ANOVA) as reported in figure 4.20.

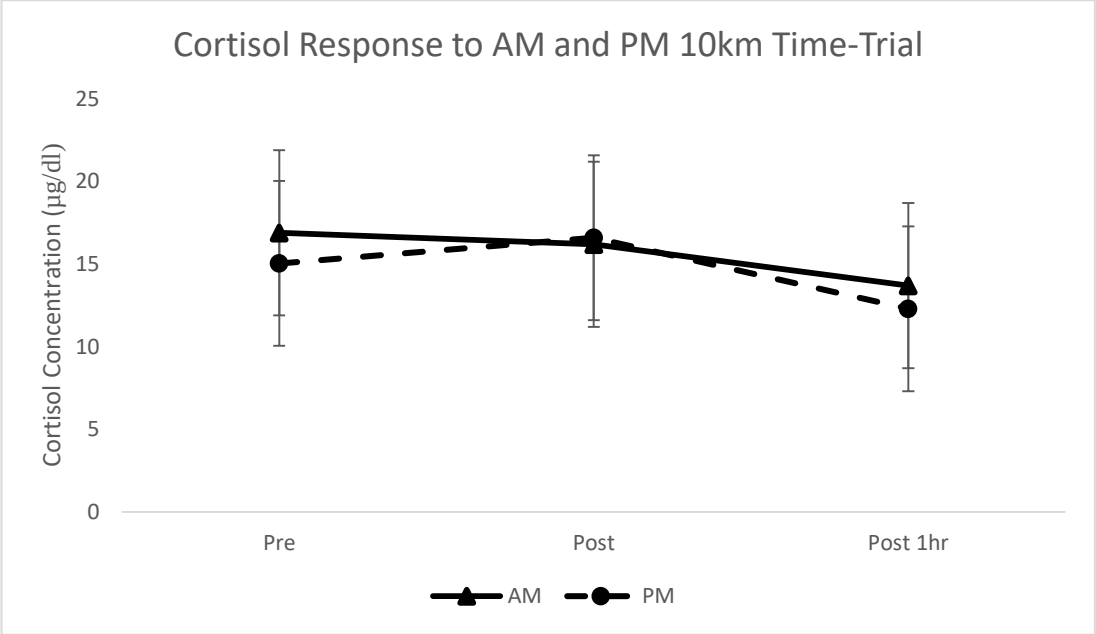


Figure 4.20. Cortisol response to high-intensity exercise at differing diurnal time-points (09.00hrs - AM and 16.00hrs - PM). Values are mean ±SD, p value set at .05 (n=6).

Displayed in figure 4.21 is the effect of exercise on adrenaline concentrations at differing times of the diurnal day. No time x exercise interaction was observed (p>.05) however an exercise effect was found (see table 4.5).

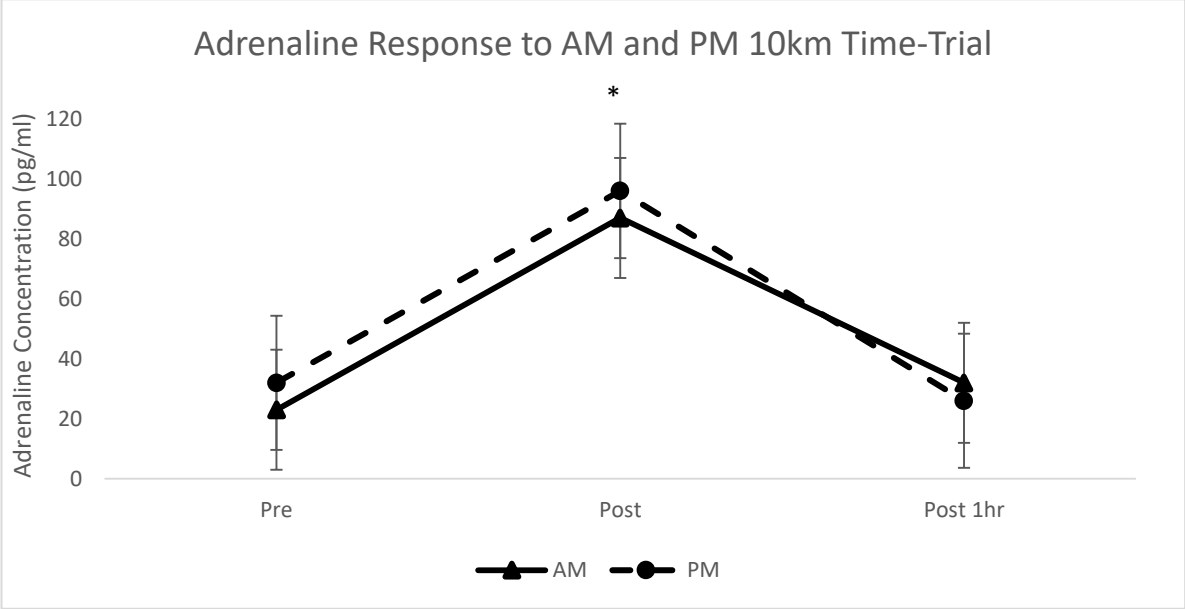


Figure 4.21. Adrenaline response to high-intensity exercise at differing diurnal time-points (09.00hrs - AM and 16.00hrs - PM). Values are mean ±SD, p value set at .05. Due to sample storage issues viable data n=7.

4.4 Discussion

The aim of this present study was to determine if high-intensity exercise completed at two differing time-points of the circadian day affected physiological and biological responses differently, in a diurnal phase response manner.

The main findings of this study were:

1. Self-paced exercise at 09.00hrs (referred to as AM) and 16.00hrs (referred to as PM) resulted in a non-significant improvement in time-trial performance in the PM. CBT as a marker of circadian phase was found to be significantly higher in the PM pre-exercise (rest) and at the 1km stage of the time-trial (compared to AM values). This finding, and the apparent lack of diurnally different effect of exercise on CBT, is in agreement with previous findings (Atkinson *et al*., 2006; 2005). Strong correlations between physiology (CBT, HR and treadmill speed) were found as time trials advanced stage on stage at AM trials, while PM trials reported a similar but less pronounced relationship.
2. AM and PM high-intensity exercise performance was not affected by chronotype classification. Chronotype scoring (MEQ) grouped as according to the 50^{th} percentile (MEQ_{low} and MEQ_{high}) revealed non-significant results with the more morning oriented chronotypes MEQ_{high} performance (time to complete) enhanced at AM trials, whereas MEQ_{low} produced quicker performance times at PM trials. No chronotype influence on CBT was found at either time points.

3. Lung function capabilities remained unaffected by exercise, time of day or a combination of the two (exercise x time interaction).
4. Lymphocytes displayed a significant biphasic exercise effect characterised by an increase in circulating cell count numbers from pre to post-exercise and a decrease from post-exercise to 1hr post. Neutrophils displayed an increase from pre to post-exercise with a continued increase post to 1hr post. A significant leukocyte time by exercise interaction was predominantly due to elevated levels of neutrophils. No time by exercise interaction was found for CD8+ T-lymphocyte subsets (naïve or experienced phenotypes). Additionally, the hormones cortisol and adrenaline did not display a time by exercise interaction.

This study investigated the effect of self-paced time-trial exercise on physiological and immune parameters at diurnally different time-points. Aerobically trained, experienced distance runners completed simulated time trials (10km) in a temperature controlled environmental chamber set at a standardised 6°C on two separate occasions. Performance (time-to-complete), although not significantly different, was 29 seconds quicker in the evening trial (start time of 16.00hrs) compared to the morning one (start time of 09.00hrs). As the study population comprised of experienced distance runners who compete and train at evening and morning time-points, often on the same day, there is potential that a conditioning-effect may have negated any diurnally influenced discrepancies in performance. The experimental conditions this study was undertaken in may have presented favourable conditions for optimal performance due to limiting core temperature rises to

endogenous mechanisms. Thermo-neutral conditions are associated with an enhanced ability to maintain running velocity (Maughan *et al*., 2007; Nimmo, 2004; Weller *et al*., 1997). Ambient temperatures ranging from 5-10°C present an enhanced capacity to maintain running velocity especially by the quickest runners (Montain *et al*., 2007), with 6.2ºC reported to be the optimum environmental condition for distance running (Helou *et al*., 2012). Core body temperature has been shown to increase at a greater rate in response to morning exercise than evening exercise due to lower resting values (Aldemir *et al*., 2000).

Although non-significant, quicker performance in the evening is in agreement with a majority of research investigating diurnal rhythms in sporting performance and physiology under regular sleep-wake conditions (Atkinson *et al*., 2005; Forsyth and Reilly, 2004; Martin *et al*., 2001). Atkinson *et al*. (2005) found that cycling performance was improved in the evening compared to the morning. Martin *et al*. (2001) reported that optimal running time was in the early evening, with amplitudes in performance ranging from 2% to 11% of daily mean (Reilly *et al*., 2007). This present study found a 1.2% quicker performance time in the evening trial, however, the experimental trial was not conducted in the window of optimal diurnal phase at ~20.30hrs as summarised in Reilly and Waterhouse (2009). Potentially, this methodological design may have limited the physiological response, affecting performance and muting the effect of chronotype on performance in a *quid pro quo* manner. Furthermore, regular exercise completed at various diurnal time-points has been identified to have zeitgeber-effects synchronising circadian phase, thus limiting any

time-of-day effect between AM and PM trials (Atkinson *et al*., 2003; 1993). Exercise undertaken in the morning has demonstrated the propensity to advance circadian phase, a finding that was not replicated after evening exercise (Piercy and Lack, 1988).

This study found that the physiological parameters examined displayed a distinct exercise effect. CBT was significantly different pre to post-exercise in both trial conditions ($p<.05$) (09.00hrs and 16.00hrs), respectively. This exercise effect on CBT has been found extensively in research and is considered a fundamental response to high-intensity exercise (Reilly *et al*., 2007; Atkinson *et al*., 2005; Edwards *et al*., 2005; Aldemir *et al*., 2000). CBT showed a diurnal difference at baseline (09.00hrs and 16.00hrs, pre-exercise), with a significant difference ($p<.05$) between diurnal phases (09.00hrs m=36.48°C±0.5, 16.00hrs m=37.12±0.37). The ability to perform athletic activities optimally is correlated to circadian phase, with CBT used as a surrogate marker (Edwards *et al.,* 2013). Aldemir *et al*. (2000) reported the greater propensity to generate heat during a bout of morning exercise, due to a lower basal body temperature. A similar finding was found in this present study with an increase of 1.34°C from pre to post-exercise in the AM time-trial amounting to a 3.4% change ($p<.05$). A similar, but less profound, increase of 2.2% (0.8°C) was found in the PM experimental condition, most likely as a consequence of a circadian phase compensated mechanism with elevated resting CBT. This CBT phase response is an important finding as it supports the viewpoint that human physiology is compensated, to not only its

endogenous and exogenous environment, but also to zeitgebers in a circadian phase dependent manner.

CBT was significantly different between AM and PM trials at the 1km stage ($p = 0.05$) but at no other point. In part, this temperature response may potentially be due to the effect of exercise in the AM trial increasing at an advanced rate compared to the PM trial, as discussed earlier. Additionally, the significant difference at the 1km stage may be due to a delayed effect on the body's metabolic rate to the exercise stimulus, with the diurnal phase negating an exercise-induced increase. An elevated metabolic rate largely contributes to the increase in CBT during high-intensity exercise, however it can take up to 10 minutes of continuous exercise at room temperature to significantly increase body temperature (Racinais, 2008). Importantly, in cool environmental conditions, similar to this present study (6°C), it is quite likely that an increase in CBT potentially may be muted initially in the early stages of exercise. Moreover, CBT and self-paced motorised treadmill speed displayed a positive relationship in both AM ($r^2 = .88$) and PM ($r^2 = .85$) trials supporting the assertion that an increasing exercise load is mirrored in the body's thermoregulatory response irrespective of diurnal phase. Furthermore, as CBT was seen to increase at a greater rate at the AM trial, the body's thermoregulation could be identified as being in 'heat gain mode' as described in Aldemir and colleagues (2000).

CBT begins to rise in the hours immediately prior to waking in the morning following a night of sleep, due to distal vasoconstriction reducing the loss of heat (van Someren, 2004). However, CBT acrophase is not reached until the

evening at which point vasodilation results in a loss of heat to the periphery and subsequent decline in CBT during sleep (van Someren, 2004). The retention of heat during exercise in addition to the elevation of CBT to >39.7°C is associated with detrimental performance outcomes (Nielson *et al*., 1993), especially when environmental conditions are greater than 20°C (Helou *et al*., 2012). Ambient temperatures greater than 20°C have been reported to decrease endurance distance (marathon) performance by 17.7% compared to neutral environmental temperature (5-10°C, optimum 6.2 °C) (Helou *et al*., 2012), predominantly due a decrease in running speed (Marr and Ely, 2010). Exercise undertaken in latter parts of the diurnal day are characterised as resulting in a less pronounced increase in CBT due to higher pre-exercise temperature and greater vasodilation resulting in the propensity for an increased rate of heat loss (Aldemir *et al*., 2000). Consequently, the environmental conditions, the habitual nature of the participants' training and circadian phase in which testing was completed provided the optimal conditions for exercise to be undertaken irrespective of time-of-day negating AM or PM differences.

Competitive race start times are selected with care to consider the above mentioned factors. For example, when ambient conditions are hot and/or humid, performance capabilities are reduced due to increased physiological and thermo-regulatory demands (Jones *et al*., 2012). The incidence of runners reporting heat stress related conditions is positively correlated to higher ambient environmental conditions (Helou *et al*., 2012). The 2007 London marathon was completed in temperatures reaching 21°C and saw one runner

die and a greater than average seek medical treatment (Helou *et al*., 2012; BBC News Online, 2007). Similarly, the Boston marathon has had the start time moved to 10.00hrs from 12.00hrs due to an increase (>300 required medical treatment) in heat stress associated injuries increasing dramatically at the 2004 race where temperatures reached 22.5°C (Roberts, 2010).

Previous work (Scheer *et al*., 2010) has found no relationship between diurnal phase and heart rate (HR) response to exercise. However, similar to previous work, HR increased significantly from pre to post-exercise in both trial conditions (Borg *et al*., 1987). A strong correlation was found in the AM (r^2=.92) and PM (r^2=.90) conditions with increasing CBT closely mirrored by HR, although no diurnal divergence was observed. Similarly, a positive relationship was found between HR and the rate of perceived exertion (RPE) (Borg, 1970; Borg *et al*., 1987), with both values increasing incrementally through the 10km time-trials. Interestingly, the AM exercise bout showed a stronger correlation (r^2=.90) than the PM trial (r^2=.79). As no diurnal difference was found in HR response to exercise (P> 0.05), it may be speculated that the increased perception of exercise is possibly due to the circadian phase at which the exercise was undertaken, i.e. early part of day/diurnal phase. Although measures to control the experimental conditions were in place to standardise the trials (i.e. temperature controlled, self-paced, blinded and cross-over study design), it is possible that nutritional status may pose an effect of perceived exertion in the AM trial (fasting or low calorie intake during hours that preceded testing) (Yoshizaki *et al*., 2013). However, the purpose of the experiment was to assess diurnal variation in as close to a competitive ‘real world’ environment

with controls to standardise the conditions in place and not circadian rhythm. In addition, the participants, as competitive athletes, were instructed to follow their regular pre-race schedule (feeding habits with the exception of caffeine or listed foodstuffs).

Lung function (FVC, FEF_{25-75}, $FEV_{1,}$ and PEF) showed no exercise (pre, post, 1hr post) or diurnal significant effect ($p > .05$). Recent research has reported diurnal rhythms in a wide variety of lung function parameters (Goel *et al*., 2015; Medarov *et al*., 2008) however this was from a considerably larger sample size (n=161) (Goel *et al*., 2015) and with true resting (awakening) assessment points (Kelly *et al*., 2004). It is possible that, with such variability in lung function and out-with the optimal time of the day to test, the power of the sample size in this study was too low to report a significant value. Additionally, the highly-trained and experienced training status of the study cohort has been shown previously to limit exercise-induced impairment to lung function (McConnell *et al*., 1997). Irrespective of a lack of diurnal rhythms in lung function, it is somewhat surprising that no exercise effect was uncovered especially as a high ventilatory rate, due to the nature of the activity and cool environmental conditions, would be expected. Exercise in sub-zero (<°C) conditions increases the propensity for airway damage and as a consequence impairs lung function, causing inflammation and promoting exercised induced asthma (Kippelen *et al*., 2012; Bolger *et al*., 2011; Chimenti *et al*., 2010). However the duration of the exercise (<~40minutes), the self-paced nature of the time-trial without competitors and the stable neutral temperature may not be sufficient to affect the lungs to cause dysfunction or induced AHR.

Furthermore, post-exercise FEV_1 values support the null hypothesis as values did not meet the criteria for exercise induced asthma as set at a greater >10% reduction in lung function from pre-exercise levels (Rundell and Jenkinson, 2002).

It is well known that a distinct circadian rhythm exists within the endocrine and immune systems (Dimitrov *et al*., 2009). For this present study, a significant diurnal difference (main effect of time; $p<.05$) was observed in leukocyte, neutrophil and lymphocyte counts. As anticipated, the exercise bout was sufficient to promote a change in lymphocyte counts increasing immediately post-trial from pre-exercise resting values, in a typical biphasic lymphocytosis manner (egress/ingress) as reported previously in the literature (Campbell *et al*., 2009; 2008; Simpson *et al*., 2007; 2006). This lymphocyte response was found in both AM and PM time-trials. Similarly, CD8+ cytotoxic T-lymphocytes subsets displayed an exercise induced response that is in agreement with previous literature (Turner *et al*., 2010; Campbell *et al*., 2008), however no interaction was found between time of day and exercise.

Neutrophils displayed a robust exercise and diurnal response with values continuing to increase pre to post and post to 1hr post-exercise which accounted for the rise in leukocyte numbers into the peripheral blood compartment. This inflammatory response to high-intensity exercise has been reported in previous literature (Sureda *et al*., 2009; Yaegaki *et al*., 2007; Peake, 2002), however the greater amplitude in PM post-exercise response is a novel finding. Ackermann *et al*. (2012) observed that circulating peripheral neutrophils peaked at 15.42hrs in young, healthy men. The findings of this

study, which had a similar study demographic, are similar with elevated PM resting neutrophil numbers in comparison to the AM. Additionally, a pilot study assessing diurnal variation in neutrophils (figure 4.15, chapter 4) found higher neutrophil cell counts in the early evening concurring with previous work (Ackermann *et al*., 2012). Although speculative, an augmentation in neutrophil response to PM self-paced exercise in comparison to AM exercise of a similar intensity, is likely to be due to interactions between an upregulation in the expression of the circadian-dependent clock-gene ROR-α promoting a pro-inlammatory response in circulating peripheral cytokine profile (Kopmel *et al*., 1990). Clock output genes orchestrate circadian rhythms in constituents of peripheral blood, including neutrophils. Neutrophils, like other granulocytes, once migration to the tissues via the blood occurs they do not pocess the ability to recirculate, resulting in the exercise-induced increase displayed in this study and previously (Oishi *et al*., 2006). Limited data suggests a combination of the inherent diurnal factors and exercise-induced stress results in a more exaggerated neutrophilia in the evening (Hammouda *et al*., 2012) as reported in this present study.

The main aim for this study was to investigate a diurnal response to various parameters, that being a differential response to exercise as a consequence of the circadian phase. Neutrophils increased in circulating cell numbers from pre to post-exercise by 40.6% in the PM trial and by 20.4% in the AM trial. A second increase was observed with neutrophils continuing to increase in the peripheral circulatory by 60.4% (PM) and 21.4% (AM) from post-exercise to 1hr post-exercise. Furthermore, peripheral neutrophil numbers increased from

pre to 1hr post-exercise 129.7% due to evening exercise and by 46.6% due to morning exercise. The mechanism behind this diurnal phase response is at present unclear but factors such as damage to the airway epithelium lining, damage to exercising muscles, CBT or hormonal factors must be a consideration.

An increase in circulating neutrophil cell numbers, similar to those as a result of a bout of exercise, have been found after cortisol (Tonnesen *et al*., 1987) and adrenaline (Kappel *et al*., 1991; Tonnesen *et al*., 1987) infusion. Therefore, circulating stress hormones, cortisol and catecholamines (adrenaline and noradrenaline), have been considered as potential mediators of exercise-induced neutrophilia after high-intensity exercise especially of a prolonged nature (Laing *et al*., 2008; Kappel *et al*., 1991; Tonnesen *et al*., 1987). No relationship between stress hormones and leukocytes was discovered in this present study although this is most probably due to low statistical power (n=6) for the statistical model and not necessarily due to the hormonal mechanisms. Furthermore the half-life of adrenaline in peripheral blood ranges from seconds to minutes once stress is ceased (exercise) and approximately 10 minutes for cortisol (Laing *et al*., 2008). Therefore adrenaline as the driving force behind 1hr post-exercise neutrophil elevated levels seems unlikely. Moreover blocking adrenaline receptors using propranolol (beta blocker) has been shown to have no effect on neutrophilia after submaximal exercise (Foster *et al*., 1986). In addition, blocking of propranolol did not affect cardiac output but did lower (by 14%) HR during exercise (Foster *et al*., 1986). As a result, the sheer mechanical force due to increased cardiac output and

elevated glucocorticoid levels such as cortisol during high-intensity exercise has been proposed as a viable mechanism that causes demarginalisation of neutrophils from the peripheral pools in response to exercise (Laing *et al*., 2008; Foster *et al*., 1986).

Exercise undertaken in a cold environment has been reported to substantially reduce the rise in peripheral cortisol and adrenaline levels. This was associated with a less pronounced increase in peripheral leukocyte counts, including neutrophils, in comparison to exercise conducted thermo-neutral laboratory settings (Rhind *et al*., 1999; De Haas *et al*., 1994). Exercising in cold dry environmental conditions can lead to an increase in the incidence of upper airway inflammation and is associated with the migration of neutrophils from the peripheral blood into damaged airway tissues (Couto *et al*., 2013; Bermon, 2007). However, an increased frequency of neutrophils in the circulatorion as a response to muscle or airway damage may explain exercise-induced elevations in neutrophil numbers if it were not for the diurnal phase response as seen in this study. Instead, it suggests that upper respiratory tract inflammation of the airways is unlikely to be the main contributor to neutrophilia.

