Diurnal variation in physiological, psychological and immune responses to running 10 km time trials performed in hot and cold environments.

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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, Boukhemis Boukelia aka Zac.

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ABSTRACT

Purpose: To investigate the physiological, immunological and psychological response to an intense bout of exercise performed by highly trained individuals at 09:00 hs and 16:00 hs (Chapter 5) and at 09:00 hs and 18:00 hs (Chapter 6). **Methods**: Using a crossover randomized design, 7 and 13 well trained runners (range $\dot{V}O_2max$ 61-79 $ml \cdot kg^{-1}min^{-1}$) performed a 10 km time trial run, in two contrasting environments: cold (6°C) and hot (28°C and 70% relative humidity), at 2 different times of day (09:00 hs and 18:00 hs or 16:00 hs). Lung function tests and blood samples were taken immediately pre-, post- and 1h post-trial to determine, total WBC counts, WBC variables, total RBC counts, RBC variables, IL-6, CC16 and HSP70 levels. Nasal lavage procedure for the analysis of upper respiratory airway was conducted pre-, post- and 1h post-trial. Core body temperature, heart rate, power, strength and flexibility as well as RPE, mood, arousal and alertness were measured pre-, post-trial and 1h post- trial at both times of the day. **Results**: The time taken to complete the trial was not significantly different in both studies but was faster at 09:00 hs under hot environmental conditions. During the time trial, core body temperature was significantly higher at 18:00 hs ($P < 0.05$) under hot and humid conditions, whereas, heart rate and core body temperature were higher at 09:00 hs in the cold environment. A significant diurnal difference ($P < 0.05$) was found for total WBC, neutrophil and lymphocyte counts with higher values at the evening in both studies. Plasma CC16 and total RBC and RBC variable counts were not affected by the time of the day in the cold condition. Resting IL-6 and CC16 as well as HSP70 at 1 h post-trial were significantly higher in the morning, whereas, HSP70, total RBC and RBC variables counts were not affected by the time of the day in hot and humid conditions. Similarly no significant differences were observed in power, strength or flexibility in these conditions. Most psychological measures were not affected by the time of the day in either environmental condition. However, recovery arousal at 09:00 hs was significantly higher ($P < 0.05$) in the hot and humid condition. **Conclusion**: a 10 km time trial run, in both environmental conditions, can cause different physiological and immunological responses dependent on the time-of-day in which it is performed. Nevertheless in highly trained runners this variation was not enough to impact on their performance. Despite no statistically significant difference in diurnal running performance in the hot and humid

condition a 19 second mean difference in completion time would decide the race winner or even new records.

ABBREVIATIONS AND SYMBOLS

BRUMS	Brunel mood scales
CC16	Clara cell protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoasorbant assay
FEF_{25-75}	Forced expiratory flow during the middle half of the forced vital capacity
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
HSP70	Heat shock protein 70
IL	Interleukin
NL	Nasal lavage
PEF	Peak expiratory flow
RPE	Rating of perceived exertion
TNF-α	Tumor necrosis factor alpha
$\dot{V}O_2max$	Volume of oxygen
μg	Microgram
μl	Microlitre

DEFINITION OF TERMS:

Acrophase: the time at which the peak of a rhythm occurs (Refinetti, 2006).

Adaptation: is a physiological or behavioural changes process, which decrease the strain caused by environmental changes (Refinetti, 2006).

Amplitude: the difference between the peak and the mean value of a wave (Refinetti, 2006).

Basophils: part of human body immune system normally protects body from infection and they are involved in inflammatory reactions in the body, especially those related to allergies and asthma (Schoroeder 2009).

CC16: Clara cell protein (CC16, CC10, or CCSP) is the main constituent of secretory granules of clara cells (Arsalane et al., 2000), found in the pulmonary airways predominantly in the respiratory bronchioles and in the terminal bronchioles and are one of the main secretory proteins of the lung (McAuley and Matthay, 2009). They work to protect the respiratory system against toxic inhaled agents, repair damaged epithelium, detoxify xenobiotics and secrete proteins with important biological activities, such as surfactant-associated proteins and leukocyte-protease inhibitors (Braido et al., 2007).

Circadian rhythm: biological rhythms that run in a period of approximately of 24 hours (Refinetti, 2006).

Chronobiology: the scientific study of biological rhythms (Refinetti, 2006).

Clock: a gene that is an essential element in the molecular mechanism of circadian rhythmicity in animals (Refinetti, 2006).

Core body temperature: refers to the temperatures of the internal body organs and cavities including abdominal, thoracic and cranial cavities (Gisolfi and Mora 2000).Core body temperature has been used as a primary indicator of circadian rhythmicity in physiological variables which affect sports performance (Florida-James and Doggart, 2000; Atkinson et al., 2005).

Cortisol: a hormone (steroid) made in the adrenal glands (Hill et al., 2008). Cortisol assists in regulating cardiovascular functions, blood pressure, and body's carbohydrates, proteins and fats use.

Cytokine: a protein produced by many different cell types that mediate inflammatory and immune reactions. Cytokines are the principle mediators of communication between cells in the immune system (Takahashi et al., 2001).

Daily: having the duration of a day (24 hours) (Refinetti, 2006).

Diurnal: diurnal variation, also known as daily variation, changes with time of day and may persist when the individual is placed in an environment devoid of time cues, such as exposure to direct light or darkness (Refinetti, 2006).

Endogenous: originating or part within an organism (Refinetti, 2006).

Eosinophil: type of human white blood cells**,** produced in the bone marrow (Young et al., 2006). Helps to protect the body against diseases and fight infections. Eosinophil concentration rises in blood as a result to an allergic reaction (Young et al., 2006).