This present study reported an increase in CBT as a result of exercise, with higher values in the evening. A temperature-compensated mechanism for circulatory neutrophil numbers has been reported previously and may be an influential factor modulating a greater evening neutrophil response to exercise. Neutrophil migratory patterns have been shown to increase at higher CBTs (38°C to 39°C) in response to running at 75% $\dot{V}O_2$max for one hour (Niess *et*

al., 2003). This hypothesis would support the idea of a circadian phase influence on circulating neutrophil numbers in response to PM exercise that is more pronounced than AM exercise of a similar intensity and duration through mechanisms related to higher CBT. However, evidence to this effect is currently limited with a more probable inverse relationship between the circadian phase of cortisol, with early diurnal acrophase, and evening neutrophil counts and exercise-response. This assertion is supported for a number of reasons; lung function displayed no time by exercise interaction, and as the study population was experienced distance runners, muscle or airway damage seems unlikely to vary between AM and PM trials.

The aim of this study was to examine the effect of diurnal phase on physiological and biological parameters, in response to a bout of high intensity exercise conducted in the morning and the afternoon. No time-of-day effect in lung function, HR response or circulating lymphocyte and T-lymphocyte subsets was observed, but an exercise effect was shown in many variables. The participants in this study were well trained athletes, and their propensity to train both in the morning and evening, has been thought to act in a zeitgeber fashion, that is to entrain the physiological and immunological responses. It would appear that this training-effect, and the intensity of the time-trial have been the main drivers of the participants' response, overriding the circadian variation in performance due the exercise bout acting as a zeitgeber advancing circadian phase response consequently limiting diurnal discrepancies (Atkinson *et al*., 2003; 1993; Piercy and Lack, 1988).

Chapter 5: Diurnal Variation in Physiology and Immune Responses in Recreational and Experienced Male Distance Runners

5.1. Introduction

The previous chapter showed no diurnal difference (trained, habitual runners) in the performance of self-paced time-trials in the evening compared to morning, altough core body temperature (CBT) displayed a diurnal rhythm with elevated levels at 16.00hrs (referred to as PM) compared to 09.00hrs (referred to as AM) in experienced male distance runners. The physiology parameters of heart rate (HR), rate of perceived exertion (RPE), self-paced treadmill speed and lung function did not present with a diurnal effect at the defined time-points, but an exercise effect was observed in all with the exception of lung function. Similarly, an exercise-induced effect in immune parameters was observed in lymphocytes and lymphocyte subsets but no time of day by exercise interaction was found. Circulating peripheral neutrophil numbers however were found to display a diurnal phase response that differed in morning (also referred to as AM) and evening (also referred to as PM) exercise, with a greater amplitude in response to evening exercise.

Neutrophils comprise of up to 70% of all leukocytes found in the blood and are essential in combating invading pathogens providing an unspecialised innate defence (Geenen and Chrousos, 2004). Neutrophils play an important role in the inflammatory response to injured tissues and during the early stages of a defence against infection, where they migrate from the blood to the site of injury or damage. In addition, neutrophils display the greatest exercise induced response of all leukocytes in terms of total cell numbers, increasing fourfold during the recovery period (Gleeson *et al*., 2013) and as demonstrated in findings of *chapter 4*.

Exercise duration and intensity however are important factors in the neutrophil response with longer duration exercise resulting in an initial peak in circulating levels post-exercise, while high intensity exercise of a shorter duration causes neutrophils to peak three hours post-exercise cessation (Robson *et al*., 1999; McCarty and Dale, 1988). As a marker of inflammation, neutrophils have been reported to increase as a consequence of exercise-induced muscle damage and lung injury (Brancaccio *et al*., 2010; Mooren *et al*., 2002; Mackinnon, 1999). High-intensity exercise increases the ventilatory load three to four fold, often resulting in injury to the bronchial epithelium lining of the lungs (Bermon, 2007), especially if exercise is conducted in polluted environments (Gomes *et al*., 2010; Chimenti *et al*., 2009; Mudway and Kelly, 2004) resulting in elevated neutrophil levels for several hours post-exercise (Simpson *et al*., 2009; Steensberg *et al.,* 2002). Findings outlined in *chapter 4* elucidate the diurnal nature in exercise-induced neutrophilia with a greater amplitude to PM compared to AM exercise of a similar load and duration. The pro-inflammatory mechanism that drives this neutrophil response is equivocal, however diurnal homocysteine factors are one possible explanation. Increased cardiac output (sheer stress) (Laing *et al*., 2008), glucocorticoids (cortisol) (Steensberg *et al*., 2003), inflammatory response to damaged tissue (Bermon, 2007) and an increase in CBT (Niess *et al*., 2003) have been proposed as mechanisms that promote neutrophilia post-exercise, however data from *chapter 4* does not fully support this assertion.

Endurance trained individuals have been reported to display higher baseline neutrophil values or levels that are considered clinically normal (Belda *et al*., 2008; Bonsignore *et al*., 2001). However, elite standard distance runners have been shown to present with lower neutrophil counts during periods of intensive training (>100 km·wk^{-1}) (Hack *et al.,* 1994). Additionally, multiple days of high-intensity aerobic exercise depress peripheral neutrophil cell counts (Henson *et al*., 2008; Suzuki *et al*., 2003). Although not an elite level, the highly trained competitive level of the study cohort in *chapter 4* display similar charatcerisitcs; high training load and competitively active compared to the Hack *et al*. (1994) study population thus framing the question: does training load and aerobic fitness level affect the response of leukocytes, with great emphasis on neutrophils, to self-paced aerobic time-trial exercise? With regards to circadian rhythms, larger amplitudes in physiological parameters and higher self-paced work rates are found in aerobically trained compared to sedentary populations (Atkinson and Reilly, 1996).

Circulating peripheral lymphocytes display a similar response to exercise from onset to cessation, with lymphocytosis well established in the literature (Ingram *et al*., 2015; Brown *et al*., 2014; Witard *et al*., 2012; Simpson *et al*., 2009). However, exercise of an appropriate duration and intensity commonly decreases circulating lymphocyte cell numbers during the recovery phase. Lymphocytopenia occurs when lymphocytes are redistributed to parts of the body (lymph tissues, lungs, bone marrow) or undergo cell apoptosis (Simpson *et al*., 2009; 2007; 2006; Mooren *et al*., 2002). Lymphocyte subsets of a cytotoxic functionality account for a large proportion of mobilised lymphocytes

(i.e. primarily affecting effector CD8+ T-lymphocytes, gamma delta (γδ) T-lymphocytes and natural killer (NK) cells) (Anane *et al*., 2009; Campbell *et al.,* 2009), presumably acting to strengthen immune defences against infection or damage encountered during a period of stress (Lange *et al*., 2010). Furthermore, research indicates that this alteration in circulating immune cell levels may be amplified by bouts of exercise of a high intensity or by inadequate recovery periods between exercise sessions (Gleeson, 2006).

A divergent migratory pattern in T-lymphocyte subsets based on cell surface markers of nativity and senescence has been well established (Turner *et al*., 2010; Campbell *et al*., 2008; Simpson *et al*., 2007). T-lymphocyte CD8+ cytotoxic subsets can be defined as naïve, intermediate memory or senescent via the expression of cell surface markers for example CD28 and CD27 which are up or down regulated based on prior antigen exposure (Appay *et al*., 2008; Romero *et al*., 2007). CD8+ effector memory phenotypes that are functionally senescent are preferentially mobilised into the peripheral blood compartment in response to exercise, while naïve phenotypes are redeployed to lymphoid tissues (Romero *et al*., 2007; Appay *et al*., 2002; Hamann *et al*., 1997). Furthermore, fitness levels among trained and untrained populations is associated with differing circulatory patterns in naïve/senescent phenotypes (Spielmann *et al*., 2011)

Reports suggest that lymphocytes and neutrophils are significantly influenced by the effect of changing hormone levels induced by physiological or psychological stress associated with the onset of physical activity (Anane *et al*., 2009; Segerstrom and Miller, 2004). Indeed, the immune system and its

constituent circulating cells display a distinct circadian rhythm of one sort or another (Dimitrov *et al*., 2009). Research (Dimitrov *et al*., 2010; 2009; Lange *et al*., 2010; Steensberg *et al*., 2003; Elenkov *et al*., 2000; Suzuki *et al*., 1999) is unequivocal in determining the influence the two major stress-axes, the sympathetic nervous system (SNS) and the hypothalamus pituitary adrenal (HPA), exhibit on the immune system. Both of these stress-axes apply opposing governance of immune defence by their respective conflicting circadian rhythms (Dimitrov *et al*., 2009) with catecholamines (adrenaline and noradrenaline) displaying a diurnal acrophase and nadir during the hours of nocturnal sleep (Dimitrov *et al*., 2010; Lange *et al*., 2010; Dimitrov *et al*., 2009). Moreover, publications have highlighted expression of β2-adrenoceptors increases with immune cell activation and differentiation state (Dimitrov *et al*., 2010; 2009). Conversely, the HPA derived hormone, cortisol, has been shown to be responsible for the regulation of naïve lymphocytes, in a near antipodal manner to catecholamines, with acrophase during nocturnal sleep and nadir during the period of heightened daytime adrenaline levels (Dimitrov *et al*., 2009). The balance between these two opposing stress-systems and their respective products appear to be the greatest influence on circulating lymphocytes. For instance, adrenaline levels increase exponentially from the onset of exercise, gradually returning to pre-exercise levels from the cessation of exercise. Contrary to this, cortisol levels display an initial depression form the onset of exercise-stressor, with a subsequent rise in plasma levels with reduction of exercise intensity or complete cessation (Galbo, 1983).

Furthermore, during the period of heightened cortisol levels, i.e. the cessation of exercise, a greater proportion of naïve lymphocytes replace functionally cytotoxic cell types (Dimitrov *et al*., 2010). Atanackovic and co-authors (2006) demonstrate this phenomenon quite elegantly with acute stress inducing a decrease in the total number of circulating T-lymphocytes of a naïve and a central memory phenotype, in coincidence with an increase in effector memory and terminally differentiated T-lymphocyte subsets. Accordingly, the relative effects of catecholamines is determined by the type and quantity of receptors present on target cells, in addition to the local transient concentrations of adrenaline and noradrenaline (Kruger *et al*., 2008). Significantly, circulating catecholamine and cortisol levels display distinct, albeit, differing circadian characteristics (Dimitrov *et al*., 2010; 2009).

5.2 Aims

The aim of this study was to examine training status on physiological and biological responses at differing diurnal time-points.

It was hypothesised that:

1. The diurnal phase exercise is undertaken at would result in a differential physiological response in recreational and experienced distance runners.
2. A significant diurnal response to exercise would be observed in recreational and experienced runners.
3. Late differentiated CD8+ subsets would be preferentially mobilised in response to evening exercise and training status would have a significant effect on this response.

4. Lung function, in response to exercise and influenced by training status, would be subject to diurnal rhythms in peak performance.
5. Cortisol concentrations in peripheral blood compartment, and in response to exercise, are subject to diurnal factors and training status.

5.3 Methodology

Chapter 3 details the comprehensive methods used for this study. Methods specifically used in this study only are described below.

5.3.1 Participants

Nineteen healthy males (mean ± SD: age: 31.5 ± 6.9 years, body mass: 77.2 ± 8.3kg, height 181.9 ± 6.44cm, $\dot{V}O_2max$ 55.8 ± 6.1 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$) were recruited. Participants were divided into two groups based on distance running experience and $\dot{V}O_2max$ results; (i) recreationally fit (n=9, mean ± SD: age: 28.7± 5.5 years, body mass: 78.6 ± 7.4kg, height 183.2 ± 6.4cm, BMI 23.42, $\dot{V}O_2max$ 52.2 ± 6.4 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$, training volume <20 $km \cdot wk\text{-}^{1}$. (ii) experienced (n=10, mean ± SD: age: 34 ± 7.2 years, body mass: 75.9 ± 9.2kg, height 180.7 ± 6.5cm, $\dot{V}O_2max$ 59.2 ± 3.7 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$, training volume >40 $km \cdot wk\text{-}^{1}$. Time to complete $\dot{V}O_2max$ tests were 10mins 49sec ± 1min 58sec for the total study population (n=19), experienced participants (11mins 43sec ± 1min 10sec) took significantly longer ($p < .05$) to reach volitional exhaustion than recreational participants (9mins 49sec ± 2min 15sec), also reporting lower respiratory exchange ratio (RER); 1.12 ± .09 compared to recreational participants (1.18 ± .08). All participants were healthy, non-smokers and were

not taking any medication in the previous weeks. Each participant completed all prerequisite paperwork and questionnaires as described in detail in chapter 3. Additionally, participants were asked to complete an Obligatory Exercise Questionnaire prior to the time-trials (Appendix 10) and Respiratory Symptoms Questionnaire post-trials (Appendix 11). Ethical approval was granted by the ethics committee at Edinburgh Napier University and all participants provided written informed consent.

5.3.2 Experimental Procedures

The experimental procedures are described in detail in chapter 3.

Briefly, experimental days consisted of participants arriving at the Human Performance Laboratory at Edinburgh Napier University a minimum of 90 minutes prior to trial commencement (07.30hrs for AM trial and 15.30hrs for PM trial). The trial consisted of a 10km self-paced blinded (no input as to running speed) time-trial run on a motorised treadmill (Woodway, ergo ELG55, Germany) at two different times of the day (09.00hrs and 17.00hrs) on two separate occasions, with a minimum of seven days between trials during British Summer Time (BST) (March 29th – October 25th, 2015). The trials were completed in a randomised counter-balanced manner. All participants were healthy, free from injury or infection in the previous weeks. The trials were performed in an environmental chamber (Weis-Gallenkamp, UK) with the temperature set for a standardised 6°C for the duration of the exercise protocol.

Lung function and blood samples were obtained pre, post and post 60 minutes (post60), as described in chapter 3. Core body temperature (CBT) was monitored throughout the trial by way of core temperature (telemetry) pill, which was administered between 90 to 120 minutes prior to exercise-trial commencing.

5.3.3 Lymphocyte Isolation, Labelling and Analysis

Briefly, whole blood was separated, labelled with monoclonal antibodies (MAb) and analysed by FACSCalibur flow cytometry using CELLQuest Pro software (BD Biosciences, San Jose, CA, USA), as described in detail in chapter 3.

5.3.4 Statistical Analysis

SPSS software version 20 (Chicago, IL) was used to complete statistical analysis. Normal distribution was assessed using the Kolmogrov Smirnov or Shapiro-Wilk test (Newell *et al*., 2010). The effect of time (i.e. pre, post and post60) and diurnal time-point (AM and PM) were analysed using repeated measures analyses of variance (ANOVA). Only significant interactions (time x diurnal time-point) are presented unless otherwise stated. Bonferroni corrected paired T-tests were utilised when significance was found ($p < 0.01$). Assumptions of sphericity in data were checked for each ANOVA, and, where appropriate, adjustments were made to the degrees of freedom. Paired sample T-tests were used to determine parameters for pre and post-exercise and for diurnal phase (AM pre vs PM pre). Statistical significance was recorded as $p<0.05$. All results are presented as mean ± SE unless otherwise stated.

5.4 Results

Habitual exercise questionnaire responses showed that all of the experienced group competed in two or more endurance events within the past twelve months, while 44% (4 of 9) of the recreational group competed in at least one organised race.

No diurnal differences were observed in total group time to complete 5km (AM 24.27minutes, SD= ±5.17, PM 23.49, SD=±5.29) (t(18)=.715, p>0.05) or 10km (AM 45.59minutes, SD= ±11.32, PM 46.37, SD=±9.38) (t(18)=.390, p>0.05) respective stages of time-trial, as seen in figure 5.1.

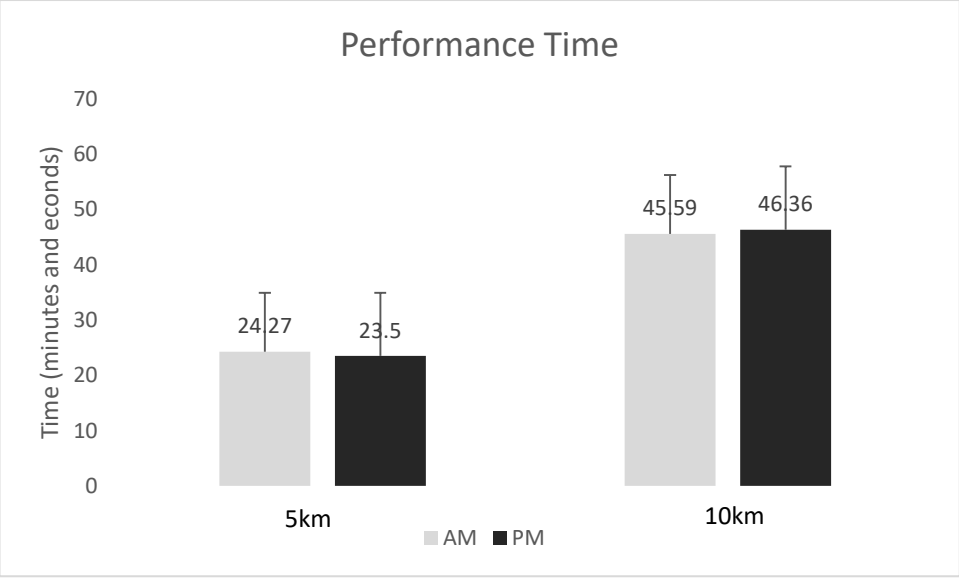


Figure 5.1. Performance (time in minutes and seconds to complete) at 09.00hrs (AM) and 17.00hrs (PM) for total group runners. Values are mean ±SD. p value set at 0.05 significance. Coefficient of variation percentage (CV%): Experienced AM = 10.37% and PM = 9.83%, Recreational AM = 12.51% and PM = 13.05%. Group CV% : 5km AM = 21.3 , 5km PM = 22.52 and 10km AM = 24.83, PM = 20.22 (n = 19).

Figure 5.2 shows performance times for 10km time-trials at 09.00hrs (AM) and 17.00hrs (PM) in experienced and recreational participants. All participants completed the exercise trials. A significant difference was observed between

experienced and recreational runners in time to complete the exercise task at AM and PM trials (independent-sample T-test; $P < 0.05$) but no difference in performance times within groups was found (paired-sample T-test; $P > 0.05$).

Experienced runners' time was 40.09 ± 4.16 minutes at AM compared to 40.38 ± 3.97 minutes at PM trial. Recreational runners' performed the exercise in 53.59 ± 6.68 minutes at AM and 54.20 ± 7.05 minutes at PM trial.

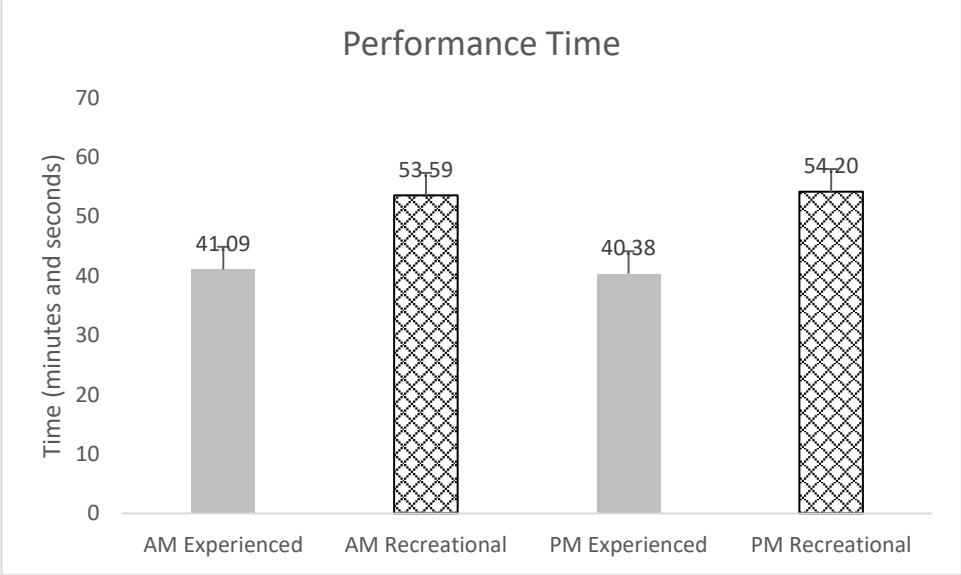


Figure 5.2. Performance (time in minutes and seconds to complete) at 09.00hrs (AM) and 17.00hrs (PM) in experienced and recreational runners. Values are mean ±SD. p value set at 0.05 significance.

Heart rate (HR) response for total study cohort (n=19), displayed in figure 5.3, did not show a time (AM/PM) by exercise interaction at pre, 5km or 10km stages (repeated measures ANOVA: $F(2,36)= 1060.717$; $p > 0.05$).

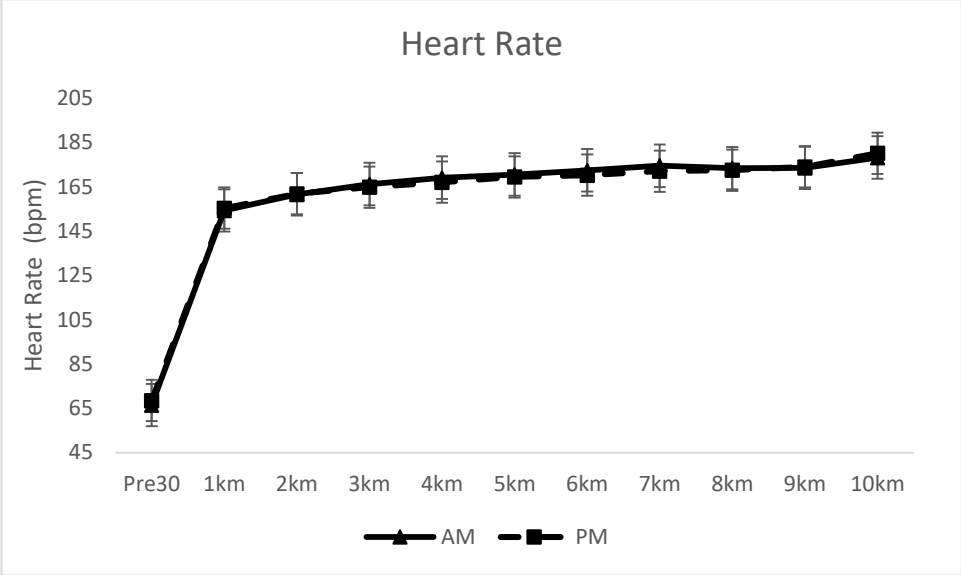


Figure 5.3. Heart rate (beats per minute) during 10km time-trial at 09.00hrs (AM) and 17.00hrs (PM) for total group runners. Values are mean ±SD.

Similarly, no time by exercise interaction was observed between AM and PM time-trials at pre-exercise, 5km or 10km stages in experienced ($F(1,9)= .013$; $p > 0.05$) (A) or recreational ($F(1,8)= .071$; $p > 0.05$) (B) groups (figure 5.4), respectively.

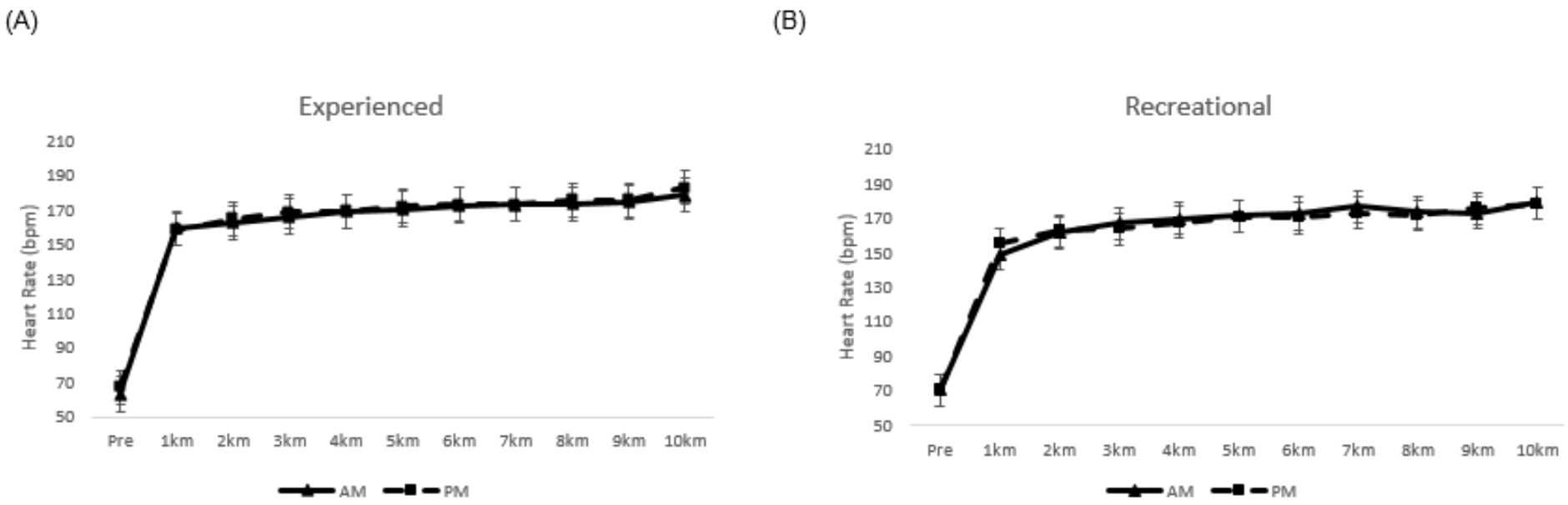


Figure 5.4. Heart rate response to 10km time-trial in experienced and recreational runners at 09.00hrs (AM) and 17.00hrs (PM). Values are mean ±SD. p value set at 0.05 significance.

Repeated measures ANOVA yielded no time (AM/PM) by exercise interaction in core body temperature (CBT) during 10km time-trial exercise at AM or PM start times ($F(1,216) = 1.456$; $p > 0.05$), as shown in figure 5.5 (below).

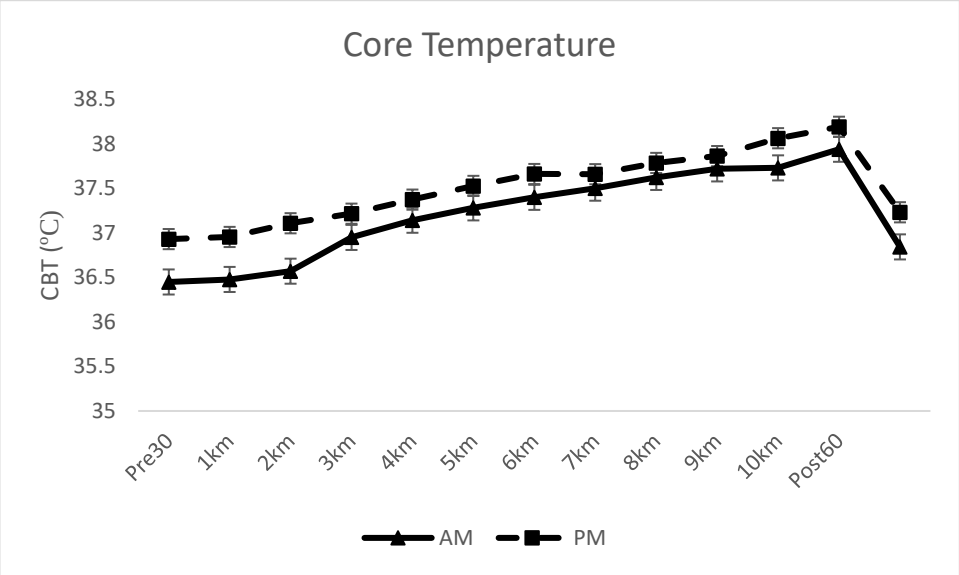


Figure 5.5. CBT (°C) 30 minutes prior, immediately pre, one hour post in response to 10km time-trial in experienced and recreational runners at 09.00hrs (AM) and 17.00hrs (PM). Values are mean ±SD. p value set at 0.05 significance.

Analysis of CBT data yielded no significant relationship between time-of-day and exercise at pre 30 (minutes), pre-exercise, 1k, 2km, 3km, 4km, 5km, 6km, 7km, 8km, 9km, 10km, post60 (minutes) stages in experienced ($F(12,108)= .642$; $p > 0.05$) (A) or recreational ($F(12,96)= 1.308$; $p > 0.05$) (B) groups (figure 5.6), respectively.

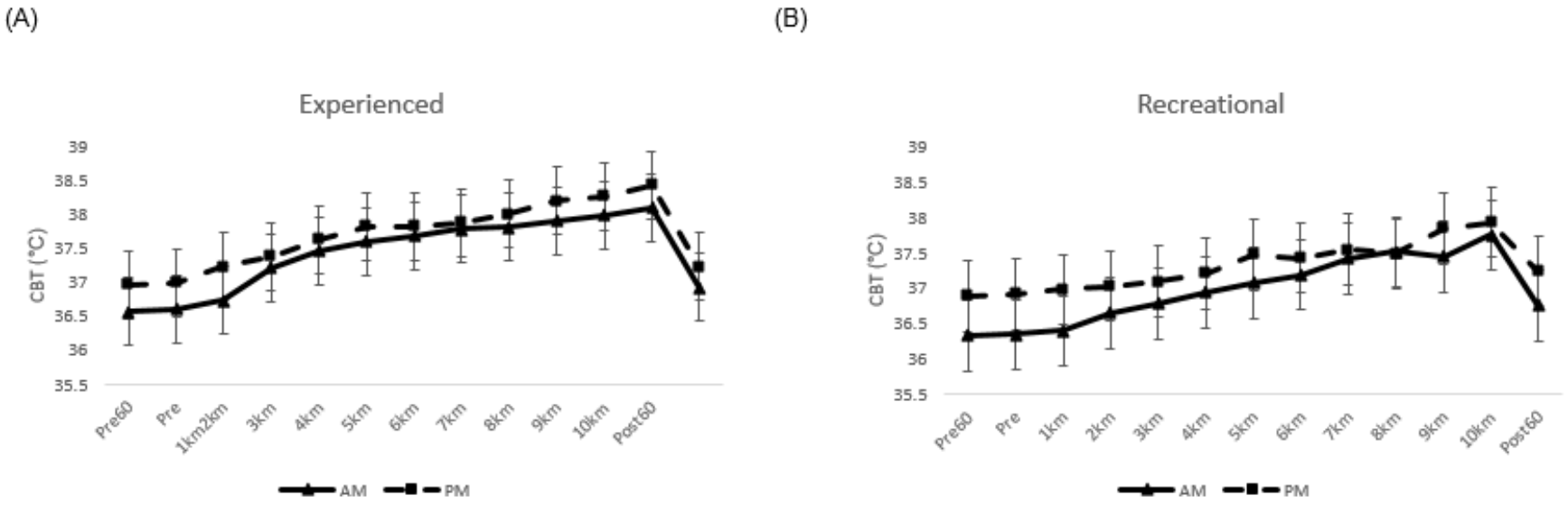


Figure 5.6. Core body temperature response prior (30 minutes prior (Pre30) and pre), during and 60 minutes post (Post60) self-paced 10km treadmill time-trial in (A) experienced and (B) recreational runners.

Assessment of lung function for forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), peak expiratory volume (PEV) and mid-expiratory flow (F_{25-75}) found no interaction between exercise and time (AM/PM) (repeated measures ANOVA) (P> 0.05) (table 5.1).

Table 5.1. Lung function measures pre, post and post60 at 09.00hrs (AM) and 17.00hrs (PM) in experienced and recreational runners in response to 10km self-paced time-trial exercise. Values are mean ±SD. p value set at 0.05 significance.

Test	AM			PM			Main Effects
	Pre	Post	Post60	Pre	Post	Post60	
FVC (L)							Main effects of time: $F_{(1,18)}$ = .793; p = NS
Experienced	5.61± 0.98	5.51± 0.85	5.65± 0.93	5.79± 1.05	5.48± 0.75	5.73± 0.99	Main effects of exercise: $F_{(2,36)}$ = 9.963; p = .000
Recreational	5.7± 0.95	5.48± 0.81	5.48± 0.94	5.89± 1.5	5.74± 1.5	5.82± 1.49	Time x Exercise interaction: $F_{(2,36)}$ = .445; p = NS
FEV_1 (L)							Main effects of time: $F_{(1,18)}$ = .541; p = NS
Experienced	4.62± 0.72	4.47± 0.57	4.57± 0.61	4.59± 0.79	4.49± 0.69	4.53± 0.75	Main effects of exercise: $F_{(2,36)}$ = 3.045; p = NS
Recreational	4.43± 0.78	4.25± 0.72	4.51± 0.88	4.56± 0.99	4.47± 0.96	4.57± 0.93	Time x Exercise interaction: $F_{(2,36)}$ = .454; p = NS
PEF ($L \cdot min^{-1}$)							Main effects of time: $F_{(1,18)}$ = 9763; p = NS
Experienced	661.9± 131	677.9± 111	673.5± 138	695.8± 130.6	678.2± 145.5	667.9± 146.9	Main effects of exercise: $F_{(2,36)}$ = 4.011; p = .027
Recreational	633.63± 90	572.4± 68	612.1± 76	643.9± 122.7	600.7± 95.8	628.9± 99.9	Time x Exercise interaction: $F_{(2,36)}$ = .705; p = NS
FEF_{25-75}							Main effects of time: $F_{(1,18)}$ = .035; p = NS
Experienced	4.24±1.2	4.19±1.2	4.22±1.2	4.24±1.31	4.39±1.23	4.36±1.31	Main effects of exercise: $F_{(2,36)}$ = .140; p = NS
Recreational	3.99±.99	4.67±.99	3.98±1.3	4.01±0.69	3.66±0.88	3.83±0.96	Time x Exercise interaction: $F_{(2,36)}$ = .717; p = NS

No main effect of time by exercise interaction was found in leukocytes or leukocyte subsets as reported in table 5.2.

Table 5.2. Peripheral leukocyte and leukocyte subset cell counts pre, post and post60 at 09.00hrs (AM) and 17.00hrs (PM) in experienced and recreational runners in response to 10km self-paced time-trial exercise. Values are mean ±SD. p value set at 0.05 significance.

Cell Type (cells/µl)	Trial	Pre	Post	Post60	Main Effects of Time	Main Effects of Exercise	Time x Exercise Interaction
Leukocytes Group	AM	4.84 ± 1.04	8.17± 1.87	7.07 ± 2.48	$F_{(1,18)}$ = 1.247; p = **.001**	$F_{(2,34)}$ = 32.778; p = **.001**	$F_{(2,34)}$ = .502; p = **NS**
	PM	6.4 ± 1.03	10.13 ± 2.57	9.2 ± 2.78			
Leukocytes Experienced	AM	4.81 ± 1.02	8.1 ± 1.6	6.53 ± 1.33	$F_{(1,9)}$ = 12.072; p = **.007**	$F_{(2,18)}$ = 34.293; p = **.000**	$F_{(2,18)}$ = .094; p = **NS**
	PM	6.4 ± .92	10.03 ± 1.99	8.33 ± 2.31			
Leukocytes Recreational	AM	4.87 ± 1.13	8.28 ± 2.26	7.77 ± 3.42	$F_{(1,7)}$ = 3.659; p = **NS**	$F_{(2,14)}$ = 14.931; p = **.001**	$F_{(2,14)}$ = .405; p = **NS**
	PM	6.44 ± 1.22	10.26 ± 3.29	10.29 ± 3.09			
Neutrophils Group	AM	2.56 ± .88	3.9 ± 1.2	5.16 ± 2.29	$F_{(1,18)}$ = 23.998; p = **.002**	$F_{(2,34)}$ = 60.923; p = **.000**	$F_{(2,34)}$ = 2.039; p = **NS**

	PM	3.78 ± .84	5.22 ± 1.8	7.01 ± 2.42			
Neutrophils Experienced	AM	2.62 ± 1.05	3.88 ± 1.25	4.7 ± 1.4	$F_{(1,9)}$ = 4.281; p = **NS**	$F_{(2,18)}$ = 28.654; p = **.000**	$F_{(2,18)}$ = .294; p = **NS**
	PM	3.76 ± .91	4.89 ± 1.58	6.14 ± 2.38			
Neutrophils Recreational	AM	2.5 ± .69	3.94 ± 1.3	5.74 ± 3.09	$F_{(1,7)}$ = 5.772; p = **NS**	$F_{(2,14)}$ = 20.277; p = **.000**	$F_{(2,14)}$ = .414; p = **NS**
	PM	3.82 ± .79	5.66 ± 2.08	8.1 ± 2.14			
Lymphocytes Group	AM	1.43 ± .28	3.15 ± 1.18	1.17 ± .33	$F_{(1,18)}$ = 23.988; p = **.000**	$F_{(2,34)}$ = 60.923; p = **.000**	$F_{(2,34)}$ = 2.039; p = **NS**
	PM	1.8 ± .47	3.76 ± 1.2	1.51 ± .39			
Lymphocytes Experienced	AM	1.41 ± .26	3.24 ± 1.22	1.17 ± .31	$F_{(1,9)}$ = 4.466; p = **NS**	$F_{(2,34)}$ = 79.037; p = **.000**	$F_{(2,34)}$ = .471; p = **NS**
	PM	1.81 ± .47	3.96 ± 1.28	1.51 ± .31			
Lymphocytes Recreational	AM	1.47 ± .32	3.05 ± 1.18	1.18 ± .37	$F_{(1,7)}$ = 1.33; p = **NS**	$F_{(2,14)}$ = 28.878; p = **.000**	$F_{(2,14)}$ = .310; p = **NS**
	PM	1.79 ± .52	3.49 ± 1.43	1.53 ± .49			

Figure 5.7 illustrates a significant effect of exercise on peripheral lymphocyte populations at morning and evening time-points ($p < 0.000$).

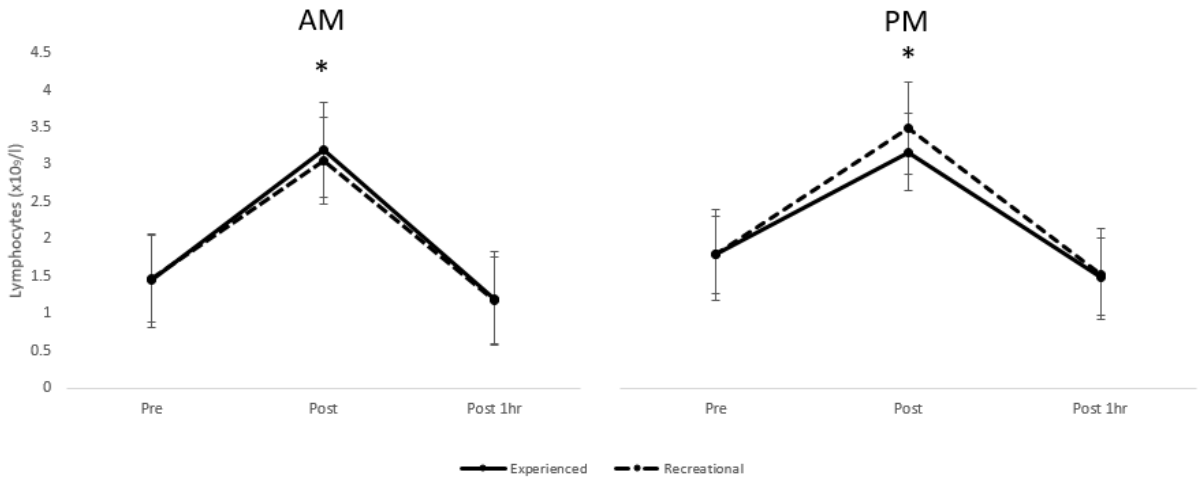
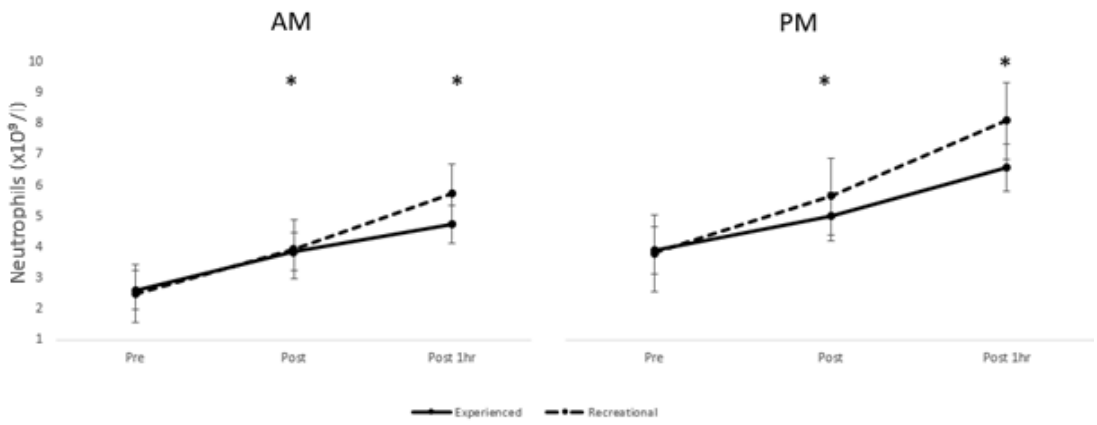


Figure 5.7. Peripheral lymphocyte counts pre, post and post60 in response to 10km self-paced time-trial exercise in experienced and recreational runners at 09.00hrs (AM) and 17.00hrs (PM). Values are mean ±SD. p value set at 0.05 significance.

Neutrophil response to high-intensity exercise is displayed in figure 5.8 where a significant effect of exercise was observed in experienced and recreational runners (figure 5.8A) ($p < 0.000$) and as a group (figure 5.8B).

(A)



(B)

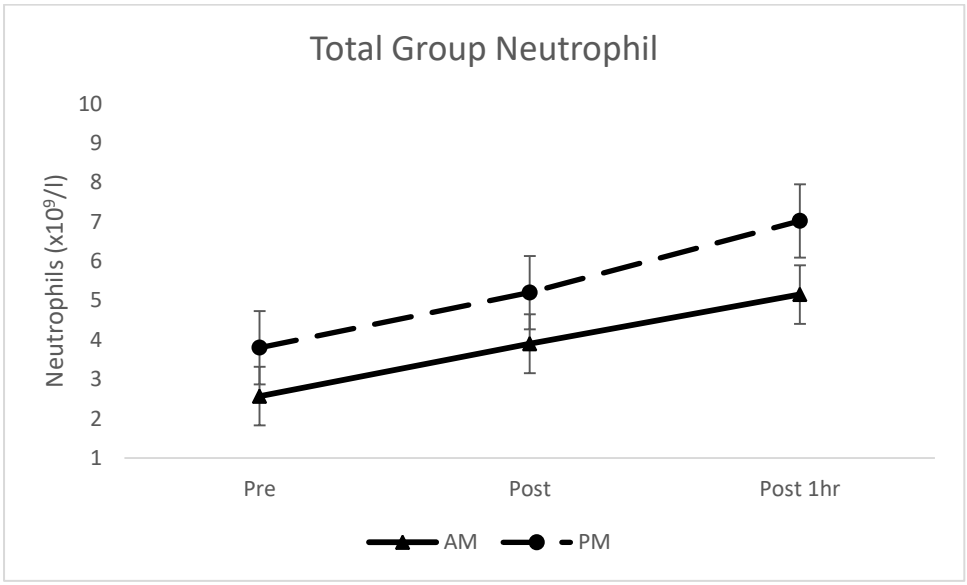


Figure 5.8. Peripheral neutrophil counts pre, post and post 1hour in response to 10km self-paced time-trial exercise in (A) experienced and recreational runners at 09.00hrs (AM) and 17.00hrs (PM), and (B) total study cohort (n=19). Values are mean ±SD. p value set at 0.05 significance.

No relationship was observed between CBT values recorded during exercise and peripheral neutrophil counts immediately post-exercise (table 5.3). Additionally, analysing the data according to experienced (n=10) and recreational (n=8) groups yielded no relationship between CBT and circulating neutrophil cell counts post-exercise (data not displayed). Furthermore, time to complete 5km and 10km stages of the time-trial, in experienced and recreational groups, and neutrophil counts post-exercise showed no relationship (Pearson's correlation, $p > 0.05$; data not shown).

Table 5.3. The relationship between core body temperature and peripheral neutrophil counts post 10km time-trial exercise.