Exogenous: originating outside or not part within an organism (Refinetti, 2006).

Free running: the period of an oscillation (rhythm) in the free run state (Refinetti, 2006).

Homeostasis: The body's tendency or ability to maintain internal stability to compensate for environmental changes in which that internal condition remains stable (Refinetti, 2006).

HSP70: part of HSP's family which is a protein group which increase when the human cells are exposed to elevated temperatures or to other stress types (Guisbert and Morimoto, 2013).

Hyperthermia: the opposite of hypothermia which is the failed thermoregulation that occurs when a body heat production is higher than the heat dissipated.

Hypothermia: the organism's condition which is the core body temperature is lower than the regular range of oscillation (Refinetti, 2006).

IL-6: an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory (Pedersen and Hoffman, 2000).

Infradian rhythms: have the longest period of greater than 24 hours lasting several days (period longer than circadian) (Refinetti, 2006).

Jet lag: a disruption of human rhythms caused by air travel across time zones (high-speed) (Refinetti, 2006).

Lymphocyte: consist of 20 to 40 percent of the total number of white blood cells found in the circulation and also are concentrated in central lymphoid organs and tissues. Lymphocytes consist of 2 primary cell T and B cells originated from steam cells, T cells and B cells are a subset of lymphocyte cells; T cells are responsible for cellular direction of the immune response, whereas B cells are responsible for humoral immunity by producing antibodies (Walsh et al., 2011).

Masking: disruption in the human circadian rhythm caused by an exogenous agent without effecting the phase of the pacemaker (Refinetti, 2006).

Monocyte: white blood cell counts consist of 1% to 3% of monocytes in the body, depending on the individual level of health. These cells come from the bone marrow and migrate to the site of infection in approximately one to three days. Monocytes act in the same way as neutrophils (McFarlin and Mitchell, 2003).

Nadir: the lowest value of an oscillatory function (Refinetti, 2006).

Neutrophil: the most abundant circulating white blood cell that is recruited to inflammatory sites and is capable of phagocytosing and digesting microbes (Abbas et al., 2012).

Nocturnal: occurring during the night-time (Refinetti, 2006).

Pacemaker: able to generate endogenous rhythmicity (Refinetti, 2006).

Phagocyte: a cell, such as a white blood cell, that engulfs and absorbs waste material, harmful microorganisms, or other foreign bodies in the bloodstream and tissues (Abbas et al. 2012).

Phase: a distinct stage of a process. (Such as the difference between resting and post-exercise phase on heart rate) (Refinetti, 2006).

Shift work: usually during the night (non-traditional working times) (Refinetti, 2006).

Ultradian rhythms: have a shorter period from seconds to less than 24 hours (Refinetti, 2006).

Zeitgeber: German world meaning ‘time giver’(Refinetti, 2006).

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CHAPTER 1:

GENERAL INTRODUCTION

In recent years Exercise Chronobiology has become a rapidly expanding discipline, attracting researchers' attention from various scientific fields, including Exercise Science. Publication in exercise science and circadian rhythmicity dates back to the early 1980's (Reilly and Brooks, 1982; Shephard, 1984). Much of the research conducted before 1985 was performed to measure the circadian rhythm effect on more basic physiological functions, such as heart rate, core body temperature and oxygen consumption at various intensity levels of exercise (Shephard, 1984; Winget, 1985). It was not until the end of 1980's and early 1990's that a significant number of scientific studies started to appear (Aschoff, 1988; Waterhouse and Minors, 1988; Reilly, 1990; Minors and Waterhouse, 1993; Atkinson and Reilly, 1996; Atkinson and Spiers, 1998).

Abnormal circadian rhythms have been linked to illnesses such as diabetes (Gale et al., 2011), depression (Salva et al., 2011), bipolar disorder (Harvey, 2008), seasonal affective disorder (SAD) (Johansson et al., 2003) and even obesity (Kalra et al., 2003). Furthermore, circadian rhythms can affect body function and health due to sleep-wake cycles (Florida-James et al., 1996), body temperature (Atkinson et al., 2005), hormone release (Czeisler and Klerman, 1999), and other important body functions. More recent studies have investigated other performance parameters that have also been found to possess a circadian rhythmicity, such as, stroke volume, cardiac output, blood pressure, vascular blood flow, metabolic rate, sweat rate and trunk flexibility (Jones et al., 2009; Atkinson et al., 2010; Atkinson et al., 2014).

The link between circadian rhythmicity and sport performance has been extensively studied (Reilly, 1990; Atkinson and Reilly, 1996; Atkinson and Speirs, 1998; Atkinson et al., 2005; Waterhouse et al., 2005) with several contributing factors having been identified. These factors can be endogenous, including physiological changes such as core body temperature; and exogenous, including environmental changes such as sleep deprivation, and daily training time (Atkinson and Reilly, 1996; Waterhouse et al., 2005; Atkinson et al., 2005). Identifying the cause for improved performance is often difficult because performance fluctuations can be affected by different exogenous and endogenous factors occurring at the same time of day.

The indirect evidence for the existence of circadian rhythmicity in sports performance can be examined by the time of day athletes perform best or worst in actual sporting events (Drust et al., 2005). The study of circadian rhythmicity and sport performance can be investigated in a laboratory setting directly obtaining evidence against, or in support of, this relationship. However, a great many sporting events are scheduled for the evening which may create a false circadian record. It is important therefore for coaches and athletes to be aware of how the time of day affects various and inter-related components of sport performance.