	Neutrophils Post Exercise	**CBT 30pre**	**CBT pre**	**CBT 1km**	**CBT 2km**	**CBT 3km**	**CBT 4km**	**CBT 5km**	**CBT 6km**	**CBT 7km**	**CBT 8km**	**CBT 9km**	**CBT 10km**
Pearson Correlation (r)	1	.052	.074	.001	.172	.235	.091	.057	.093	.005	.059	.032	.05
P value (AM)		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Pearson Correlation (r)	1	.139	.136	.250	.216	.272	.214	.291	.306	.274	.247	.430	.287
P value (PM)		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Repeated measures ANOVA yielded no main effect of time (AM/PM) by exercise interaction in CD8+CD27+/-CD28+/- subsets, as described in table 5.4.

Table 5.4. Percentage of CD8+ expressing cell surface markers CD27+/-CD28+/- pre, post and post60 at 09.00hrs (AM) and 17.00hrs (PM) in experienced and recreational runners in response to 10km self-paced time-trial exercise. Values are mean ±SD. p value set at 0.05 significance.

CD8+ Subset	AM			PM			Main Effects
	Pre	Post	Post60	Pre	Post	Post60	
CD8+ Group (cell count ug/ml)	386 ±73	501 ± 88	349 ± 55	437 ± 67	500 ± 78	387 ± 68	Main effects of time: $F_{(1,16)}$ = 3.458; p = NS Main effects of exercise: $F_{(2,32)}$ = 40.288; p = .000 Time x Exercise interaction: $F_{(2,32)}$ = 3.344; p = NS
Early % (CD27+CD28+)							
Experienced	71.3± 2.71	69.0± 2.62	73.3± 2.8	71.4± 2.2	68.3±1.8	73.2± 2.0	Main effects of time: $F_{(1,16)}$ = .793; p = NS Main effects of exercise: $F_{(2,32)}$ = 9.963; p = .000 Time x Exercise interaction: $F_{(2,32)}$ = .445; p = NS
Recreational	69.62± 4.9	67.7± 5.2	70.37±3.6	68.37± 3.2	67.12± 5.19	70.5± 3.5	
Intermediate % (CD27+CD28-)							
Experienced	12.3± 1.84	13.7± 1.76	12.2± 1.22	12.1± 1.3	13.5± 1.28	11.8± 1.75	Main effects of time: $F_{(1,16)}$ = .541; p = NS Main effects of exercise: $F_{(2,32)}$ = 3.045; p = NS Time x Exercise interaction: $F_{(2,32)}$ = .454; p = NS
Recreational	11.75± 2.49	12.75± 1.9	11.5±1.77	11.75± 1.9	12.75± 1.9	11.35± 1.99	
Late (CD27-CD28-)							
Experienced	16.4± 2.9	17.3± 3.2	14.5±2.4	16.5± 2.5	19± 2.5	14.9± 2.31	Main effects of time: $F_{(1,16)}$ = 9763; p = NS Main effects of exercise: $F_{(2,32)}$ = 4.011; p = .027 Time x Exercise interaction: $F_{(2,32)}$ = .705; p = NS
Recreational	18.6±3.4	19.5± 4.0	18.1±3.8	19.8± 2.4	21.1± 1.7	18.2± 2.8	

Total group cortisol (n=12) (figure 5.9) displayed no main effect of time (AM/PM) by exercise interaction ($F_{(1,11)}$ = 2.009; p = >0.05) (main effect of exercise: $F_{(2,22)}$ = 7.721; p = 0.03, main effect of time (AM/PM): $F_{(1,11)}$ = 30.737; p = 0.00).

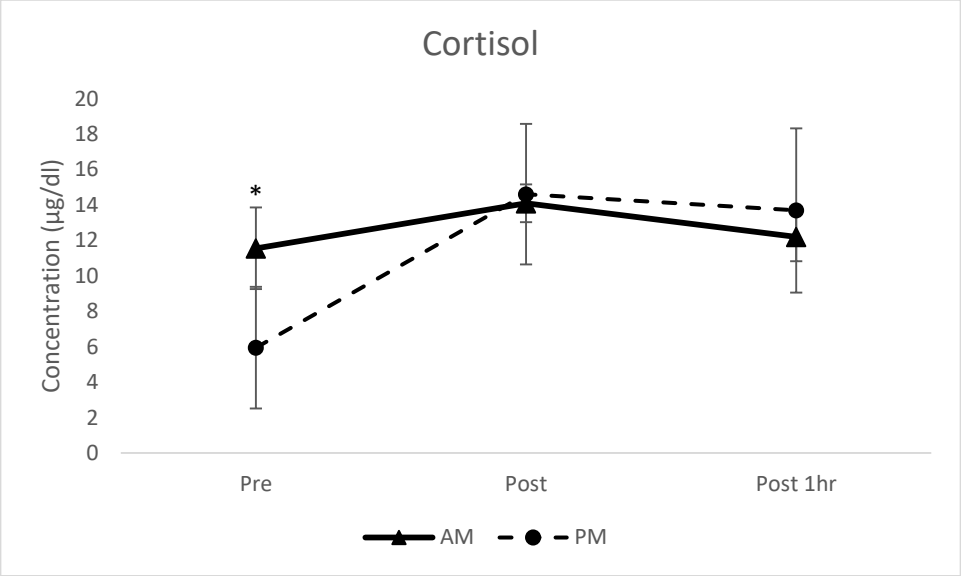


Figure 5.9. Grouped (n=12, experienced and recreational) peripheral cortisol concentrations pre, post and post60 in response to 10km self-paced time-trial exercise in experienced and recreational runners at 09.00hrs (AM) and 17.00hrs (PM). Values are mean ±SD. p value set at 0.05 significance.

Figure 5.10 (A) experienced (n=7) displayed a main effect of time (AM/PM) ($F_{(1,6)}$ = 22.806; p = 0.003) and exercise ($F_{(2,12)}$ = 5.996; p = 0.16) but no main effect of time by exercise interaction was observed ($F_{(2,22)}$ = 2.052; p >0.05) and (B) recreational (n=5) displayed a main effect of time (AM/PM) ($F_{(1,4)}$ = 22.708; p = 0.009) and but no main effect of exercise ($F_{(2,8)}$ = 3.528; p > 0.05) or time by exercise interaction was observed ($F_{(2,8)}$ = .284; p >0.05).

(A) (B)

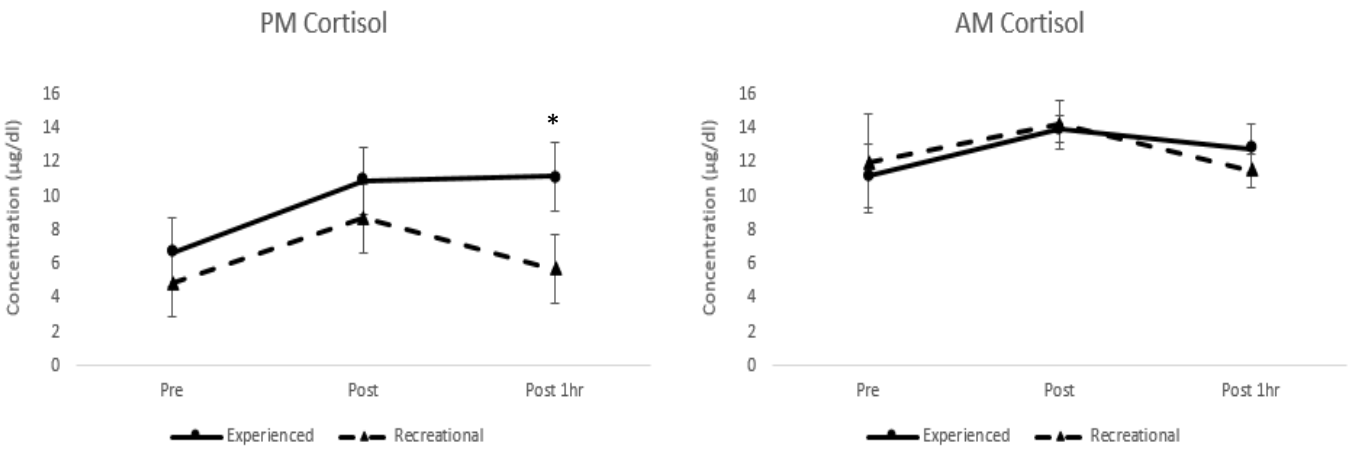


Figure 5.10. Peripheral cortisol concentrations in response to exercise in experienced and recreational runners at 09.00hrs (AM) and 17.00hrs (PM). Values are mean ±SD. p value set at 0.05 significance.

Paired sample T-tests reported a statistically significant difference in peripheral cortisol concentrations between resting (pre) AM (m=11.22, SD=1.88) and PM (m=6.69, SD=3.92) t(6)=3.159, p.0.025 in the experienced group. Similarly, the recreational group showed a significant diurnal difference at resting (pre) in AM (m=11.96, SD=2.92) and PM (m=4.9, SD=2.84) t(4)=5.23, p=0.006. Independent T-tests were used to assess means between experienced and recreational groups 1hr post-exercise. A significant difference was observed with the recreational runners in the PM (m=5.7, SD=4.1) and the experienced group in PM trials (m=11.15, SD=3.64) t(11)=2.336, p=0.044 (figure 5.9 and 5.10).

5.5 Discussion

This study investigated the influence of training status on physiological and immunological responses to a bout of high intensity exercise (10km time-trial run) performed at two different time points of the diurnal day.

The main findings were:

1. Neutrophils displayed a greater amplitude at rest and in response to self-paced 10km run in the evening with a more pronounced effect in recreational athletes. Lymphocytes exhibited a biphasic response to a bout of exercise at morning and evening time-points with recreational runners displaying greater post-exercise values after a bout of evening exercise.
2. The redistribution of late, intermediate and early differentiated CD8+T-lymphocyte subsets had no combined effect due to time of day and exercise (main effect of time x exercise interaction; $p > 0.05$) significance, although the percentage of early and late differentiated CD8+ subsets in the periphery altered due to exercise. The recreational runners group displayed a higher percentage of terminally differentiated late phenotypes compared to the experienced group at the three experimental time-points, remaining elevated one-hour post trial completion.
3. The assessment of AM and PM lung function ability; pre, post and one hour post-exercise did not yield a significant exercise by time of day interaction. However, forced vital capacity (FVC) and peak expiratory flow (PEF) did show a significant main effect of exercise.
4. The glucocorticoid hormone cortisol did not show a time (AM/PM) by exercise interaction.

This present study showed that a bout of self-paced exercise of ~40 to ~55 minutes duration in the morning and evening resulted in an increase of peripheral lymphocytes and neutrophils immediately post-exercise, with lymphocytes egressing to below resting levels while neutrophils continued to increase one hour post-exercise cessation. These findings are in agreement with the established consensus on leukocyte response to exercise (Simpson *et al*., 2009; Sureda *et al*., 2009; Yaegaki *et al*., 2007; Mooren *et al*., 2002).

Lymphocyte populations of cytotoxic nature, expressing late differentiated and/or senescent phenotypes are preferentially mobilised in response to exercise-induced stress (Simpson, 2011; Turner *et al*., 2010; Campbell *et al.,* 2009; 2008; Simpson *et al*., 2008; 2007) into peripheral blood for immuno-surveillance purposes (Kruger *et al.,* 2008; Kruger and Mooren, 2007; Dhabhar, 2000). This study observed similar findings with the percentage of late differentiated CD8+ (CD27-CD28-) increasing immediately post-exercise, with a similar reduction in percentage of early differentiated phenotypes. Moreover, phenotypic expression of naïve, central memory, effector memory and RA-effector memory phenotypes by way of using the RA isoform cell surface expression has been used in research previously (Turner *et al*., 2010; Campbell *et al.,* 2009; Simpson *et al*., 2009; 2007), however considerable overlap with expression of CD28 phenotype exists (Appay *et al*., 2008; van Lier *et al*., 2003).

Percentage of CD8+ T-lymphocytes was used to standardise comparison across the four conditions (AM/PM and experienced/recreational). The influx of late differentiated CD8+ T-lymphocytes was slightly elevated to a greater

extent in the recreational athlete group. Spielmann *et al*. (2011) found that elevated training status (>47ml.kg^{-1}) was correlated with higher numbers of naïve and a reduction in the expression of senescent CD8+ phenotypes, which was replicated by both groups comprising this study. Experienced runners group had a maximal aerobic capacity of 59.2mlO_2·kg^{-1}·min^{-1} while recreational group were lower (52.2mlO_2·kg^{-1}·min^{-1}) but within the bounds of trained status as characterised previously (by Spielmann *et al*., 2011), which goes some way to explain the not so dissimilar resting and exercise-induced CD8+ subset response. In addition, senescent CD8+ subsets are considered to increase approximately by ~5% in the peripheral pool per decade (Spielmann *et al*., 2011), potentially contributing to the similar finding between study groups. The recreational runner group displayed a higher percentage of late-differentiated CD8+ subsets compared to the experienced habitual competitor group, which is in agreement with previous work (Brown *et al*., 2014; Witard *et al.*, 2012). However, as the total study population were actively training, and in many cases, competing in endurance events (14 of 19 participants reported taking part in organised races in the last 12 months), temporal elevation in late-differentiated CD8+ phenotype due to heavy training load must not be discounted as being a confounding limiting factor (Cosgrove *et al*., 2012).

Although exercise elicited a similar response in recreational and experienced runners, no interaction was found between the effect of time (AM/PM) and exercise suggesting that no diurnal effect is present in exercise undertaken at 09.00hrs and 17.00hrs. Importantly, CD8+ T-lymphocyte subsets display an

acrophase between 22.00hrs and 02.00hrs (Ackermann *et al*., 2012; Dimitrov *et al*., 2009) with the exception of the senescent effector memory subset which display a daytime acrophase (15.34hrs ± 116 minutes) (Dimitrov *et al*., 2009). As such, exercise conducted at the time-points implemented by this current study (09.00hrs and 17.00hrs) would be considered to be at, or close, to nadir of the respective early and intermediate subsets and as such no time by exercise interaction or diurnal phase response was discovered.

Neutrophils increased at a greater rate in recreational athletes at both 09.00hrs (AM) and 17.00hrs (PM) exercise bouts compared to experienced runners. A greater amplitude in neutrophil response to exercise was seen after the PM time-trial with an increase of 28.2% in experienced runners and 48.3% in recreational runners from pre to post-exercise. Similarly, this trend of increased neutrophilia continued from post-exercise to one hour post-exercise with values rising by 40.3% in the experienced group and 64.3% in the recreational group. An exercise-induced rise in circulating neutrophil numbers has been linked to a number of factors (Steensberg *et al.,* 2002) including; inflammation of the airways (Bermon, 2007), muscle damage (Brancaccio *et al*., 2010; Mooren *et al*., 2002), peripheral hormone concentrations (Steensberg *et al*., 2003), a rise in core body temperature (Foster *et al*., 1986) and cytokine levels having all been previously proposed.

A redistribution of neutrophils to the airways is one possible factor behind an elevated neutrophil number in the peripheral blood compartment post-exercise with recreational athletes who are unaccustomed to exercise mode, intensity and environmental conditions displaying a greater magnitude of damaged lung

or muscle tissue. PEV and FVC were significantly lower in the recreational athlete group following a self-paced exercise bout. PEF reflects the functionality of large airways whereas FEF represents both large and small calibre airways (Mead, 1979). PEF and FVC were impaired post-exercise in the recreational athletic group, potentially due to injury to the airways due to a combination of the cold environmental condition, increased ventilary load and prolonged duration of the exercise resulting in damage to small and larger calibre bronchial (Goel *et al*., 2015; Kippelon *et al*., 2012). Neutrophils migrate from endothelium tissue to sites of injury and inflammation within the bronchioles or damaged muscle tissue causing an increase in peripheral numbers. This process is mediated in part by cortisol, and other glucocorticoids, which are known to decrease gene transcription of adhesion molecule L-selectin on the surface of neutrophils detaching them from endothelial (McCarthey *et al*., 1992).

CBT, under regular sleep-wake conditions, is an established analogue for circadian phase with values rising as the day progresses, which the resting pre-exercise values are in accordance with a rise in CBT has been proposed as a mechanism in the mobilisation of neutrophils in response to exercise (Foster *et al*., 1986). However, the findings of this study do not support this assertion as no correlation was found between CBT pre and at 1km stages during an exercise bout and circulating neutrophil cell counts immediately post-exercise in the total study population, experienced or recreational groups ($P > 0.05$). Furthermore, the recreational group reported lower CBT post-exercise at PM trials which would be contrary to assertions (Foster *et al*, 1986)

that higher CBT induced greater peripheral neutrophil numbers. However this does go someway to support the notion that higher work rate (exercise intensity) is conducive to a greater increase in CBT in thermal-neutral controlled environmental conditions. Similarly environmental conditions, by way of cold-water immersion, resulted in no effect on circulating peripheral neutrophil numbers supporting the argument that the associated rise of CBT in response to exercise is due to internal physiological measures and not due to environmental conditions (Laing *et al.*, 2008). Although data is limited, it would appear that the transient temporal rise in CBT during exercise between ~40 to ~55 minutes duration is not responsible for modulating post-exercise neutrophilia but may be correlated with the physiology that modulates such responses.

Neutrophils are highly responsive to exercise duration and intensity (Peake *et al*., 2002) with trained populations showing lower cell counts than healthy counterparts (Sasaki *et al*., 2013). A similar training effect is seen in this study with a greater response in the healthy recreational athletes compared to their experienced peers. However, despite this it remains unclear as to why a greater amplitude of neutrophils is seen one hour post-exercise between experimental groups and trial-times (AM/PM). Shear stress due to increased cardiac output and an initial increase in peripheral catecholamine during exercise have been postulated as mechanisms that release neutrophils from epithelium tissue causing demarginalisation to occur (McCarthey *et al*., 1992). This phenomena would go some way to explain the immediate exercise-induced response seen in peripheral neutrophil numbers post-exercise but not

the one hour post disproportional increase after PM trials and is this unlikely to modulate diurnal phase response.

Peripheral cortisol concentrations have an inverse relationship with circulating neutrophil cell numbers by inhibiting the binding of neutrophils to the endothelial, disrupting the trafficking into damaged or injured tissue (Steensberg *et al*., 2003). Furthermore, peripheral cortisol concentrations are positively correlated with exercise intensity (Lovallo *et al*., 2006). Cortisol displays a robust circadian rhythm with acrophase in the morning and an awakening response prevalent (Dimitrov *et al*., 2009) which may account for a less pronounced rise in neutrophils when comparing AM to PM trials. Although no significant correlation was reported in this current study, cortisol levels in recreational and experienced groups displayed dissimilar values especially after PM testing with experienced athletes showing higher concentrations of cortisol than recreational peers. Concomitantly, greater elevation in peripheral neutrophils was found in recreational athletes. It is suggested that higher peripheral concentrations of cortisol are inversely related to peripheral neutrophil numbers. This is displayed with elevated morning cortisol levels observed in both experienced and recreational groups. Furthermore, only the experienced group reported similar cortisol levels post-PM exercise. Interestingly this was not found in results for PM exercise for the recreational group post-exercise. This, and the effect of catecholamines post-exercise, could possibly be a mechanism for exaggerated neutrophil response seen in the recreational group post-PM trial. Supporting this assertion, moderate intensity exercise decreases cortisol levels while high intensity of

70% maximum aerobic capacity increases cortisol (Lovallo *et al*., 2006). Potentially the intensity of exercise in the experienced group, although not monitored, may be higher than recreational athletes as seen in performance time, resulting in higher concentrations of cortisol and lower neutrophils values due to an altered catecholamines-cortisol ratio. Similarly, the circadian phase governed cortisol awakening response with elevated levels pre-exercise in both study groups muted neutrophil cell counts in the recreational group at AM compared to PM trials. Cortisol has been reported to reach acrophase at ~10.40hrs (Dimitrov *et al*., 2009). If repeated in this study, this time would correspond with the AM exercise trial and post-exercise sampling and hence account for elevated cortisol levels in experienced and recreational groups post-AM testing. If accurate, a finely-tuned relationship between stress hormones (catecholamines and cortisol) and their circadian secretory patterns would explain the diurnal phase response seen in recreational athletes post-exercise in neutrophils at the PM time-point.

Limited data (Hammouda *et al*. 2011; 2012) suggests muscle damage, by way of proportionally elevated evening homocysteine concentrations, displays distinct time-of-day response to high-intensity exercise. Although no direct relationship has been elucidated between homocysteine and circulting neutrophil numbers, as both are markers of inflammation, potentially a biological clock dependent mechanism may exist which would explain the findings of this paper. Providing some anecodatal evidence to support this assertion, the recreationally trained athletic group displayed an exaggerated evening neutrophil response in comparison to the more experienced and

conditioned 10km runners, who may haave generated less muscle damage and inflammation. Alternatively, a yet to be determined mechanism may modulate an elevated neutrophil response to evening exercise in recreationally trained athletes.

In conclusion, self-paced exercise undertaken at 09.00hrs and 17.00hrs performed on separate days resulted in quicker performance in experienced, trained athletes. Recreational athletes displayed a greater inflammatory response due to elevated neutrophil counts in response to exercise in the evening compared to experienced peers, possibly due to reduced cortisol levels. Cortisol concentrations in the periphery displayed a diurnal response with elevated ‘awakening response’ in the morning pre, post and one hour post-exercise, limiting neutrophil cell counts. Greater clarity as to immune, and especially neutrophil response, to exercise at divergent times of the diurnal phase is warranted as, to date, the overwhelming majority of literature has focused concertedly on too narrow a window of time and is not aligned with when physical activity generally takes place.

Chapter 6: Chronotype, Diurnal Phase and Performance in Recreational and Experienced Male Distance Runners

6.1 Introduction

Human physiology and biology display distinct circadian rhythms, with the purpose to entrain internal timing clocks to the external environment to maintain homeostasis (Ackermann *et al*., 2012; Dimitrov *et al*., 2010; 2009; Drust, 2005; Waterhouse, 2005). Biologic functions such as the regulation of hormone secretion, circulatory patterns of immune cells, physiology, the sleep-wake cycle, and intellectual activities are governed by the circadian timing system (Bron and Furness, 2009; Hoogerwerf, 2006). Indeed, human behaviour is similar in this regard with inherent differences in the population as to a preference for physical or intellectual activity based on one's innate circadian rhythm, people typically display preferences for activity at either early or later times of day (Oginska, 2011; Bailey and Heitkemper, 2001). This innate circadian phenotype for activity is specific to an individual and is referred to as chronotype (Horne and Ostberg, 1976) that is a genetically determined redistribution (Roenneberg *et al*., 2004).