A strong correlation has been established between core body temperature and performance, with better performance generally occurring in the early evening when the core body temperature is higher. It has been proposed that a higher core body temperature represents physiological arousal that enhances human performance (Atkinson et al., 2005; Waterhouse et al., 2005). A review by Drust et al. (2005) observed that most athletic world records have been set in the evening: suggesting a link between the times of the day, core body temperature and sport performance. Heart rate has also been shown to oscillate during the day with a range of 5 to 15% and a peak around 15:00 hours (Reilly, 1990). Atkinson et al. (2005), demonstrated better afternoon performance than the morning after 16-km cycling races. Pereira et al. (2011) also showed a better evening performance than morning for maximum isometric voluntary knee extensions. In addition, improved performances have been observed for both 100 m and 400 m swims in the afternoon (Kline et al., 2007).

The circadian rhythm of the immune system and sport performance is still a new field of research, although it has been reported that circadian rhythmicity exists in the human immune system for some time (Petrovsky and Harrison, 1998; Haus and Smolensky, 1999; Miles et al., 2008). Kimura et al. (2009) found that the circulation of the white blood cells involved in the defence of the human body show high-amplitude circadian rhythmicity: being lower in the morning and higher in the afternoon.

The immune system plays an important role in human health and the prevention of illness. Preventing athletes from illness during heavy training periods and/or races is a high

priority for athletes, coaches and exercise researchers. A perception exists that some athletes, especially those engaging in prolonged intensive exercise and competition, such as running, cycling and swimming, may show an increased rate of upper respiratory tract infection (URTI) during intense training periods and competition (Nieman et al., 2011; Gleeson et al., 2013). Given the considerable economic and personal investment in preparing athletes for events, there is a need to identify strategies to improve host resistance whilst minimising the risk of illnesses that may affect athletic performance. An agreement between scientists exist in that moderate exercise enhances the athlete's immunity (Simpson et al., 2006; Warburton et al., 2006), but high-intensity prolonged exercise impairs, temporarily, immune competence (Mooren et al., 2012). A relationship between immune-depression and risk of URTI has been established following heavy exercise (Teixeira et al., 2011).

The inverse relationship between exercise workloads and immune system function has been studied intensively (Nieman et al., 2011; Gleeson et al., 2013). A number of studies have reported that exercise leads to a considerable physiological change in the human immune system (Simpson et al., 2006, for a review see Walsh et al., 2011). However, immune suppression is higher in some athletes compared to general active population (Gleeson, 2006), while there does appear to be a secondary response in truly elite athletes which reverses this theory. Although modest exercise may enhance the function of the immune system above sedentary levels; prolonged high-intensity exercise can weaken the immune competence of trained athletes (Simpson et al., 2006). It may be that this relationship is most likely affected by individual determinants such as circadian rhythmicity, dietary status, allergies or environmental conditions.

As stated previously, the relationship between circadian rhythmicity and the immune system has been investigated previously (Haus and Smolensky, 1999; Habbal and Al-Jabri, 2009). Miles et al., (2008) observed diurnal variation in Interleukin 6 (IL-6), with a decrease at 12:00 hs, 16:00 hs and 20:00 hs compared to 07:00 hs during high-force eccentric resistance exercise using the elbow flexor muscles. In contrast DeRijk et al. (1997) found plasma concentrations of IL-6 were almost identical in the morning and evening, and the difference was not significant. Heat shock protein (HSP70) shows a

diurnal variation which strongly correlates with core body temperature with maxima reported in the evening and nadir in the early morning (Sandstrom et al., 2009). Moreover, Clara Cell Protein (CC16) concentration shows a diurnal variation with a decrease during daytime (Helleday et al., 2006; Andersson et al., 2007).

Athletes may sometimes have to perform to the best of their ability at a specific time of day and under extreme environmental conditions (Lane et al., 2005). Kobrick and Johnson (1991) suggested that effects of environmental change tend to influence psychological functioning before they affect physiological factors. These therefore, provide a useful early indicator of the adverse effects of environmental stress that can lead to poor performance. It would appear that there is a lack of literature investigating the effect of circadian rhythmicity and environment (hot and cold) on athletic performance. Ultimately, an athletes' understanding of the risks to the immune system associated with high level activity at specific times of the day and the environmental conditions within which they train, can lead to improved performances. When combining extreme environments with exercise, these two forms of physiological stress may produce changes and function in white blood cells compared to either stressor alone (McFarlin and Mitchell, 2003).

Exercising in hot conditions was found to result in elevated core temperature (over 39°C), that evokes an increase in white blood cells with an increase in circulating neutrophils, lymphocytes and eosinophils and a decrease in monocyte counts (McFarlin and Mitchell, 2003). In contrast another study has shown that increased core body temperature to 38°C by exposure to 40°C in a climatic chamber without exercise for duration of 3 hours had limited effect on circulating white blood cells (Cross et al., 1996). Cold temperatures can damage normal physical barriers to infection, such as increased mucus viscosity and decreased ciliary action in the upper respiratory system (Beachey, 2012). Hence, the body increases hormone release during exposure to cold air, leading to an increase in circulating white cells, and a reduction in the production of an inflammatory mediator (Brenner et al., 1999).

Long distance air travel is a frequent occurrence for modern elite athletes and causes a shift in the internal biological clock called ‘jet lag’. Athletes may experience some symptoms during the period of jet lag that include sleep dysfunction, lack of concentration, depression, fatigue, loss of appetite, and gastrointestinal disturbance. In addition athletes will also face the additional negative consequences of a shift from the optimal circadian window of performance (Refinetti, 2006). This rapid air travel across several time zones can hinder athletic performance and certain precautions need be taken by athletes or coaches until the body’s rhythm is re-phased to the new environmental conditions (Leatherwood and Dragoo, 2012).

The main aim of the research presented in this thesis was to evaluate the impact of time of day on 10 km running performance by considering physiological, immunological and psychological measures. A second aim was to investigate if changes in environment had an additional impact on the above variables simulated in two contrasting conditions: UK Winter (6°C and 60% relative humidity) and overseas summer (28°C and relative humidity of 70%).

Finally, generally all participants whether at a major or minor competition will experience stress pre-event and this stress severity differs from one person to another. Indeed a little stress improves performance, whereas uncontrollable or severe stress decreases performance and may eventually cause athlete break down (Hamilton, 2000). The thesis is comprised of seven chapters:

Chapter 1 - Introduction: a general introduction to the research.

Chapter 2 - Literature review: a review of the main literature encompassing the research topics.

Chapter 3 - General methodology: the general materials and methods used in the experimental part of the studies. Any specific methodology is described in the relevant chapter.

Chapter 4 - Measuring body temperature in athletes using different devices.

Chapter 5 - Investigating the diurnal immune response and physiological differences in a cold environment in well trained runners. This chapter investigates the effect of circadian rhythmicity on the physiological and immunological responses to a 10 km run in highly trained athletes at 6°C.

Chapter 6 - Investigating the diurnal immune response and physiological differences in a hot and humid environment in well trained runners. This chapter investigates the effect of circadian rhythmicity on the physiological and immunological responses to a 10 km run using highly trained athletes in an environment of 28°C and 70% relative humidity.

Chapter 7 - Psychological circadian rhythmicity in highly trained athletes in two different environmental conditions. This chapter provides the athletes' psychological data from the two contrasting studies (Chapters 5 and 6), and the results are discussed.

Chapter 8 - General discussion: this last chapter has a general discussion of the findings elucidating the main conclusions and relevance of the studies. In addition, the limitations and key areas for future research are presented.

CHAPTER 2:

REVIEW OF LITERATURE

The purpose of this literature review is to introduce the reader to the area of circadian rhythmicity and the concept of diurnal variation and sport performance. This includes immunological, physiological and psychological rhythmicity in athletes.

2.1 Circadian rhythm overview

Biological rhythms are the ways in which organisms adapt and live with environmental rhythms, such as the earth's orbit around the sun, the earth's rotation and the moon's orbit around the earth (Refinetti, 2006). Biological rhythms consist of 3 types according to the cycle length: diurnal rhythms run in a period of approximately 24 hours; ultradian rhythms have a shorter period from seconds to less than 24 hours; and lastly, infradian rhythms have the longest period of greater than 24 hours lasting several days. Biological rhythms that repeat approximately every 24 hours are termed circadian (from the Greek circa- about, dias –a day) (Vitaterna et al., 2001). It is these circadian biological rhythms that are of most interest in this thesis, particularly in respect to the diurnal variation that they cause in human sports performance. Diurnal variation, also known as daily variation, changes with time of day and may persist when the individual is placed in an environment devoid of time cues, such as exposure to direct light or darkness. In humans, almost every physiological, immunological and behavioural system shows a level of circadian rhythmicity, and the mechanism behind any of these rhythms can be viewed as exogenous, endogenous or a combination of both (Figure 2.1).

Endogenous rhythmicity exists because of the earth's geophysical 24 hour cycle; however, circadian rhythmicity has been recorded in humans in space (Gundel et al., 1997) as well as underground (Colin et al., 1968). In addition to the above, free-running rhythms are also reported in humans living within the Antarctic Circle or Arctic Circle where sunlight exists seasonally (Arendt 2012). Free running is a self-sustaining rhythm in absence of effective zeitgeberg or other exogenous agents that may effect the oscillation period. Thus, diurnal variations can be driven either by light or by the central clock (free running) (Vitaterna et al., 2001). Almost all diurnal rhythms that occur under natural conditions continue to cycle under laboratory conditions without any external physical environment effect.

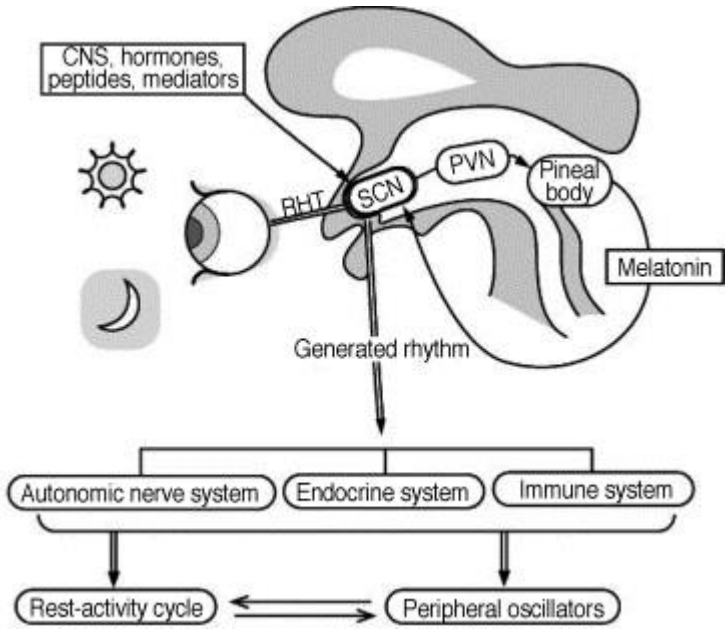

Figure 2.1: Schema of human circadian system showing the location of the SCN in the human brain, (CNS; central nervous system, RHT; Retinohypothalamic tract, SCN suprachiasmatic nuclei, PVN; paraven- tricular nucleus) (Eriguchi et al., 2003).