The distribution of chronotypes are affected by factors such as age (Cavallera and Giudici, 2008; Diaz-Morales and Sanchez-Lopes, 2004), gender (Randler, 2011; Zimmermann, 2011) and geographic location (Borchers and Randall, 2012). However, it is generally accepted that the global population of chronotypes lie between moderately-morning type, intermediates (neither-types) and moderately-evening types with approximately 10% of a given population accounting for extremely morning or evening-types (Kabrita *et al*., 2014; Kudielka *et al*., 2006; Zavada *et al*., 2005; Adan and Natale, 2002). Chronotypes are determined by a variety of endogenous and exogenous

factors (Adan *et al*., 2010), with some evidence that climate, ambient temperature and photoperiod (i.e. 12 hours (hrs) of sunlight and 12 hrs of darkness on the equator) influencing the local distribution of chronotypes (Borchers and Randler, 2012; Tonetti *et al*., 2012; Randler, 2008; Smith *et al*., 2002). Since sunlight is considered the most influential of all zeitgebers directly communicating with the central pacemaker, the suprachiasmatic nuclei, entraining circadian rhythm, it is considered plausible that the length and strength of sunlight exposure and latitude position influences chronotype development (Wright *et al*., 2013; Corbett *et al*., 2012; Duffy and Czeisler, 2008).

Research suggests individual chronotypes tend to partake in sports that are historically competed or practised during periods of the day optimal for their own personal preference. For example, sports which are typically practised and competed in the evening, such as water polo and volleyball, tend to be played by evening chronotypes (Zani *et al*., 1984). Whereas sports that are competed in the morning, usually due to their outdoor nature requiring sufficient daylight, such as distance running (Henst *et al*., 2015; Kunorozva *et al*., 2012), triathlons, cycling (Kunorozva *et al*., 2012) shooting and golf (Zani *et al*., 1984) tend to be performed by more morning chronotypes. Morning chronotypes were also found to be more proficient in golf and shooting, with intermediate chronotypes less so (Rossi *et al*., 1983). Recently, research found that marathon runners tended to be more inclined to be morning chronotypes than evening chronotypes, however this was most probably due to the early event start times (05.15hrs to 08.00hrs) dissuading evening

chronotypes from competing (Kunorozva *et al*., 2012). Additionally, a training effect due to repetitive early morning training and competing may condition individuals, who otherwise may not be morning chronotypes, for exercise in the early morning but this idea remains controversial (Henst *et al*., 2015).

Many physiological parameters essential for sporting performance exhibit a diurnal (time-of-day) or circadian rhythm (Drust *et al*., 2005; Atkinson and Reilly, 1996). Research investigating circadian performance has reported significantly improved evening performance in lung function ability (Goel *et al*., 2015; Goyal *et al*., 2008), isokinetic leg strength (Wyse *et al*., 1994), jump distance (Reilly and Down, 1992), and maximal power (Deschodt and Arsac, 2004).

Chronotypes of an evening-incline phenotype display higher $\dot{V}O_2$max values when testing is completed in the hours of the evening, with a similar trend in morning chronotypes not found (Hill *et al*., 1988). However, no consensus on the effect of chronotype on performance has been met with rowing (Forsyth and Reilly, 2004), treadmill running (Burgoon *et al*., 1992) and cycling (Hill *et al*., 1988) where a chronotype effect on performance at differing times of the circadian day was not found. It has been postulated that these equivocal findings may be multifaceted with a combination of many factors including habitual exercise routines, the experimental time the exercise protocol takes place, masking effects from internal or external factors, gender, age or from a yet to be identified factor (Reilly *et al*., 1997). Chronologic age is considered the most prominent factor effecting chronotype and physiological/sporting performance studies due to chronotype being vastly influenced by age, with

adolescents tending to be evening types (Kim *et al.,* 2002*;* Smith *et al.,* 2002*)* and morning phenotype developing with advancing age (Cavallera and Giudici, 2008; Taillard *et al.*, 2004; Carrier *et al*., 1997).

Recent research by Facer-Childs and Brandstaetter (2015) and Vitale *et al*. (2014) identified that, in addition to individual chronotypes, sleep quality and time since entrainment (awakening) were influential in physiological performance parameters. Similarly, Rossi *et al.* (2015) found differences between chronotype and RPE is affected by the time of day (circadian phase) exercise is undertaken suggesting entrainment to circadin phase plays a crucial role in performance.

In recent years, many studies have examined circadian rhythms and the sleep-wake cycle on peripheral immune parameters (Ackermann *et al*., 2012; Bollinger *et al*., 2009; Dimitrov *et al*., 2009; Benedict *et al*., 2007; Dimitrov *et al*., 2007; 2004; Born *et al*., 1997). Lymphocytes typically peak at night between the hours of 22.00hrs and 02.00hrs (Mazzoccoli *et al*., 2010; Dimitrov *et al*., 2009; 2007; 2004) with CD8+ effector-memory (senescent) subset displaying a diurnal acrophase between mid-afternoon and early evening (Dimitrov *et al*., 2009). Neutrophil cell counts typically peak in the early evening but greater variability in their acrophase has been reported (Selmaoui *et al*., 1998; Dimitrov *et al*., 2007; Haus and Smolensky, 1999; Born *et al*., 1997). It has been suggested that a daytime acrophase in circulating lymphocyte CD8+ effector-memory subset and neutrophils may be an evolutionary legacy to enhance immunosurveillance with cell types that do not require prior antigen exposure primed during times of activity and potential injury or infection, i.e.

the active diurnal phase. Similarly, hormones that modulate immune parameters display circadian patterns, for example cortisol displays a robust awakening response in the morning and daytime acrophase (Lammers-van der Holst and Kerkhof, 2015; Dimitrov *et al*., 2009; Lovallo *et al*., 2006). Importantly, a chrontype-influenced phase-response has been observed with morning chronotypes, rising from sleep and reaching an acrophase in cortisol earlier than evening types.

The aim of this study was to investigate:

1) The association between Morningness-Eveningness Questionnaire (MEQ) score and parameters of physiology at rest and in response to exercise at morning and evening time-points.
2) The association between MEQ score and parameters of the immune system (leukocytes, lymphocytes, CD8+ T-lymphocyte subsets and neutrophils).

6.2 Methodology

Chapter 3 details the comprehensive methods used for this study. Methods specifically used in this study only are described in chapter 4 and briefly below.

6.2.1 Participant Recruitment and Assessment of Chronotype

Seventeen healthy males (mean ± SD: age: 31.5 ± 6.9 years, body mass: 77.2 ± 8.3kg, height 181.9 ± 6.44cm, $\dot{V}O_2max$ 55.8 ± 6.1 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$) were recruited. Participants were divided into two groups based on distance running experience and $\dot{V}O_2max$: recreationally fit (=9, mean ± SD: age: 28.7± 5.5 years, body mass: 78.6 ± 7.4kg, height 183.2 ± 6.4cm, $\dot{V}O_2max$ 52.2 ± 6.4

$mlO_2 \cdot kg^{-1} \cdot min^{-1}$) or experienced (n=10, mean ± SD: age: 34 ± 7.2 years, body mass: 75.9 ± 9.2kg, height 180.7 ± 6.5cm, $\dot{V}O_2max$ 59.2 ± 3.7 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$). All participants were healthy, non-smokers and were not taking any medication in the previous weeks. Each participant completed all prerequisite paperwork and questionnaires as described in detail in chapter 3.

To identify individual chronotypes (i.e. definitely morning-type, moderately morning-type, intermediate (or neither-types), evening-type and definitely evening-type), the Morningness-Eveningness Questionnaire (MEQ) (Horne and Ostberg, 1976) (Appendix 3) was completed and returned by all participants (n=19). Ethical approval was granted by the ethics committee at Edinburgh Napier University and all participants provided written informed consent.

6.2.2 Experimental Procedures

The experimental procedures are described in detail in chapter 3.

Briefly, experimental days consisted of participants arriving at the Human Performance Laboratory at Edinburgh Napier University a minimum of 90 minutes prior to trial commencement (07.30hrs for morning (AM) trial and 15.30hrs for evening (PM) trial). The trial consisted of a 10 kilometer (km) self-paced blinded (no input as to running speed) time-trial run on a motorised treadmill (Woodway, ergo ELG55, Germany) at two different times of the day (09.00hrs and 16.00hrs) on two separate occasions, with a minimum of seven days between trials. The trials were completed in a randomised counter-balanced manner. All participants were healthy, free from injury or infection in

the previous weeks. The trials were performed in an environmental chamber (Weis-Gallenkamp, UK) with the temperature set at a standardised 6°C for the duration of the exercise protocol.

Lung function and blood samples were obtained pre, post and 1hr post, as described in chapter 3. Core body temperature (CBT) was monitored throughout the trial by way of a core temperature (telemetry) pill, which was administered between 90 to 120 minutes prior to exercise-trial commencing.

6.2.3 Lymphocyte Isolation, Labelling and Analysis

Briefly, whole blood was separated, labelled with monoclonal antibodies (MAb) and analysed by FACSCalibur flow cytometry using CELLQuest Pro software (BD Biosciences, San Jose, CA, USA), as described in detail in chapter 3.

6.2.4 Statistical Analysis

SPSS software version 20 (Chicago, IL) was used to complete statistical analysis. Normal distribution was assessed using the Kolmogrov Smirnov or Shapiro-Wilk test (Newell *et al*., 2010). Circadian and chronotype effects on 10km time-trials were evaluated using a repeated-measures analysis of variance (ANOVA) with a repeated-measures time-of-day effect (AM/PM) and a grouping factor for chronotype (evening, morning, neither and/or MEQ_{low} (lowest 50^{th} percentile scoring) or MEQ_{high} (highest 50^{th} percentile scoring)). A one-way ANOVA was used to compare the descriptive characteristics and chronotype scores between experienced and recreational running groups in normally distributed data. Normally disturbed data was analysed using

Pearson's correlation to measure the linear correlation between two MEQ scores and variables. Spearman's rank order correlation was utilised for non-normally distributed data. Statistical significance was recorded as $p<0.05$. All results are presented as mean ±SD unless otherwise stated.

6.3 Results

6.3.1 Study Population Chronotype Demographics

Table 6.1 illustrates chronotype demographics of the study cohort assessed on completion of all three experimental trials using Horne and Ostberg's (1976) Morningness-Eveningness Questionnaire (MEQ). MEQ scores were categorised into morning-type (n=6), neither-type (n=10) and evening-type (n=1). No extreme morning or evening-types were found and 17 of 19 participants returned the MEQ.

Table 6.1. Chronotype, physiological parameters and performance time, values are mean ±SD. p value set at 0.05 significance.

Chronotype	**Age (years)**	**$\dot{V}O_2$**	**Study Group**	**Systolic**		**Diastolic**		**Resting HR**		**Resting CBT**		**10km Time**	
				AM	**PM**	**AM**	**PM**	**AM**	**PM**	**AM**	**PM**	**AM**	**PM**
Morning-type (n=6)	34.8	57.4	Experienced n=3, Recreational n=3	120± 10	122 ± 9	76 ± 6	72 ± 6	67 ± 10	68 ± 8	36.53	36.76	44:36 ± 00:49	44:18 ± 00:50
Neither-type (n=10)	29.4	54.6	Experienced n=5, Recreational n=5	129 ± 7	127 ± 7	76 ± 5	77 ± 7	70 ± 11	71 ± 10	36.41	37.03	49:13 ± 00:85	49:06 ± 00:82
Evening-type (n=1)	38	56.5	Experienced n=1	124	134	71	80	54	57	37.21	36.4	44:22	44:45

As previously reported in Chapter 5, performance time to complete the 10km time-trials were non-significantly quicker in the morning (53.59 minutes, SD± 700s) compared to the evening (54.20 minutes, SD± 710s) in the recreational group (paired sample T-test; $p > 0.05$); and the experienced runner group performed non-significantly quicker in the evening trial (40.38 minutes, SD± 238s) compared to morning (41.09 minutes, SD± 250s) (paired sample T-test; $p > 0.05$), as seen in figure 5.2.

No difference between MEQ score and AM or PM time-trial at 5km or 10km performance was found (Pearson's correlation, $p > 0.05$) (figure 6.1).

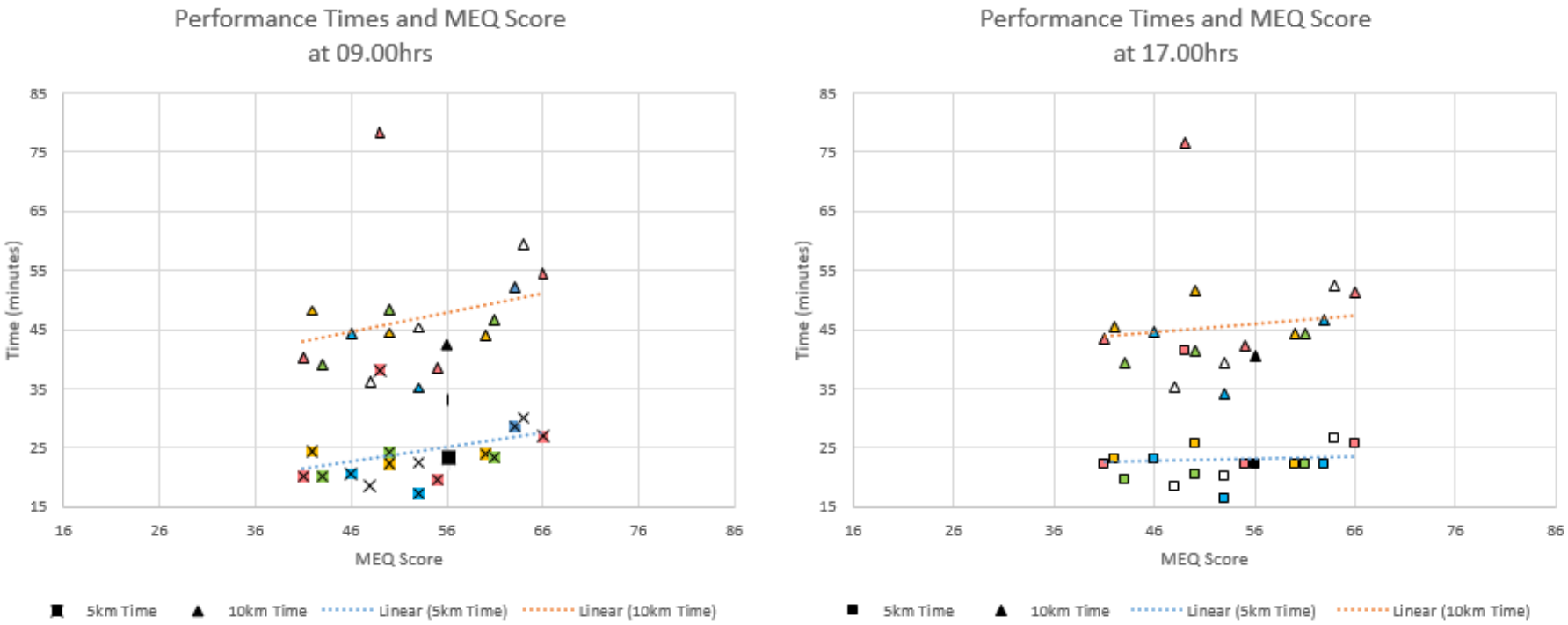


Figure 6.1. Time-trial performance times (y axis) and MEQ scores (x axis) at 5km and 10km stages. MEQ scoring: 16-41 evening-type, 42-58 neither-type, 59-86 morning-type.

Figure 6.2 illustrates a weak association between advancing chronological age and the propensity for greater morning-type MEQ score (Pearson's correlation, $p > 0.05$, $r^2 = .15$).

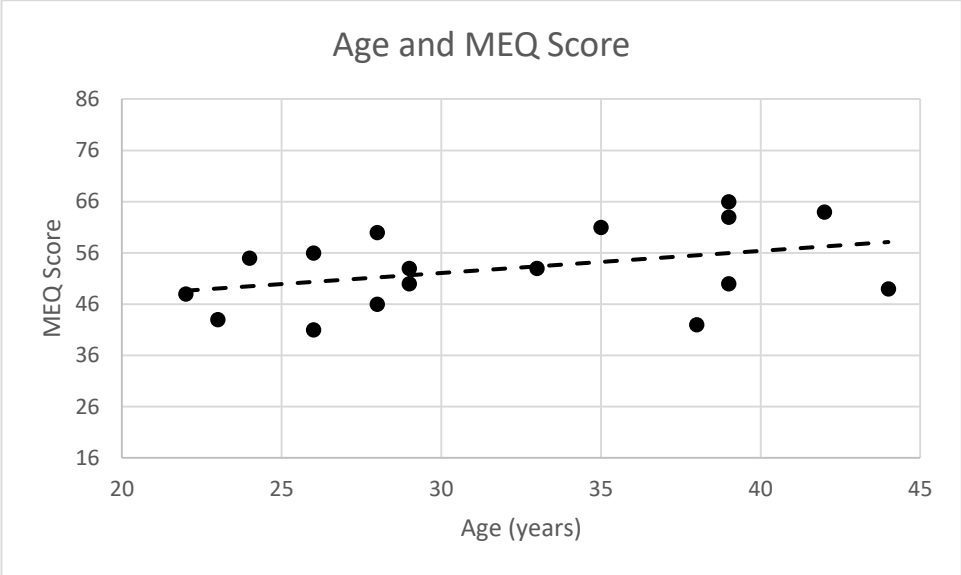


Figure 6.2. MEQ score (y axis) and age (x axis). MEQ scoring: 16-41 evening-type, 42-58 neither-type, 59-86 morning-type.

Chronotype and core body temperature (CBT) are displayed in table 6.2. Morning chronotypes displayed a main effect of time (AM/PM) by exercise interaction and displayed higher resting, exercise and 60 minutes post exercise CBT than neither-types and evening-type at AM testing. Evening-type (n=1) displayed the highest CBT values pre, post and 60 minutes post exercise at PM trial.

Table 6.2. CBT response to AM and PM time-trials and chronotype, values are mean ±SD. p value set at 0.05 significance

Core Body Temperature	Trial	Pre30	Pre	Post	Post60	Main Effects of Time	Main Effects of Exercise	Time x Exercise Interaction
Morning Chronotype	AM	36.53± .37	36.56± .39.	38.18± .74	36.95 ± .49	$F_{(1,5)}$ = 9.252; p = **NS**	$F_{(3,15)}$ = 48.109; p = **.000**	$F_{(3,15)}$ = 5.075; p = 0.008
	PM	36.76± .46	36.78± .54	37± .73	37.21± .19			
Neither Chronotype	AM	36.41± .61	36.44± .66	37.86± .84	36.79± .43	$F_{(1,9)}$ = 6.272; p = **0.032**	$F_{(3,27)}$ = 56.215; p = **.000**	$F_{(3,27)}$ = .175; p = **NS**
	PM	37.03± .48	37.05± .4	38.43± .78	37.23. ± .24			
Evening Chronotype	AM	36.4	36.08	36.72	36.34	N/A	N/A	N/A
	PM	37.21	37.24	38.48	37.34			

Figure 6.3 shows CBT chronotype categories 30 minutes pre, pre, post and 60 minutes post AM and PM time-trials. Morning-types displayed the highest CBT throughout AM testing and lowest at PM testing, while evening-types displayed the highest recorded values during PM testing and lowest at AM testing.

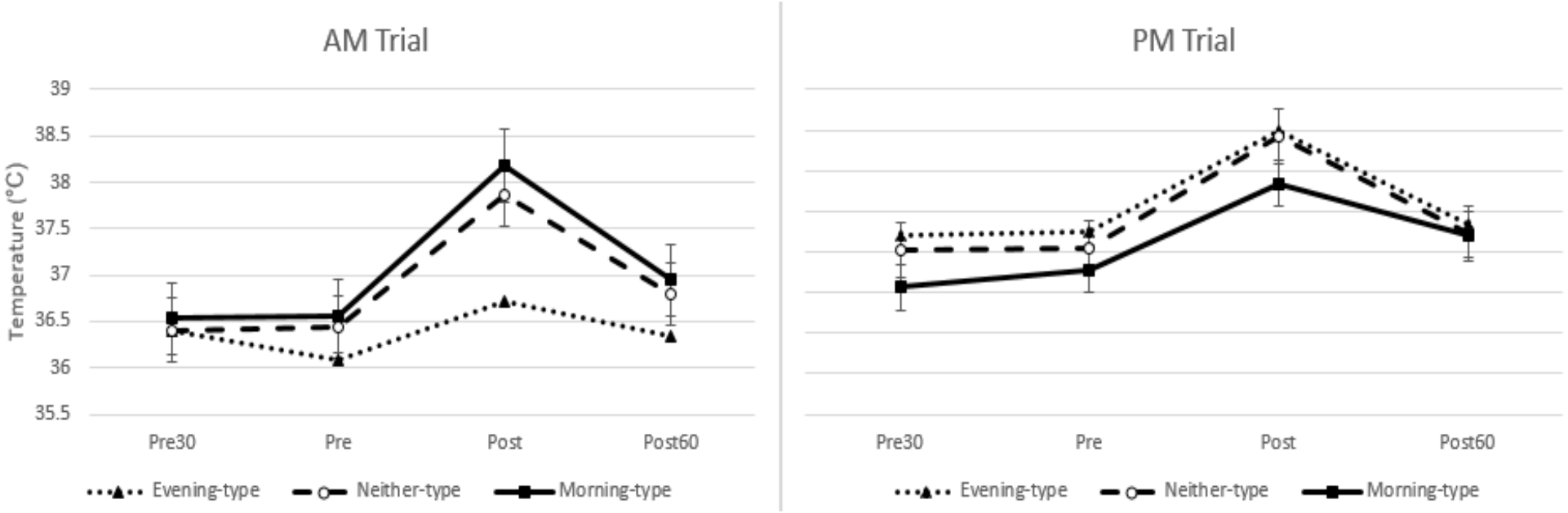


Figure 6.3. CBT and chronotype at AM and PM time-trials.