To improve circadian data statistical analysis, Halberg et al. (1973) developed a statistical technique known as cosinor fitting by least squares. A circadian rhythm is identified by Midline Estimating Statistic of Rhythm (MESOR), amplitude and period. Amplitude is the difference between the peak and the mean value of a wave, the period is the time elapsed for one complete fluctuation or the difference between two consecutive peaks (measured by time) (Refinetti, 2006). MESOR is the midway value between the highest and the lowest values of the (cosine) function best suited to the data, and therefore gives an unbiased and circadian rhythm corrected mean value compared to a regular (non-MESOR) mean value (Portaluppi et al., 2008). Notwithstanding this, circadian rhythms free run under constant environmental conditions, and after a period of time differ slightly from 24 hours (Gillette and Abott, 2006). This rhythmicity is affected by constant light and/or constant darkness. Furthermore, species that demonstrated free-running rhythmicity in regular light are affected by the intensity and duration of the light received (Aschoff's rule) (Aschoff, 1988). Another effect that may confound interpretation of rhythm waveforms in humans is masking, where a circadian rhythm is temporarily influenced by exogenous components (Minors and Waterhouse, 2013). Masking refers to the effects of non-circadian factors that cover circadian rhythmicity and make detection or identification of this rhythmicity difficult (Kryger et al., 2005). Eliminating masking

from the body rhythm is known as purifying the rhythm (Minors and Waterhouse, 1993). Frequently conditions such as the environmental and experimental set up in which circadian rhythmicity measurements are taken are the main causes of masking and can obscure or modify the daily pattern rhythms and/or create circadian rhythm appearance (Kryger et al., 2005; Wirz-Justice, 2007). Other masking factors include: diet, ambient temperature (Minors and Waterhouse, 2013), lightness condition (light saturation) (Wright et., 2013) and physical and psychological activities (Florida-James et al., 1996).

Circadian rhythms in sport performance can be masked by exogenous influences, such as exercise and sleep (Minors and Waterhouse, 2013). Moreover, there is a physiological response resulting from exercise which may mask part or all of the underlying mechanisms. Depending on the physiological variable measured in any research, this masking can be acute or prolonged (Edwards et al., 2005). Examples of physiological parameters include heart rate and core body temperature, both of which can be elevated by exercise and reduced by rest/sleep. This masking component leads scientists to investigate the cause of this rhythmicity in sport performance.

Generally, biological rhythms consist of endogenous and exogenous rhythms (Florida-James et al., 1996). Exogenous rhythms are a result of direct environmental or other external influences, such as light (Refinetti, 2006). The endogenous rhythms occur even when environmental cues are removed; they are the result of internal biological clocks, controlled by the suprachiasmatic nuclei (SCN) (Bloch et al., 2005). The SCN is a group of cells (tiny region) located in the hypothalamus of the brain situated directly above the optic chiasm and consists of an array of genes, protein products they encode and group of nerve cells. This consequently regulates various physiological processes throughout the human body during the daily cycle (Vitaterna et al., 2001) (Figure 2.1 and Figure 2.2). Endogenous biological rhythms include heart rate and core body temperature, whereas exogenous rhythms include light-dark cycles and meal times.

Biological clocks can be characterised by having an endogenous rhythm, a temperature rhythm and by having the ability to be reset in order to maintain a relationship with environmental cues (Refinetti, 2006). Additionally, the biological clock is involved in

two main functions: the biological clock's capacity to free run (absence of any entraining stimuli, i.e. operate without external cues), and timing signals ability, known as zeitgeber "time-giver" (Refinetti, 2006). Zeitgebers, include environmental temperature, light and physical contact, which entrain the biological system to a 24 hour period, whereas the average free running period for circadian rhythms in humans is 25 hours (Dijk and von Schantz, 2005). An example of this is with a new born, whose circadian rhythms can free run significantly, disrupting the sleeping patterns of the guardian. However, at the time that infants become responsive to zeitgebers, such as light and dark, they gradually adopt the 24-hour schedule (McGraw et al., 1999).

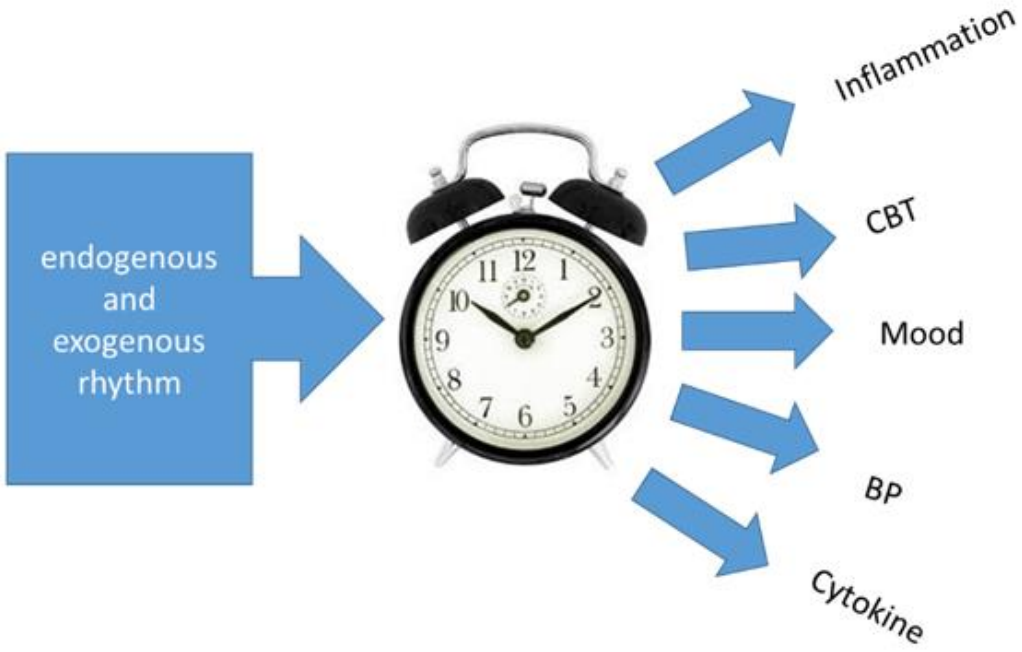

Figure 2.2: The effect of circadian rhythms on different physiological and immunological variables.