Percentage change (responsiveness) of CBT at AM and PM time-trials is shown in figure 6.4. Morning-types displayed the greatest percentage increase pre to post exercise at AM while evening-types were more responsive pre to post at PM trial. Neither-types displayed a similar CBT response pre to post and post to post60 at both time-trials.

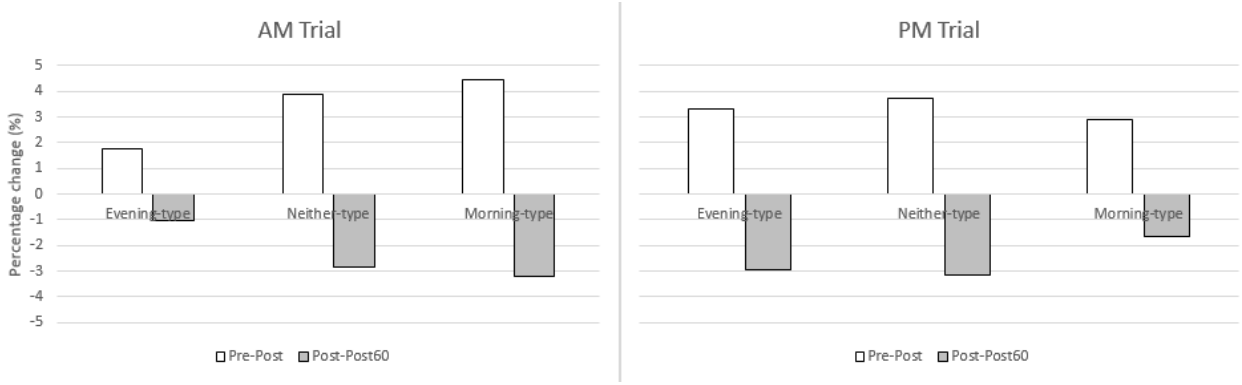


Figure 6.4. Percentage change in CBT at pre to post and post to post60 and chronotype.

Chronotype showed no influence on lung function at AM or PM testing (paired sample T-test $P > 0.05$) or within AM or PM trial time-points (pre, post, post60) (repeated mesure ANOVA yield no time x exercise effect). Data not displayed.

6.3.2 Chronotype and Immune Parameters

Table 6.3 illustrates no significant effect of chronotype and circulating leukocyte subsets (no time by exercise interaction, repeated measure ANOVA p> 0.05).

Table 6.3. Leukocyte subset response to AM and PM time-trials and chronotype, values are mean ±SD. p value set at 0.05 significance.

Cell Type (cells/µl)	Trial	Pre	Post	Post60	Main Effects of Time	Main Effects of Exercise	Time x Exercise Interaction
Leukocytes Morning Chronotype	**AM**	5.24 ± 1.05	8.91 ± 2.28	8.88 ± 3.16	$F_{(1,5)}$ = 2.31; p = **NS**	$F_{(2,10)}$ = 16.888; p = **.001**	$F_{(2,10)}$ = .272; p = **NS**
	PM	6.95 ± .89	10.98 ± 3.32	11.88 ± 2.31			
Leukocytes Neither Chronotype	**AM**	4.7 ± 1.1	8.01 ± 1.7	6.18 ± 1.67	$F_{(1,9)}$ = 15.557; p = **.003**	$F_{(2,18)}$ = 36.722; p = **.000**	$F_{(2,18)}$ = .236; p = **NS**
	PM	6.41 ± .89	10.25 ± 1.85	8.32 ± 2.32			
Leukocytes Evening Chronotype	**AM**	4.57	6.62	6.69	N/A	N/A	N/A
	PM	5.33	7.85	6.78			
Neutrophils Morning Chronotype	**AM**	2.69 ± .7	4.39 ± 1.1	6.7 ± 2.9	$F_{(1,5)}$ = 3.651; p = **NS**	$F_{(2,9)}$ = 31.208; p = **.000**	$F_{(2,9)}$ = .753; p = **NS**
	PM	4.07 ± .55	6.1 ± 2.18	8.9 ± 1.74			
Neutrophils Neither Chronotype	**AM**	2.62 ± 1.05	3.88 ± 1.25	4.7 ± 1.4	$F_{(1,9)}$ = 6.493; p = **0.031**	$F_{(2,18)}$ = 22.251; p = **.000**	$F_{(2,18)}$ = .425; p = **NS**
	PM	3.76 ± .91	4.89 ± 1.58	6.14 ± 2.38			

Neutrophils Evening Chronotype	**AM**	2.63	3.56	5.37	N/A	N/A	N/A
	PM	2.96	3.52	4.96			
Lymphocytes Morning Chronotype	**AM**	1.54 ± .34	3.06 ± 1.41	1.23 ± .42	$F_{(1,5)}$ = 1.072; p = **NS**	$F_{(2,10)}$ = 18.73; p = **.000**	$F_{(2,10)}$ = .114; p = **NS**
	PM	1.97 ± .44	3.67 ± 1.54	1.61 ± .55			
Lymphocytes Neither Chronotype	**AM**	1.41 ± .28	3.32 ± 1.17	1.18 ± .28	$F_{(1,9)}$ = 3.841; p = **NS**	$F_{(2,18)}$ = 91.627; p = **.000**	$F_{(2,18)}$ = .525; p = **NS**
	PM	1.78 ± .53	4.08 ± 1.26	1.49 ± .32			
Lymphocytes Evening Chronotype	**AM**	1.19	2.19	.74	N/A	N/A	N/A
	PM	1.61	3.23	1.12			

No relationship between MEQ score and circulating leukocytes was discovered at 09.00hrs or 17.00hrs, figure 6.5.

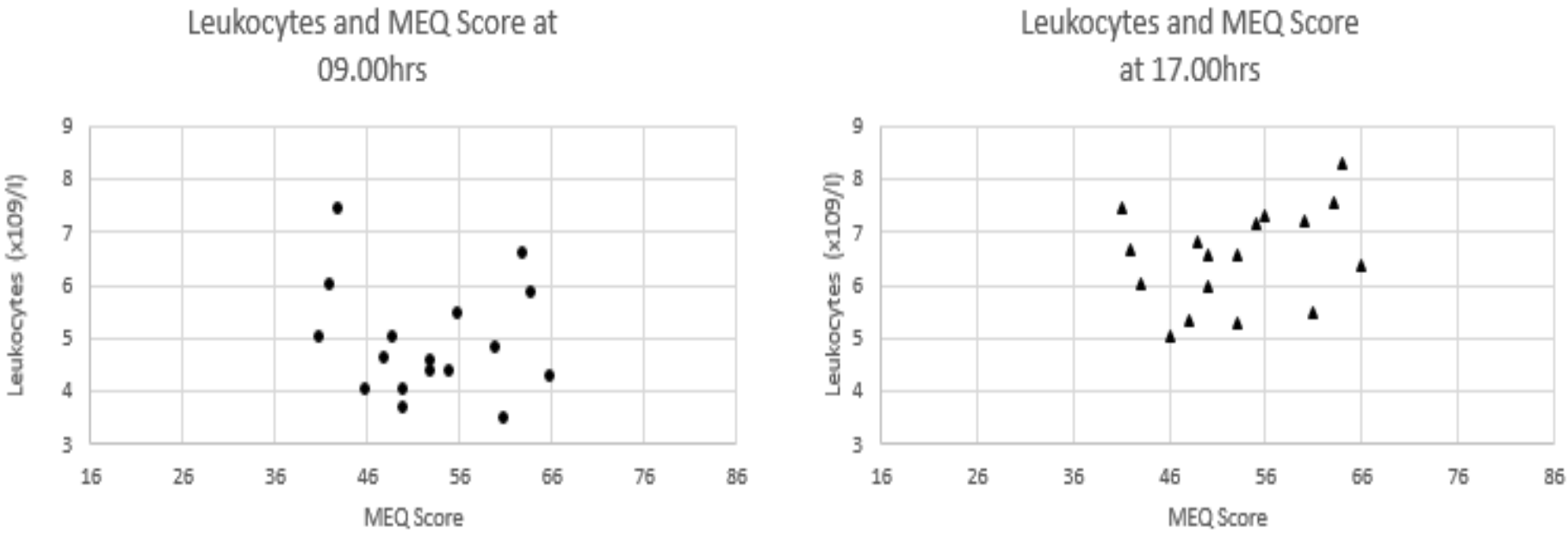


Figure 6.5. Resting leukocyte (y axis) cell counts (x10^9/l) at 09.00hrs and 17.00hrs and MEQ (x axis). MEQ scoring: 16-41 evening-type, 42-58 neither-type, 59-86 morning-type.

MEQ score displayed no infuence on circulating neutrophils at 09.00hrs or 17.00hrs, figure 6.6.

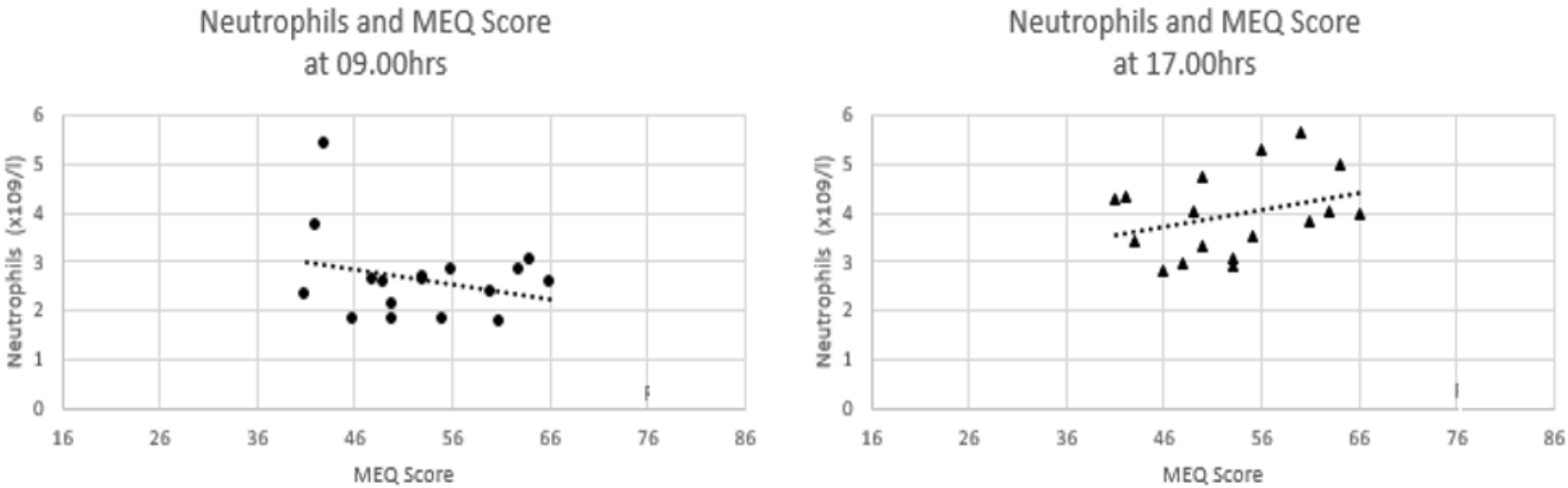


Figure 6.6. Resting neutrophil (y axis) cell counts (x10^9/l) at 09.00hrs and 17.00hrs and MEQ (x axis). MEQ scoring: 16-41 evening-type, 42-58 neither-type, 59-86 morning-type

6.3.3 Chronotype and Cortisol Concentrations

As illustrated in figure 5.9, peripheral cortisol concentrations for the total study population (n=12) irrespective of study group (experienced or recreational) or chronotype (morning, neither, evening-types) was significantly different due to exercise but no main effect of time-of-day (AM or PM) by exercise interaction was observed. Experienced (n=7) and recreational (n=5) group cortisol concentrations, displayed in figure 5.9, report similar values between groups (experienced runners and recreational runners) at AM (09.00hrs). Experienced runners displayed a continued increase from post-exercise to post 60 minutes while the recreational group displayed a biphasic cortisol response.

Figure 6.7 illustrates peripheral cortisol response in morning chronotypes and neither chronotypes to 10km time-trials at AM and PM. No evening chronotype serum was sampled.

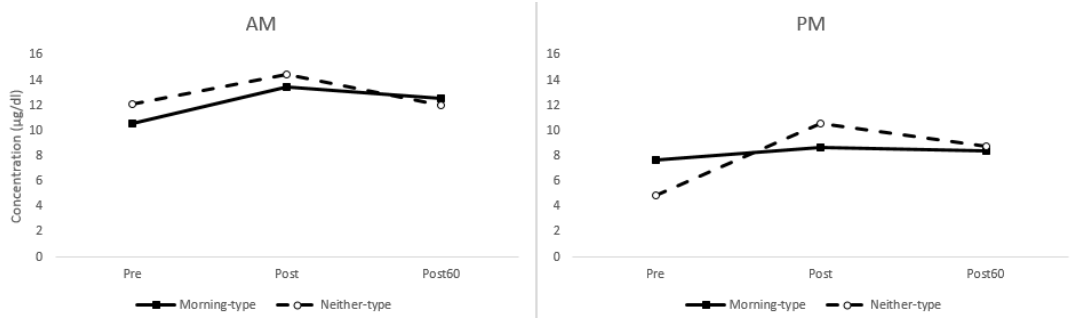


Figure 6.7. Peripheral cortisol concentrations pre, post and post60 at AM and PM time-points in morning-chronotypes (n=4) and neither-chronotypes (n=7). Values are mean ±SD. p value set at 0.05 significance.

6.4 Discussion

This study investigated chronotype using Horne and Ostberg's (1976) Morningness-Eveningness Questionnaire (MEQ), to categorise into morning-types, neither-types or evening-types on MEQ score. Chronotype has been shown to influence circadian phase response (Facer-Childs and Brandstaetter, 2015a; 2015b; Novakova *et al*., 2013; Brown *et al*., 2008; Drust *et al*., 2005; Forsyth and Reilly, 2004; Roenneberg *et al*., 2003). Within the participant population, six were morning-types, one was an evening-type and ten were neither-types. The main findings of this present study, although not reaching statistical significance, suggest morning chronotypes performed a 10km time-trial quicker in the morning than in the evening. This finding is in agreement with similar research (Brown *et al*., 2008). Recent work has suggested that a disproportionate number of individual endurance athletes were more likely to be morning-types compared to age matched controls, with a training effect of habitual early morning activity or a leaning to sports conducted early in the day (Henst *et al*., 2015). This current study found that not only did morning-types perform better in the morning but they also displayed quicker times in the evening. Potentially, this may be due to a number of reasons including experience of training at morning and evening times of the day or the evening trial undertaken too early to be detrimental to performance. Higher $\dot{V}O_2$max values were found in the morning-type (57.4%) compared to neither-type (54.6%). This may give weight to the assertion that morning-types trained habitually at differing diurnal time-points, were aerobically fitter and were more inclined to distance running as reported (Henst

et al., 2015). However, due to the sample size of this current study, statistical power is lacking and no definitive assumption can be made.

Furthermore, previous chronotype and athletic performance studies using trained (Atkinson *et al*., 2005; Hill *et al*., 1988) and untrained participants (Forsyth and Reilly, 2004) reported no chronotype influenced differences. Potentially, it may be hypothesised that extreme chronotypes that account for approximately 10% of the population (Kabrita *et al*., 2014; Kudielka *et al*., 2006; Zavada *et al*., 2005) may be more susceptible to performance decrements when exercise is undertaken out-with their preferred circadian phase. Forsyth and Reilly (2004) found that one extreme evening-type performed a rowing task optimally at 02.00hrs, which supports the assertion that, as for the case of this study, two time-points may be inadequate to thoroughly investigate chronotype-performance relationship.

Core body temperature (CBT) displayed a greater increase from pre to post-exercise in the morning-type cohort during the AM trial, increasing by 1.65°C or over 4%. Considering CBT remains relatively stable between ~36.5°C and ~37.5°C depending on circadian phase and values of ~35°C and ~40°C are a threat to mortality, a 4% rise is of relative significance. Similarly, the one evening-type showed a greater increase in CBT at the PM trial, with a similar pattern observed in both trials in the neither-type group. An exercise-induced rise in CBT is well established in literature (Edwards *et al*., 2013; Benloucif *et al*., 2005; Waterhouse *et al*., 2004; Reilly *et al*., 1997) with greater increase to morning exercise (Aldemir *et al*., 2000). Irrespective of chronotype, a ‘heat

gain' mode (as described by Aldemir *et al*., 2000) was seen in all chronotype categories in this present study.

Leukocyte subsets showed no chronotype effect. An increase in neutrophils at PM trial (17.00hrs) is most likely due to their own innate circadian acrophase (Ackermann *et al*., 2012) and not due to a chronotype-phase advancement mechanism (in morning-types), although it may be advantageous to explore chronotype as a zeitgeber in the future.

An awakening response in cortisol concentrations (Lammers-van der Holst and Kerkhof, 2015; Dimitrov *et al*., 2009) was seen at AM trials irrespective of group status (experienced or recreational) or chronotype. This response was anticipated as cortisol reaches peak levels in the morning and the early part of afternoon which may negate, if any, influence chronotypes present.

In conclusion, chronotype appears to have limited effect on an array of physiological and biological parameters. Factors such as age and habitual exercise routines may limit the effect of chronotype or alter it, posing difficult methodological issues for researchers to overcome. A wide and varied study demographic, multiple testing points equally distributed throughout a 24-hour day (minimum of six) and an appropriately matched control group would be advantageous to investigate chronotype influence on physiology, biology and effect on circadian phase.

Chapter 7: General Discussion

This chapter summarises the key findings of the studies undertaken, a general discussion of these results and wider implications for the disciplines of chronobiology, exercise immunology and exercise physiology. Finally, future research directions and study limitations will be discussed.

7.1 Findings

The studies undertaken for this thesis primarily aimed to investigate the effect of exercise and diurnal phase on immune and physiology parameters. Data was collected from highly-trained, experienced male distance runners and recreationally healthy males at morning and evening time-points. Each aim and its results are summarised as follows:

Aim 1: To examine if the diurnal phase exercise is undertaken at influences macro physiology parameters, including rate of perceived exertion (RPE), heart rate (HR), core body temperature (CBT) and exercise performance in trained male distance runners (chapter 4).

Main findings:

1. A significant elevation in CBT was observed at rest and early into a self-paced 10 kilometre (km) time-trial (1km stage) in the evening, confirming circadian rhythmicity but exercise attenuated time-of-day differences in CBT.
2. No differences in HR or RPE were observed in morning or evening exercise.
3. Performance was non-significantly quicker in evening exercise in comparison to morning exercise.

Hypothesis: Parameters of physiology display circadian variation with a divergent diurnal phase response to morning and evening exercise (*reject*).

Aim 2: To determine whether a relationship between diurnal phase and chronotype classification may effect physiological responses (chapter 6).

Main findings:

1. Chronotype was not associated with different physiological responses to exercise.
2. Chronotypes separated by morning or evening orientation (MEQ_{low}/MEQ_{high}) performed the time-trial run non-significantly quicker at their performed time of day.

Hypothesis: Chronotype score and categorisation (based on the Morningness-Eveningness questionnaire scoring) would significantly impact exercise performance and physiology (*reject*).

Aim 3: To assess lung function in response to an acute bout of self-paced high-intensity exercise in a cold environment (6°C) at morning and evening time-points (chapter 4).

Main findings:

1. Lung function was not affected by exercise in the cold.
2. No diurnal effect on lung function was observed.

Hypothesis: Lung function would be negatively affected by an acute bout of self-paced high-intensity exercise in a cold environment (6°C) and diurnal variation (*reject*).

Aim 4: To investigate immune (leukocytes and lymphocyte subsets) and endocrine (catecholamines and cortisol) parameters in response to high-intensity exercise and diurnal phase (chapter 4).

Main findings:

1. A significant interaction between time of day and exercise was observed in leukocytes.
2. This time by exercise response in leukocytes was driven by neutrophils.
3. Lymphocytes displayed a significant effect from exercise and time but no interaction between both variables.
4. T-lymphocyte subsets with naïve and experienced cell surface markers displayed an exercise response; experienced RAEM subsets (CD8+CD27-CD45+) increased in percentage terms immediately post-exercise while concomitant to this, naïve (CD8+CD27+CD45RA+) subsets decreased in percentage, but no diurnal effect was observed.
5. Cortisol and adrenaline concentrations in peripheral blood increased in response to exercise but no time by exercise effect was observed.

Hypothesis: Leukocytes (*accept*), lymphocyte/lymphocyte subsets (*reject*) and catecholamines and cortisol (*reject*) would be effected by high-intensity exercise and diurnal phase.

Aim 5: To investigate training status and diurnal phase on exercise response and physiological parameters (chapter 5).

Main findings:

1. Physiological parameters CBT, HR, RPE and lung function displayed effect of time-of-day and exercise response.
2. No within group differences (recreational and experienced) were observed in physiology.

Hypothesis: The diurnal phase exercise is undertaken at would result in a differential physiological response in recreational and experienced distance runners (*reject*).

Aim 6: To investigate neutrophil and lymphocyte responses to exercise at diurnally different time-points in recreational and experienced distance runners (chapter 5).

Main findings:

1. Both experimental groups displayed neutrophilia post and one hour post-exercise. Recreational runners displayed greater elevation in neutrophils. Lymphocytes displayed a biphasic response in both experimental groups.
2. Despite a greater amplitude in response to evening exercise in both experimental groups (neutrophils and lymphocytes), no time by exercise interaction was observed.

Hypothesis: A significant diurnal response to exercise would be observed in recreational and experienced runners (*reject*).

Aim 7: To examine the effect of exercise and diurnal phase on CD8+ T-lymphocyte subsets of early, intermediate and late phenotypes in experienced and recreational runners (chapter 5).

Main findings:

1. Recreational runners displayed a higher percentage of CD8+ late differentiated (CD8+CD27-CD28-) phenotypes than experienced peers. Both experimental groups saw an increase in the percentage of late differentiated phenotypes post-exercise with the percentage of early differentiated CD8+ phenotypes (CD8+CD27+CD28+) returning to pre-exercise levels one hour post-exercise.
2. No significant diurnal effect in the percentage of early, intermediate (CD8+CD27+CD28-) or late CD8+ subsets was observed.

Hypothesis: Late differentiated CD8+ subsets would be preferentially mobilised in response to evening exercise and training status would have a significant effect on this response (*reject*).