Biological rhythms in relation to human health are not fully understood. It is known that hormone secretion and sleep-wake cycles are some of the biological clocks which influence the functioning of various systems within the human body, i.e. the immune, cardiovascular, and muscular systems. A healthy biological clock is important for regulating sleep patterns, this has an impact on different aspects of daily life such as,

alertness, job performance, interpersonal relationships and psychological health (particularly in the regions with decreased light during winter months) (Corbett et al., 2012). The disassociations of the endogenous biological clock and the external environmental cue can lead to phase shifts causing desynchronisation of the rhythms which can often occur with rapid travel across time zones (jet lag) and shift work. Fatigue, indigestion, and nausea are a common result that occurs due to these phase shifts (Florida-James et al., 1996). In a work place setting where alertness is necessary these symptoms can have serious consequences: for example, both Chernobyl and Three Mile Island disasters occurred on the night shift (Folkard et al., 2005).

2.2 Evidence of circadian rhythmicity in sports performance

The concept of circadian rhythms in human physical performance has been extensively researched (Atkinson et al., 2005; Drust et al., 2005; Martin et al., 2007; Taylor et al., 2011). Much of the research conducted before 1990 was performed to measure the circadian rhythmicity effect on more basic physiological functions, including heart rate, core body temperature and oxygen consumption at various intensity levels of exercise (Shephard, 1984; Winget, 1985). Other performance parameters have been found to possess a circadian rhythmicity such as stroke volume, cardiac output, blood pressure, vascular blood flow, metabolic rate, sweat rate, and trunk flexibility (Jones et al., 2009; Atkinson et al., 2010; Atkinson at al., 2014). Core body temperature has been used as a primary indicator of circadian rhythmicity in physiological variables which affect sports performance (Florida-James and Doggart, 2000; Atkinson et al., 2005).

There are many aspects that contribute to circadian rhythmicity in physical performance (Atkinson et al., 2005). These can be internal (physiological) or external (environmental) changes that occur throughout the day. In sports performance and exercise Reilly and Waterhouse (2009) identified three major determinants of circadian rhythms. The first are the external influences (exogenous) such as ambient temperature, physical and/or psychological arousal affected by the environment which is usually uncontrollable. The second determinant are physiological (endogenous) such as sleep/wake cycle and core

body temperature. Lastly, there are the lifestyle influences affecting timing preference in activities, including sleep.

2.2.1 Indirect and direct evidence for circadian rhythm in sports performance

There is a correlation between circadian variation in some variables and sport performance, where morning lows and evening peaks are demonstrated in many laboratory and field tests (Atkinson et al., 2005; Reilly and Waterhouse, 2009; Waterhouse, 2010; Atkinson et al., 2014, and see Table 2.1). Traditionally, core body temperature has been used as a primary indicator of circadian rhythmicity in physiological variables which affect sports performance. Starkie et al. (2005) stated that an increase in core body temperature may lead to an increase in carbohydrate utilisation over fat as a fuel source, possibly facilitating actin-myosin cross bridge mechanics within the musculoskeletal unit (Teo et al., 2011b). For this reason, peak performances have been suggested to occur around the early evenings as it coincides with peak core body temperature (Reilly and Garrett, 1998; Atkinson et al., 2005). Atkinson (2002) defines performance in the context of a sporting action, however, it has a broader meaning, and a successful performance requires a combination of additional factors, such as motor skills, gross motor performance, and cognitive function.

Circadian rhythms exist in several physiological elements such as in sensory motor, psychomotor, perceptual, and cognitive function (Karatsoreos et al., 2011; Kwon and Nam, 2014). Reilly et al. (1997) found that reaction time was higher in the evening coinciding with a peak of core body temperature. Furthermore, Taylor et al. (2011) found reaction times better in the morning, when considering fine motor controls by examining one's hand steadiness and one's ability to balance. On the other hand, Atkinson and Spiers (1998) found speed and accuracy was at its worst in the early evening when both factors were considered together. Diurnal variation in short-term performance (less than 60 seconds) are present but are controversial and this depends on the type of muscle group tested and the type of exercise performed (Teo et al., 2011b). Reilly et al. (2000); Edwards et al. (2013) and (Souissi et al., 2002) showed that the contraction of muscle strength, back strength, peak torque, average power, and maximal work also peaked in the evening (between 14:00 hs and 19:00 hs). Circadian rhythmicity in prolonged exercise varies with

the time of the day and also how the individuals control the endogenous factors (Weterhouse et al., 2007).