Aim 8: To investigate the effect of exercise and diurnal phase on lung function ability in recreational and experienced runners in cold environmental conditions (chapter 5).

Main findings:

1. An exercise effect in peak expiratory flow (PEF) and forced vital capacity (FVC) was observed.
2. No diurnal interaction with exercise was discovered.

Hypothesis: Lung function in response to exercise would be subject to circadian rhythms with higher capabilities in the evening (*reject*) and recreational runners would report significant impairment of lung function and diurnal phase (*reject*).

Aim 9: To assess whether a cortisol response to exercise trial would be influenced by training status (chapter 5).

Main findings:

1. Cortisol concentrations in experienced runners were lower one hour post-exercise.
2. Higher cortisol concentrations were found in both experimental groups at rest and in response to exercise in the morning.

Hypothesis: Cortisol concentrations in peripheral blood displays a circadian pattern with higher values in the morning (*accept*) and cortisol concentrations would respond differently according to training status (*accept*).

Aim 10: To investigate the association between Morningness-Eveningness Questionnaire (MEQ) score and parameters of physiology at rest and in response to exercise at morning and evening time-points (chapter 6).

Aim 11: To investigate the association between MEQ score and parameters of the immune system (leukocytes, lymphocytes, CD8+ T-lymphocyte subsets and neutrophils) (chapter 6).

Main findings:

1. Neither MEQ score nor chronotype category was associated with physiology at rest or in response to morning and evening exercise in recreational or experienced runners.
2. Neither MEQ score nor chronotype category was associated with leukocyte subsets at rest or in response to morning and evening exercise in recreational or experienced runners.

Hypothesis: MEQ score, due to known advancement of circadian phase, would significantly influence physiological and immunological parameters at rest and in response to exercise (*reject*).

The main findings of this thesis suggest that the effect of high-intensity exercise at diurnally different time-points (morning and early evening) on immune parameters is equivocal, with lymphocytes and neutrophils displaying distinct responses. The intensity and duration of exercise, the training status of the exerciser and resting immune values appear to influence exercise-induced immune response as described extensively in previous literature (Witard *et al*., 2012; Campbell *et al*., 2009; Simpson *et al*., 2009; 2008; 2007). Lymphocytes and CD8+ lymphocyte subsets displayed a biphasic response to exercise in recreational and experienced runners, while neutrophils increased immediately post-exercise with this neutrophilia continuing one hour into the recovery process. Although not reaching statistical significance, a greater amplitude in neutrophil cell counts was observed one hour post-evening testing with recreational runners reporting the highest increase. It appears

unlikely that this inflammatory response is due to the early stages of infection or stimulation from pathogen. However, the mechanism driving this inflammatory response can only be speculated with circadian associated factors such as a change in hormone concentrations, oxidative stress due to muscle damage, pro- and anti- inflammatory cytokine environment, or core body temperature being likely factors to consider. Alternatively a yet to be discovered factor, such as molecular clock gene expression in neutrophils/immune cells may be pivotal in understanding this response to a stress stimulus. Recently, Geiger *et al*. (2015) proposed a model of how the circadian clock, through the influence of zeitgebers, regulates the immune system (figure 7.1).

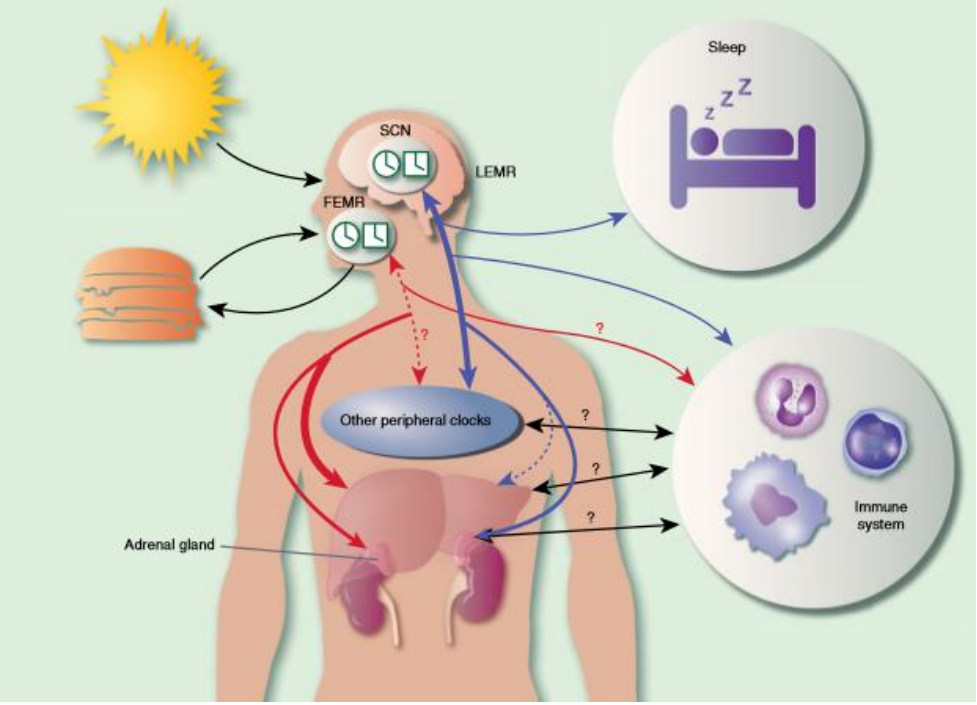


Figure 7.1. The body clock. Zeitgebers, such as food and light, entrain master regulators, which communicate with peripheral clocks in a bi-directional fashion to sustain clock alignment. Light stimuli are processed in the light-entrained master regulator (LEMR) in the suprachiasmatic nucleus (SCN), whereas food signals are conveyed to the food-entrained master regulator (FEMR), whose location is not yet known. LEMR regulates sleep and immune response, as well as other peripheral clocks (blue arrows). FEMR strongly controls the liver clock among others (red arrows). The question marks indicate a proposed, but not yet proven, interplay and the black arrows represent general crosstalk. Round clocks indicate cellular clock involvement, whereas rectangular clock symbols reference a system that is independent of the molecular clock (taken from Geiger *et al*., 2015, pp.355).

Recently, inflammatory monocytes, which are similar to neutrophils, are phagocytes and part of the innate immune system, have been shown to be regulated by the circadian gene BMAL1 (Nguyen *et al*., 2013). Potentially, neutrophils' daytime acrophase may be due to a similar circadian clock gene mechanism which may explain a phase response to evening exercise. Inflammation is tightly regulated within the immune system with pro-inflammatory cytokines displaying a nocturnal acrophase whereas anti-inflammatory cytokines peak during the diurnal phase (Spengler and Kuropatwinski, 2012; Petrzilka *et al*., 2009). Although cytokines were not examined as part of this thesis, their role in inflammation and circadian secretory patterns should not be ignored. Exercise is generally considered to increase adrenaline and cortisol peripheral concentrations and promote anti-inflammatory cytokines such as IL-1ra, IL-6 and IL-10 and suppress pro-inflammatory cytokines like TNF- α and IL-1β (Gleeson, 2013). Similarly, a biochemical marker of oxidative stress and muscle damage, homocysteine, has been shown to display a distinct circadian profile in response to high-intensity anaerobic exercise of a short duration and at rest (Hammouda *et al*., 2012; 2011). It was postulated by the authors (Hammouda *et al*. 2012; 2011), as a result of an augmentation of muscle damage (as seen with increased evening homocysteine levels) and as a consequence of the counteracting effect of antioxidants reaching an acrophase during the hours of the morning, a resultant exaggeration in inflammation occurs. Although, homocysteine response to exercise at differing diurnal time points and high-intensity anaerobic exercise was not investigated as part of this thesis, therein lies the

possibility that a similar biological environment conducive to an inflammatory response may be present in the evening resulting in the neutrophil response of recreational and experienced runners alike one-hour post PM exercise.

The immune system is a complex network of cells with each subset displaying robust circadian rhythm. Lymphocyte subsets typically display nocturnal acrophase, however CD8+ T-lymphocyte subsets of an experienced phenotype (CD27-CD45RA+) peak during the diurnal phase. Similarly, neutrophils peak during the day at approximately 16.00hrs (Ackermann *et al*., 2012). Both these cytotoxic cell types displayed the greatest mobilisation in response to diurnal exercise stimulus. Similar findings have been reported in the literature in response to exercise (Turner *et al*., 2010; Campbell, *et al*., 2009; 2008; Simpson *et al*., 2008; 2007) and psychological stress (Anane *et al*., 2009; Bosch *et al*., 2009). The majority of this research has been conducted in either the morning or early afternoon and not at an evening time-point. As such, the rejection of the hypothesis concerning a differential mobilisation of CD8+ T-lymphocytes is a novel finding.

Adrenaline has previously been shown to increase above-resting values pre-exercise, in response to exercise and for up to five minutes from exercise cessation (French *et al*., 2007). Furthermore, adrenaline displays a distinct circadian rhythm (Dimitrov *et al*., 2009) and is known to decrease cellular adhesion (Benschop *et al*., 1996) promoting the redistribution of cell expression high density of β2 adreno-receptors for example cytotoxic lymphocytes and L-Selectin expression on neutrophils (Miles *et al.*, 1998). Chapter 4 investigated adrenaline response to 10km time-trials in

cardiovascularly-trained men and found an exercise-induced increase immediately post-exercise but no time of day effect. Previous findings have reported that training status does not affect the magnitude of adrenaline response to exercise with values quickly diminishing towards pre-exercise concentrations (Kraemer *et al*., 1999). Furthermore, the effects of an exercise-induced increase in peripheral cortisol concentrations typically display a lag of between two to three hours depending on exercise intensity and duration (Nieman *et al*., 1994; McCarthey *et al*., 1992). Concomitantly, adrenaline rapidly recruits effector memory T-lymphocytes post-exercise and in the morning from the marginal, cortisol increases the number of circulating naïve and memory T-lymphocytes in the blood (Dimitrov *et al*., 2009). The delay in cortisol response to exercise and the finding of divergent responses of neutrophils one hour post-exercise in chapter 5 suggests that cortisol may be influential, especially considering the short half-life of adrenaline, two to three hour delay in cortisol response to exercise and the robust morning acrophase of cortisol (Lovallo *et al*., 2006). Furthermore cortisol and adrenaline have been used as proxies to link involvement of circadian rhythms in immune cells (Dimitrov *et al*., 2009).

Previous research has shown that physiological measures and cells of the immune system display distinct circadian rhythms. The findings from this thesis found that diurnal phase, as assessed at 09.00hrs and 16.00hrs/17.00hrs does not affect exercise-induced responses. Chapter 4 sought to investigate physiological responses in experienced distance runners. CBT was significantly different between at rest and at 1km stage of

the 10km time-trial run at AM (09.00hrs) and PM (16.00hrs) time-points. This finding is in agreement with previous literature (Atkinson *et al*., 2015; Reilly *et al*., 1997), however no significant difference was observed for the subsequent stages of exercise trial suggesting that high-intensity exercise negates time-of-day differences in CBT. Similarly, Hammouda *et al*. (2011) found an increase in markers on inflammation and core body temperature in the evening. Additional physiology parameters of heart rate and rate of perceived exertion (RPE) demonstrated an effect due to exercise but no interaction with time-of-day (AM or PM) in agreement with previous findings (Faria and Drummond, 1982). These findings, combined with no diurnal or exercise effect found in lung function ability, suggest that the physiology of highly trained ($\dot{V}O_2max$ >60ml$O_2 \cdot kg^{-1} \cdot min^{-1}$) habitual distance runners is unaffected by morning or evening exercise in cold environmental conditions possibly due to conditioning/training effect. It is common practise for competitive runners to train and race at varying times of the day; races frequently start earlier than the 09.00hrs trial start time implemented in this study, with modest adjustments in circadian phasing previously elucidated (Piercy and Lack, 1988). Furthermore it is common place for distance runners to train in the early evening after work, social, family commitments and other are fulfilled. Taking these factors into consideration, a non-significant improvement in performance at PM trial is reasonable.

Dietary or nutritional status have previously been proposed as a main driver of circadian variation in human performance and physiology (Youngstedt and O'Connor, 1999). Evidence does not support this assertion with an abundance

of literature reporting nutrition status as a weak zeitgeber (Waterhouse, 2010; Reilly *et al*., 1997). Anaerobic exercise, which is less reliant on nutritional intake than aerobic activity, displays distinct time-of-day effects on performance (Abedelmalek *et al*., 2013). Similarly, decreased morning exercise capabilities have also been linked to immobility during the hours of nocturnal sleep limiting performance (Youngstedt and O'Connor, 1999), however no improvement in morning performance was found despite the implementation of a thorough warm up (Atkinson *et al*., 2005).

Finally, chronotype did not yield a statistically significant effect on biologic or physiologic parameters. This may be due to a number of reasons; firstly low statistical power due to an inadequate data set; secondly, the experimental times were not 'extreme' enough, i.e. on awakening or pre-nocturnal sleep; thirdly, due to experienced and recreational participants being habitually active at the times the trial were conducted at resulting in pre-conditioning. When chronotype scores were divided according to mid-scoring ranges, a trend of improved performance was observed with evening-orientated runners quicker in the evening and *vice versa* with morning-orientated.

7.2 Limitations of the Studies Comprised within this Thesis

One of the key limitations of the studies contained in this thesis lies in the timing of the experimental times. It would have been advantageous, if possible, to conduct the experimental trials earlier and later in the day respectively. To investigate chronotype and circadian rhythm, a larger study cohort and six evenly separated trials over a 24-hour period (on separate days) would allow

for MESOR, acrophase and nadir time-points to be identified as used previously (Forsyth and Reilly, 2004). In addition, lung function, which has previously been shown to display a circadian rhythm (Goel *et al*., 2015) could be optimally assessed at multiple times of the day.

Furthermore, if such a circadian study was completed, a greater understanding of immune response to exercise would be revealed, including the effect of exercise on naïve lymphocyte subsets during the period of their acrophase (00.00hrs to 02.00hrs) and the effect of exercise on cortisol and melatonin concentrations during the nocturnal phase.

Additionally, the inclusion of a recreationally active group presented limitations as a wide variation in performance times and aerobic fitness levels were observed in this group. A sedentary group would help to avoid issues around cross-over in fitness levels between participant and groups. A control group would have provided an insight into diurnal phase at rest which would strengthen the study's internal validity.

Finally, screening participants for cytomegalovirus (CMV) would also have been useful as CMV has been shown to increase senescent and late-differentiated CD8+ subsets (Turner *et al*., 2010; Colonna-Romano *et al*., 2007; Koch *et al*., 2007). This study used a cross-over design with each participant controlling for themselves, however the number of CMV+ individuals between groups (chapter 5) would allow for a confident analysis between groups.

7.3 Future Research

Future research should look to explore diurnal variations at greater divergent time-points, i.e. at earlier and later times of the day. Exercise, and especially competitive races, commonly take place in the early morning with high-intensity training sessions regularly undertaken late into the evening. The effect of such exercise stress on neuroendocrine-immune parameters may have implications on immunity and circadian rhythmicity in many biological systems. Furthermore, further investigation into whether specific populations (such as chronological age, gender, morbidity) are affected by the diurnal phase exercise is undertaken at may be warranted.

In addition, the effect of exercise at differing diurnal phases and training status warrants further investigation. This would be to conclusively determine whether a training effect or conditioning occurs, limiting neutrophilia and inflammation as demonstrated in recreational runners in this thesis. Similarly, the extent of the anti-inflammatory effect of exercise into the nocturnal phase, where pro-inflammatory cytokines reach acrophase, in response to late evening exercise requires more consideration. The effect on peripheral T-lymphocyte subsets, especially naïve phenotypes, in response to a bout of evening exercise would provide greater insight as to their response at a time of peak circulating cell numbers also.

The monitoring of core body temperature over a full circadian cycle, incorporating pre and post-exercise periods, would also provide further clarification as to potential correlations between physiology and biology, including chronotype, physiological performance, exercise recovery and sleep.

Finally, regular exercise of an appropriate intensity is known to have beneficial effects on health. However, considering the finely-tuned nature of the body's internal timing system, it would be advantageous to determine whether there is an optimum time of day for activity.

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Appendices

Appendix 1: Research Participant Information Sheet

Supervisors: Dr Geraint Florida-James, Dr Eva Malone, Edinburgh Napier University

Protocol Title: The effect of acute exhaustive aerobic exercise on circulating blood T lymphocytes and catecholamine, at two diurnal time points in high level endurance runners.

Investigator: *Peter Tormey*

You are asked to voluntarily participate in a research study that aims to examine the effect of aerobic endurance exercise influences circulating blood T lymphocytes.

Your participation will involve completing a self-paced 10km time-trial. The testing will take place on two separate days, and at either one of two time-points, namely, at 9am or 4pm. The exercise protocol will take place in a temperature controlled environmental chamber on a treadmill. A blood sample will be taken before and after the exercise trial by a trained technician, which may result in brief discomfort. You will be told about any new information that might change your decision to be in this study. You may not receive a direct benefit if you agree to participate. However, people in the future may benefit from the information obtained from this research.

Contact Peter Tormey at p.tormey@napier.ac.uk for questions about the research.

All data/information about you that will be collected for this research will be stored on a secure, password-enabled USB storage device. All participants' information will be confidentially stored using abbreviations, numerical codes or pseudonyms. On completion of the study, the collected data will be destroyed appropriately. Data/information may be shared with Edinburgh Napier University Sport and Exercise Science staff, to oversee and monitor the conducted research.

This permission will not end unless you cancel it. You may cancel it by sending written notice to Peter Tormey (by the above means).

Your decision to be in this study is voluntary. You will not be penalised if you decide not to participate or if you decide to stop participating. Your part in this study may be stopped at any time by the researcher without your consent for any of the following reasons:

- if it is in your best interest;
- you do not consent to changes made in the study plan;
- you do not adhere to the criteria required;
- or for any other reason.

Signed Date

Appendix 2: Study Consent Form

School of Life Sciences,

Sport and Exercise Science,

Edinburgh Napier University.

Study participant reference number: _____

Title: The effect of acute exhaustive aerobic exercise on circulating blood T lymphocytes and catecholamine, at two diurnal time points in high level endurance runners.

Principal Investigator: Mr Peter Tormey **Please initial to indicate agreement**

1. I confirm that I have read and understood the information sheet dated _____, for the above study and have had the opportunity to ask questions [ ].

2. I understand that my participation in this study is voluntary and that I am free to withdraw at any time, without giving any reason [ ].

3. I understand that data collected during the study may be looked at by responsible and authorised personnel from the study. I give permission for these individuals to have access to this information [ ].

4. I understand that data collected may be looked at by responsible representatives from the sponsor (Edinburgh Napier University) for the purposes of monitoring and auditing to ensure that the study is being conducted proper manner. I give permission for these individuals to have access to relevant information. [ ]

5. I agree to take part in the above study [ ].

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Researcher/Investigator	Date	Signature

Appendix 3: Morningness-Eveningness Questionnaire

A SELF-ASSESSMENT QUESTIONNAIRE TO DETERMINE MORNINGNESS-EVENINGNESS IN HUMAN CIRCADIAN RHYTHMS

International Journal of Chronobiology, Vol. 4, 96-110, (1976) Gordon and Breach, Science Publishers Ltd.
J.A. Horne and O. Ostberg
Department of Human Sciences, University of Technology, Loughborough, Leicestershire, LE11 3TU, ENGLAND and Department of Occupational Health, National Board of Occupational Safety and Health, Fack, S-100, 26 Stockholm, SWEDEN

Scoring

For questions 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16 and 19, the appropriate score for each response is displayed beside the answer box.

For questions 1, 2, 10 and 18, the cross made along each scale is referred to the appropriate score value range below the scale.

For question 17, the most extreme cross on the right hand side is taken as the reference point and the appropriate score value range below this point is taken.

The scores are added together and the sum converted into a five point Morningness-Eveningness scale:

	Score
Definitely Morning Type	70-86
Moderately Morning Type	59-69
Neither Type	42-58
Moderately Evening Type	31-41
Definitely Evening Type	16-30

The Final Questionnaire

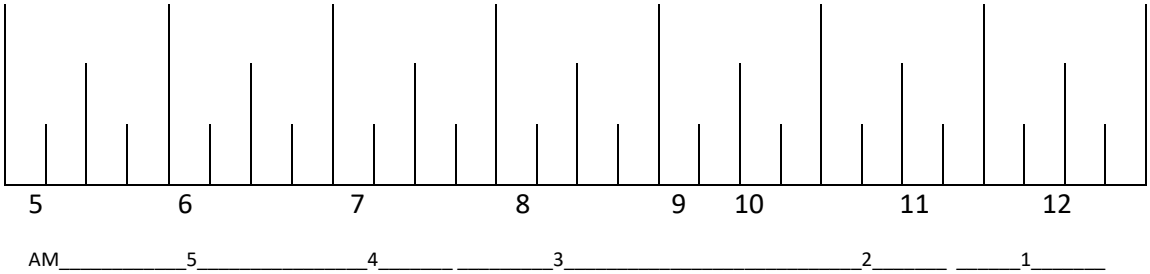
Instructions:

1. Please read each question very carefully before answering.
2. Answer ALL questions.
3. Answers questions in numerical order.
4. Each question should be answered independently of others. Do NOT go back and check your answers.
5. All questions have a selection of answers. For each question place a cross alongside ONE answer only. Some questions have a scale instead of a selection of answers. Place a cross at the appropriate point along the scale.
6. Please answer each question as honestly as possible. Both your answers and the results will be kept, in strict confidence.

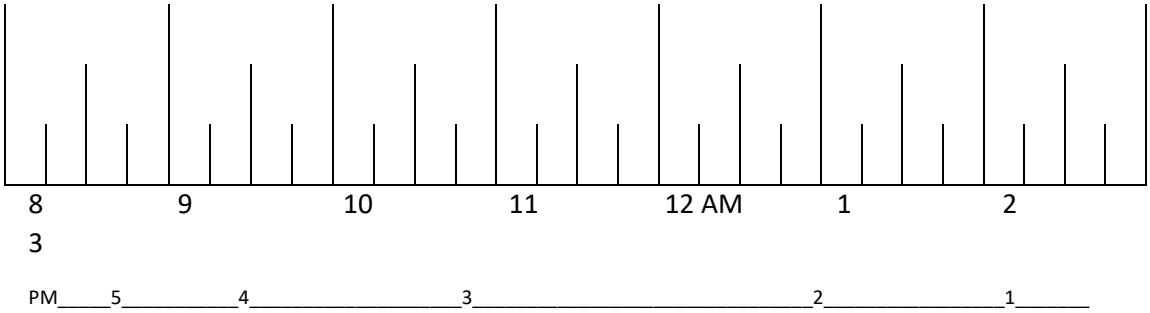
7. Please feel free to make any comments in the section provided below each question.