A strong correlation exists between muscle fatigue and sport performance, although the underlying mechanisms for diurnal variation in muscle fatigue is not fully understood (Hammouda et al., 2012). Furthermore, Hammouda et al. (2012) found biochemical markers of football players (i.e. lactate, muscle damage, oxidative stress), displayed a diurnal variation when measured at rest. They concluded that inflammation and muscle damage could be higher in the evening, whereas, antioxidant status is higher in the morning. Biomarkers of cellular damage were higher in the evening after a 30 second Wingate test (Hammouda et al., 2012) and during a repeated sprint test (Hammouda et al., 2011). Thus, short duration maximal exercise performed during evening hours leads to higher muscle fatigue possibly due to the higher levels of homocysteine, (oxidative stress indirect marker) or other muscle injury biological markers and a nadir of resting antioxidant status in the evening. Additionally, higher muscle fatigue was observed in the afternoon when two separate 10 × 6 second maximal cycling sprints with a recovery of 30 seconds between each were observed in the morning between 08:00-10:00 hs and then separately in the afternoon between 17:00-19:00 hs. Higher muscle power and strength were reported in the evening (Racinais et al., 2010). Chiba et al. (2011) further suggested that core body temperature could affect the production of lactate during exercise. In addition, Hammouda et al. (2011) showed that homocysteine levels (muscle damage) and core body temperature (oral) were higher in the evening. This higher value of the enzyme during exercise in the evening is due to the highest value at rest, especially with short bouts of exercise.

The exact underlying mechanisms of muscle fatigue diurnal variation are still debated with a link between changes in muscle contractile capacity and diurnal variation in muscle fatigue during exercise having been made (Nicolas et al., 2005). This link however, is not universally accepted. Lericollais et al. (2011) reported that during a 1 minute Wingate test, evening muscle fatigue was observed by a reduction of the range of motion of the ankle angle which could be due to biochemical factors such as muscle fatigue and oxidative stress. In addition, the production of free radicals can be impaired

directly by respiratory and motor activities, and are reported to show diurnal variation (Borisenkov et al., 2007). This indicates antioxidants are less efficient in the evening compared to the morning; however, fluctuations in antioxidant capacity remain unclear.

It has been suggested that the morning oxidative stress nadir can be attributed to total antioxidant capacity (Borisenkov et al., 2007), rate of lipid peroxidation (Cardona 2004) and the activities of some antioxidant enzymes (Hardeland et al., 2003). Furthermore, it has been documented that resting values of homocysteine levels, of white blood cells and of biomarkers of cellular damage peak in the evening (Hammouda et al., 2011). This higher evening value was linked to a peak in core body temperature (Hammouda et al., 2012). Moreover, Dalton et al. (1997) thought that the higher evening core body temperature would increase the activity of some human body enzymes. Therefore, it could be that one of the factors causing fatigue and inflammation to occur in the evening is due to a lower antioxidant status efficiency compared to the morning (Hammouda et al., 2011).

Core body temperature increases when muscle temperature rises above rectal temperature, in this instance homeostasis preserves the thermoregulatory system. During exercise of long duration in thermo neutral environmental conditions the heat dissipation is facilitated by evaporation, whilst heat loss by other methods (convection, conduction, and radiation) is minimised (Racinais, 2008). During exercise, muscle contraction produces heat, and muscle heat production has been reported from the first minutes of exercise (Krustrup et al. 2001). However, during moderate exercise, muscle temperature rises quickly and can increase above core body temperature (rectal) after 10 minutes of exercise (Racinais, 2008).

Florida-James and Doggart (2000) found that participants who undertook a 10 minute warm up run prior to the test increased their bench press performance by 2.5% and their squat performance by 15% at 08:00 hs compared to no pre-exercise warm up. They also observed a peak in bench press and squat performances at 14:00 hs and 20:00 hs after 10 minutes warm up. Taylor et al. (2011) demonstrated the effects of increased core body temperature on exercise performance. They showed that warm-up in morning test

sessions had a positive effect on power and force loss in countermovement jumps. Participants were able to increase core body temperature to a level that was comparable to that of an afternoon session by adding an additional 20 minutes of active warm-up to a controlled warm-up program. The conclusion was that an increase in core body temperature had an effect on ballistic power output and other jump variables. The results of Taylor et al. (2011) are in partial agreement with Atkinson et al. (2005) who examined the effect of warm up on a 16.1 km cycling time trial. Results confirmed that warming up generally improved time trial performance at both periods of the day, however, mean cycling time was still slower in the morning (07:30 hs) compared to the afternoon (17:30 hs) (At 07:30 hs, cold 1426±104 seconds, warm 1405±97 seconds and at 17:00 hs, cold, 1370±99 seconds, hot, 1362±91 seconds). This leads to the hypothesis that increased core body temperature may improve sport performance as demonstrated by enhanced performance following a warm up.

Eccentric and concentric strength has been reported to show a peak value that coincides with peak core body temperature in the evening (Souissi et al., 2002). Souissi et al. (2007) supported their previous findings and investigated differences in peak power, mean power, total work done, and oxygen consumption between a morning (06:00 hs) and evening (18:00 hs) testing session using a 30 seconds Wingate protocol against resistance of 0.087 kg · kg(-1) body mass. The results were better in the afternoon compared to the morning (anaerobic performances in the morning = 3.9±0.9w; evening = 4.2±0.9w). Furthermore, power loss was greater in the morning than the afternoon (39.3±4.5% in the morning and 34.5±2.8% in the evening). This may, therefore, highlight the importance of warming up in the mornings or in colder climates to improve exercise performance. Nevertheless as can be seen from the above mentioned results there is not complete agreement with this conclusion.

The traditional view which correlates core body temperature and exercise performance has been challenged. Teo et al. (2011a) and Teo et al. (2011b) analysed neuromuscular performances and the immune system circadian rhythm which have revealed a distinct circadian rhythm in physiological variables independent of temperature changes (Teo et al., 2011a). Martin et al. (1999) investigated the effect of circadian rhythmicity on neural

activation and contractile properties of the human adductor pollicis muscle in 13 healthy participants at two different times of the day at 07:00 hs and 18:00 hs. The study showed that the force produced during a maximal voluntary contraction was 8.9% higher in the evening compared to the morning. Martin et al. (1999) suggested an existence of a correlation between diurnal fluctuations in force and the modulation of peripheral contractile mechanisms. They suggested that the production of force increases in the afternoon possibly due to sarcoplasmic reticulum release of enhanced calcium, increased calcium sensitivity of the contractile proteins and altered myosin ATP activity in the muscle.