<u>The Questionnaire, with scores for each choice</u>

1. Considering only your own “feeling best” rhythm, at what time would you get up if you were entirely free to plan your day?



2. Considering only your own “feeling best” rhythm, at what time would you go to bed if you were entirely free to plan your evening?



3. If there is a specific time at which you have to get up in the morning, to what extent are you dependent on being woken by an alarm clock?

Not at all	4	dependent.....
Slightly	3	dependent.....
Fairly	2	dependent.....
Very	1	dependent.....

4. Assuming adequate environmental conditions, how easy do you find getting up in the mornings?

Not at all	☐ 1	easy.....
Not very	☐ 2	easy.....
Fairly	☐ 3	easy.....
Very	☐ 4	easy.....

5. How alert do you feel during the first half hour after having woken in the mornings?

Not at all	☐ 1	alert.....
Slightly	☐ 2	alert.....
Fairly	☐ 3	alert.....
Very	☐ 4	alert.....

6. How is your appetite during the first half-hour after having woken in the mornings?

Very	☐ 1	poor.....
Fairly	☐ 2	poor.....
Fairly	☐ 3	good.....
Very	☐ 4	good.....

7. During the first half-hour after having woken in the morning, how tired do you feel?

Very	☐ 1	tired.....
Fairly	☐ 2	tired.....
Fairly	☐ 3	refreshed.....
Very	☐ 4	refreshed.....

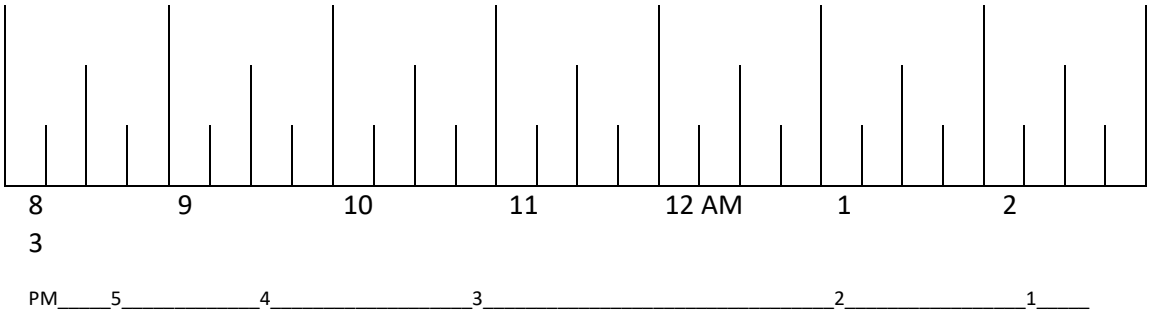
8. When you have no commitments the next day, at what time do you go to bed compared to your usual bedtime?

Seldom or *never later...	☐	4
Less than 1 hour later...	☐	3
1-2 hours later...	☐	2
More than 2 hours later...	☐	1

9. You have decided to engage in some physical exercise. A friend suggests that you do this one hour twice a week and the best time for him is between 7-8am. Bearing in mind nothing else but your own “feeling best” rhythm, how do you think you would perform?

Would be on good form...	☐	4
Would be on reasonable form…	☐	3
Would find it difficult...	☐	2
Would find it very difficult**...	☐	1

10. At what time in the evening do you feel tired and, as a result, in need of sleep?



11. You wish to be at your peak performance for a test which you know is going to be mentally exhausting and lasting for 2 hours. You are entirely free to plan your day and considering only your own “feeling best” rhythm, which ONE of the four testing times would you choose?

8am – 10am...	6
11am – 1pm...	4
3pm – 5pm...	2
7pm – 9pm...	0

12. If you went to bed at 11pm, at what level of tiredness would you be?

Not at all tired...	0
A little tired...	2
Fairly tired...	3
Very tired...	5

13. For some reason you have gone to bed several hours later than usual, but there is no need to get up at any particular time the next morning. Which ONE of the following events are you most likely to experience?

Will wake up at usual time and will NOT fall asleep...	4
Will wake up at usual time and will dose thereafter…	3
Will wake up at usual time but will fall asleep again...	2
Will NOT wake up until later than usual...	1

15. One night you have to remain awake between 4am-6am in order to carry out a night watch. You have no commitments the next day. Which ONE of the following alternatives will suit you best?

Would NOT go to bed until watch was over...	1
Would take a nap before and sleep after...	2
Would take a good sleep before and nap after...	3
Would take ALL sleep before watch...	4

16. You have to do 2 hours hard physical work. You are entirely free to plan your day and considering only your own “feeling best” rhythm which ONE of the following times would you choose?

8am – 10am...	4
11am – 1pm...	3
3pm – 5pm...	2
7pm – 9pm...	1

17. You have decided to engage in hard physical exercise. A friend suggests that you do this for one hour twice a week and the best time for him is between 10-11pm. Bearing in mind nothing else but your own “feeling best” rhythm how well do you think you would perform?

Would be on good form...	1
Would be on reasonable form…	2
Would find it difficult....	3
Would find it very difficult**...	4

18. Suppose you can choose your own your own work hours. Assume that you worked a FIVE hour day (including breaks) and that your job was interesting and paid by results. Which FIVE CONSECUTIVE HOURS would you select?

12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12

MIDNIGHT NOON MIDNIGHT

_____1_____5_____4_____3_____2___ _____1_____

19. At what time of the day do you think that you reach your “feeling best” peak?

12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12

MIDNIGHT NOON MIDNIGHT

_____1_____5_____4_____3_____2_____ _____1____

20. One hears about “morning” and “evening” types of people. Which ONE of these types do you consider yourself to be?

Definitely a “morning” type...	6
Rather more a “morning” type than an “evening type”...	4
Rather more an “evening” type than a “morning” type...	2
Definitely an “evening” type…	0

Appendix 4: Venepuncture Blood Donation Declaration Form

Edinburgh Napier University School of Life Sciences

You have consented to donate blood in the School of Life Sciences. The School phlebotomists have all undergone an approved training course and have Hepatitis B immunity. The blood you are donating will be used for the investigation of sleep disruption on immune function. Your blood will not be screened for pathogenic organisms that could adversely affect the health of any exposed person. It is therefore important that you do not donate blood if any of the risk factors listed below apply to you. At the end of the experiment the cells will be disposed of and not stored for future experiments. Please read the list below and think very carefully if any apply to you. If any factors do apply please do not sign the declaration and do not offer your services as a donor. You do not have to say which risk factors apply.

Risk Factors

Recent –

Ill-Health
Contact with infectious diseases
Vaccinations or immunisations

In the last year –

Tattoo or body piercing
Childbirth
Blood transfusion
Tissue or skin graft
Hormone treatment
Major surgery
Travel to a malarial area or in sub Saharan Africa, Asia or South America

At any time –

If you have lifestyle factors which would pose a risk please do not donate blood.

Declaration

I have read the risk factors and have considered my lifestyle factors and to the best of my knowledge none of them apply to me and I am in good health. I understand that my blood will be used for research purposes.

Name of Donor: ..... Name of Phlebotomist: .....

Signature of Donor:..... Signature of Phlebotomist:.....

Date:..... Date:.....

Appendix 5: Physical Activity Readiness Questionnaire (Par-Q)

Please read the questions carefully and answer each one honestly, ticking the appropriate box or adding information if necessary. Your responses will of course be kept in the strictest confidence.

This form must be completed, returned to a Fitness Advisor and assessed prior to availing of any induction services.

Name: _____ **Participant Reference No.** _____

Postcode: _____

Has your doctor ever said that you have had a heart problem?

No ☐ Yes ☐

In the past month have you had any chest pain when…

You were doing any activity No ☐ Yes ☐ You were resting No ☐ Yes ☐

Are you currently taking medication for…

A heart condition No ☐ Yes ☐

Any other problems No ☐ Yes ☐

Do you suffer from any bone or joint problems?

No ☐ Yes ☐

In the past year have you had any major illness or major surgery?

No ☐ Yes ☐

Have you ever been diagnosed with…

Diabetes No ☐ Yes ☐	Asthma No ☐ Yes ☐
Epilepsy No ☐ Yes ☐	Other problems No ☐ Yes ☐

Do you ever…

lose your balance because of dizziness or lose consciousness No ☐ Yes ☐

Are you feeling unwell at present due to cold, etc

No ☐ Yes ☐

If you have answered YES to one or more questions we may need to contact your doctor before you can start to exercise. If your health changes so that you may then answer YES to any of these questions, tell a member of staff as soon as possible.

I have read, understood and completed this questionnaire ☐

Any questions that I had were answered to my full satisfaction ☐

Signature: Date:

Appendix 6: Caffeine Consumption Diary (CCD)

Please answer the following questions as completely and honestly as you can. This information is STRICTLY CONFIDENTIAL. Thank you for your cooperation.

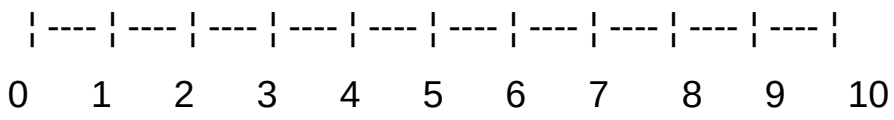
	MORNING 6am-12nn	AFTERNOON 12nn-6pm	EVENING 6pm-2am	NIGHT 2am-6am
COFFEE (140g/5oz servings) Regular brewed Percolated Drip-brewed Espresso shot Regular instant Decaffeinated Brewed Instant	_____ _____ _____ _____ _____ _____ _____ _____	_____ _____ _____ _____ _____ _____ _____ _____	_____ _____ _____ _____ _____ _____ _____ _____	_____ _____ _____ _____ _____ _____ _____ _____
TEA (140g/5oz servings)				
COCOA (140g/5oz servings)				
CHOCOLATE (224g/8oz servings)				
SOFT DRINKS (233g/12oz servings) Coca-Cola Diet Coca-Cola Red Bull (or similar)	_____ _____ _____	_____ _____ _____	_____ _____ _____	_____ _____ _____
OVER-THE-COUNTER DRUGS (Tablet) Please state type and caffeine content				

Appendix 7: Arousal and Alertness Questionnaire

Read the statement below and ring the number that best describes how you feel at the moment.

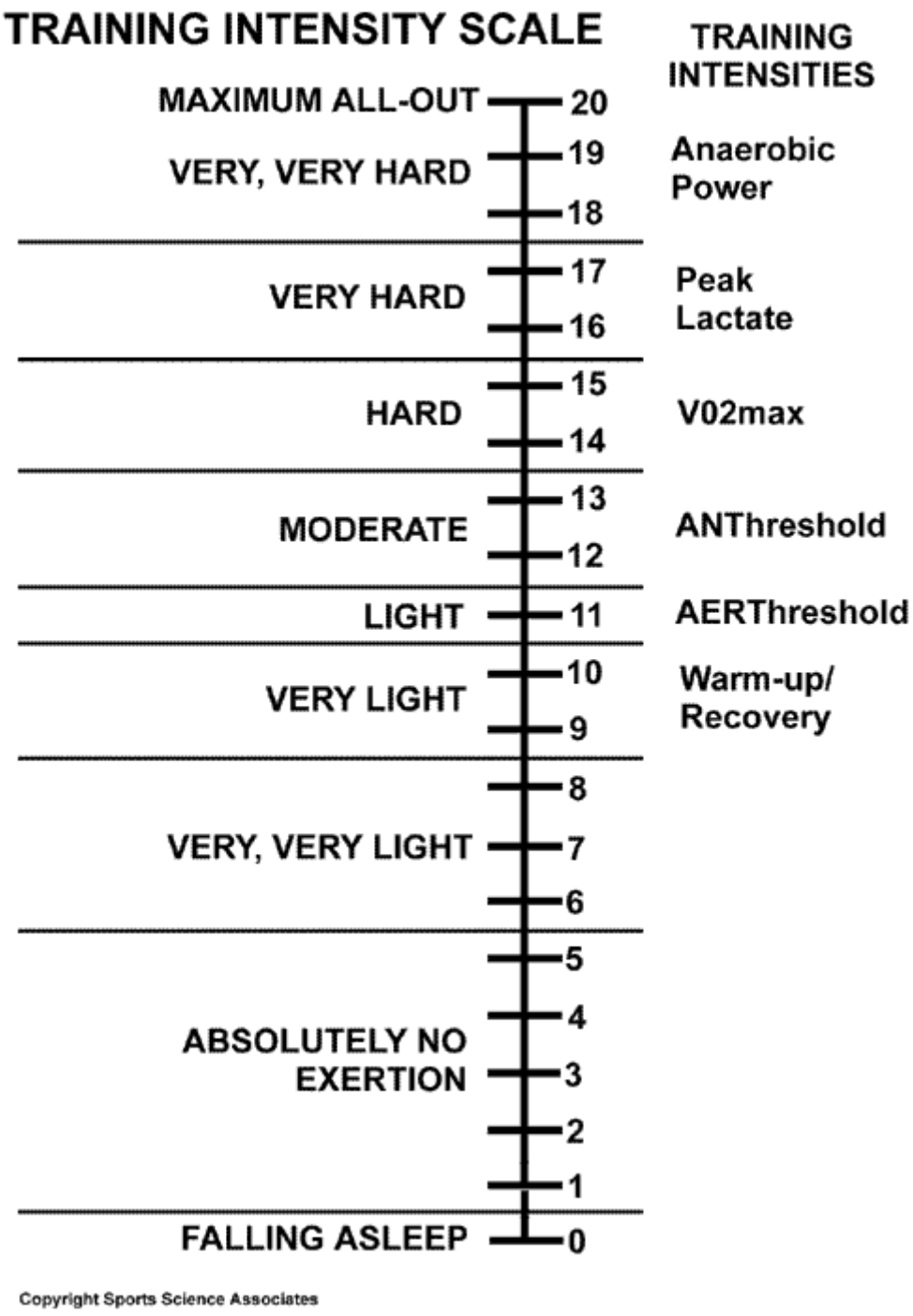
1. Feeling active and vital, alert and awake.
2. Functioning at a high level but not at peak. Able to concentrate.
3. Relaxed, awake but not at full alertness. Responsive.
4. A little foggy, not at peak, let down.
5. Fogginess, beginning to lose interest in remaining awake, slowed down.
6. Sleepiness, prefer to be lying down, fighting sleep, woozy.
7. Almost in reverie, sleep onset soon, lost struggle to remain awake.

Place an “x” on the scale below to describe your alertness. A mark to the extreme left of the line indicates ‘as tried as I’ve ever felt’, the extreme right indicates ‘ as alert as I’ve ever felt’ and the intermediate position indicates intermediate feelings of alertness.



Adapted from Reilly *et al*., 1997

Appendix 8: Rate of Perceived Exertion Scale



Adapted from Borg, G, "Perceived Exertion as an indicator of somatic stress", Scandinavian journal of Rehabilitation Medicine 1970, 2(2), 92-98.

Appendix 9: Post-Trial Respiratory Symptoms Questionnaire

Name: Date:

Trial:

Did you experience any of the following during the trial? Please choose one of the following options for each symptom.

0 = Not present 1 = Minimal 2 = Mild 3 = Moderate

4 = Severe 5 = Incapacitating

Shortness of breath	0	1	2	3	4	5
Cough	0	1	2	3	4	5
Excess sputum	0	1	2	3	4	5
Throat tickle	0	1	2	3	4	5
Raspy throat	0	1	2	3	4	5
Wheezing	0	1	2	3	4	5
Congestion	0	1	2	3	4	5
Pain on deep inspiration	0	1	2	3	4	5
Headache	0	1	2	3	4	5
Nausea	0	1	2	3	4	5
Eye irritation	0	1	2	3	4	5

Please detail any other symptoms:

_____

_____

_____

_____

During the test did you feel you would be able to perform maximally in competition?

Please circle your answer

Yes No

Appendix 10: The Obligatory Exercise Questionnaire

By Thompson, J. K. & Pasman, L.

Directions:

Listed below are a series of statements about people's exercise habits. Please circle (highlight in yellow) the number that reflects how often you could make the following statements:

1 – NEVER 2 – SOMETIMES 3 – USUALLY 4 – ALWAYS

Statement	**Scoring**
1. I engage in physical exercise on a daily basis	**1 2 3 4**
2. I engage in one/more of the following forms of exercise: walking, jogging/running or weightlifting.	**1 2 3 4**
3. I exercise more than three days per week	**1 2 3 4**
4. When I don't exercise I feel guilty.	**1 2 3 4**
5. I sometimes feel like I don't want to exercise, but I go ahead and push myself anyway.	**1 2 3 4**
6. My best friend likes to exercise.	**1 2 3 4**
7. When I miss an exercise session, I feel concerned about my body possibly getting out of shape.	**1 2 3 4**
8. If I have planned to exercise at a particular time and something unexpected comes up (like an old friend comes to visit or I have some work to do that needs immediate attention) I will usually skip my exercise for that day.	**1 2 3 4**
9. If I miss a planned workout, I attempt to make up for it the next day.	**1 2 3 4**
10. I may miss a day of exercise for no good reason.	**1 2 3 4**
11. Sometimes, I feel a need to exercise twice in one day, even though I may feel a little tired.	**1 2 3 4**
12. If I feel I have overeaten, I will try to make up for it by increasing the amount I exercise.	**1 2 3 4**
13. When I miss a scheduled exercise session I may feel tense, irritable or depressed.	**1 2 3 4**
14. Sometimes, I find that my mind wanders to thoughts about exercising.	**1 2 3 4**
15. I have had daydreams about exercing.	**1 2 3 4**
16. I keep a record of my exercise performance, such as how long I work out, how far or fast I run.	**1 2 3 4**
17. I have experienced a feeling of euphoria or a "high" during or after an exercise session.	**1 2 3 4**
18. I frequently "push myself to the limits'.	**1 2 3 4**

19. I have exercised when advised against such activity (i.e. by a doctor, friend, etc.).	**1 2 3 4**
20. I will engage in other forms of exercise if I am unable to engage in my usual form of exercise.	**1 2 3 4**
Supplementary information i). Do you compete in organised, competitive distance/endurance races?	**Yes or No**
Supplementary information ii). If so, please provide details as to what distance(s) and how often (in the last 12 months).	

Thank you for your time and consideration. Peter.

*Items 8 and 10 are reverse-keyed.

The scale takes approximately 5 minutes to complete. Items 8 and 10 are reverse scored. All other items indicate higher endorsement of and engaging in obligatory exercise behaviours. This measure has been psychometrically validated on ninety subjects. The internal consistency ratio was 0.96 and the test-retest reliability (two weeks) was also 0.96.

For information on the scale, please contact:
J. Kevin Thompson, Ph.D.
Department of Psychology
University of South Florida
PCD 4118G
4202 East Fowler Avenue
Tampa FL 33620-8200.

Appendix 11: Respiratory Symptoms Questionnaire

Name: Date:

Trial:

Did you experience any of the following during the trial? Please choose one of the following options for each symptom.

0 = Not present 1 = Minimal 2 = Mild 3 = Moderate

4 = Severe 5 = Incapacitating

Shortness of breath	0	1	2	3	4	5
Cough	0	1	2	3	4	5
Excess sputum	0	1	2	3	4	5
Throat tickle	0	1	2	3	4	5
Raspy throat	0	1	2	3	4	5
Wheezing	0	1	2	3	4	5
Congestion	0	1	2	3	4	5
Pain on deep inspiration	0	1	2	3	4	5
Headache	0	1	2	3	4	5
Nausea	0	1	2	3	4	5
Eye irritation	0	1	2	3	4	5

Please detail any other symptoms:

_____

_____

_____

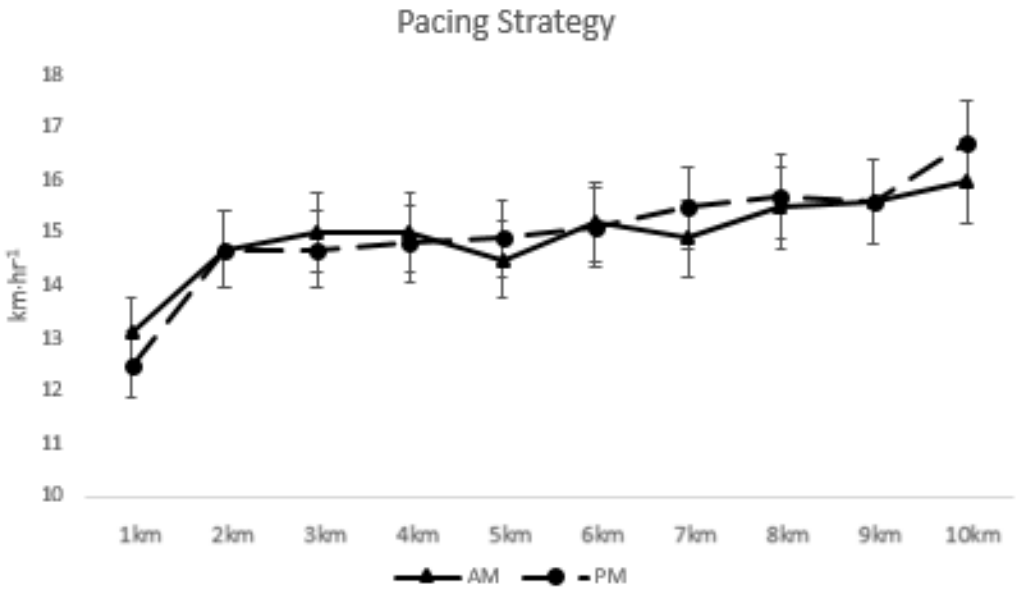
_____

During the test did you feel you would be able to perform maximally in competition?

Please circle your answer

Yes No

Appendix 12: **10km Self-Paced Time-Trial Pacing Strategy**



Appendix 12. Self-paced pacing strategy at 1km intervals throughout a 10km time-trial at 09.00hrs (AM) and 16.00hrs (PM). Values are mean ±SD (partial dataset, n=9).