Guette et al. (2005) reported that diurnal fluctuations in force may be due to the changes at the human muscular level. They showed a significant time-of-day effect on maximal voluntary contraction torque of the knee extensors for both dominant and non-dominant legs, the highest result being in the late afternoon (18:00 hs). The researchers suggested that this circadian rhythm in maximal voluntary contraction may be due to the actin-myosin cross bridge process where it is largely affected by the concentration of inorganic phosphate and that inorganic phosphate presents rhythmicity too. However, it is known that the human metabolic system consists of energy release and fuel reserve, with blood glucose influenced by multiple metabolic phenomena that regulate it and keep it balanced for a period of 24 hs (Yu et al., 2011).

Scientists have speculated that sport performance is affected by, and varies with, time of day (Table 2.1). However, diurnal differences in athletic performance could also be attributed to a number of environmental and physiological factors, such as hot and cold environments and body temperature. Furthermore, poor performance in the morning rather than in the evening could be attributed to dietary status (fasted, dehydrated), stiffness of joints following sleep, sleep inertia on arising, environmental temperature and lack of muscle warm-up (Waterhouse, 2010). In addition, previous studies may have underestimated the evening athletic performance due to the increases in physical and mental fatigue that occur through daytime activities (e.g. work and studies). The training status of participants involved (non-elite) may have contributed to reduced accuracy of these studies (Youngstedt and O'Connor, 1999).

Athletic performance should not be linked to diurnal variation alone, but rather it should be related to other endogenous and exogenous factors; i.e. sleep, diet, environmental temperature. Moreover, as indirect evidence, it has been observed that world records tend to be achieved in the early evening; the time of the day when core body temperature is at its highest level (Chtourou et al., 2011). British middle-distance runners Sebastian Coe and Steve Cram achieved world records in the evening time between 19:00 hs and 23:00 hs. Notwithstanding this, many sporting events tend to be scheduled for the evening which may create false evidence that circadian rhythmicity is the reason for the record. However, in support of this hypothesis Atkinson et al. (2005) and Kline et al. (2007) showed that performance of young cyclists and swimmers (cyclist undertaking a 16 km road race and swimmers 200m swim) was superior when the race or the trial was held in the afternoon or evening compared to the morning (cyclist, 1426±104 seconds at 07:30 hs versus 1362±91seconds 17:30 hs, swimming performance was significantly worse at 0200, 0500, and 0800 than at 1100, 1400, 1700, 2000, and 2300 hs). Irrespective of this, Kline et al. (2007) reported that swimming performance is independent of environmental (exogenous) and psychological (endogenous) masking effects.

Most researchers agree that sport performances are better in the evening than in the morning (Table 2.1), and that better performance in the evening is often linked to when core body temperature peaks occur. The lack of control over environmental influences such as environmental temperature, wind speed and water density, can nevertheless increase the masking effect of the circadian rhythm and affect the accuracy of studies. The failure to control the masking factors such as intensity of exercise, the fitness of the participants and diet has a large impact on the result of each study, and hence the true influence of circadian rhythmicity on sporting performance.

Table 2.1: List of studies showing circadian rhythmicity within different sport disciplines.

Exercise experiment	Studies	Summary
Cycling/ Wingate test	(Atkinson et al., 2005), (Chtourou et al., 2011) , (Souissi et al., 2002), (Atkinson et al., 2005), (Edwards et al., 2005), (Bessot et al., 2006), (Bessot et al., 2007), (Souissi et al., 2007)	A positive correlation between an increase in body temperature and an increase in sport performance was reported over the time of the day. Training at a specific time of the day may help to improve the performance at that particular time; however, better evening performances were reported.
Running	(Martin et al., 2001)	Body temperature shows a diurnal variation, physiological responses to running at lactate threshold speed are unaffected including heart rate, minute ventilation, oxygen uptake, carbon dioxide expired, respiratory exchange ratio.
Football	(Reilly et al., 2007) (Hammouda et al., 2013)	Specific skills in football show a consistency during the day: shooting accuracy and dribbling ability are better in the evening.
Tennis and Badminton	(Atkinson and Speirs, 1998) (Edwards et al., 2005)	There is an increase in the velocity of the serve shot and the racquet handgrip over the day. However, the accuracy of the serve was not affected by circadian rhythm.
Swimming	(Arnett, 2001) (Martin et al., 2007)	Swimming performance was found to be consistently greater in the evening and in correlation with body temperature.
Resistance	(Teo et al., 2011b) (Taylor et al., 2011)	Performances were higher during the day light in strength, power and anaerobic exercise.

2.2.2 Evidence of circadian rhythmicity in the cardiovascular and respiratory systems

The existence of circadian rhythms in cardiovascular function have been reported (Lemmer, 2007). However, most cardiovascular functions and their daily pattern have been examined by symptoms of disease. Several studies have reported a link between an animal's circadian system disruption and the development of cardiovascular illness (Martino et al., 2007; Bray et al., 2008). In humans the rate of the risk of cardiovascular events is higher in the morning compared to the rest of the day (around 09:00 hs and second peak at around 20:00 hs), and the circadian system alone has a large influence on these events (Elliott, 2001). Furthermore, the endogenous circadian system, including the wake-sleep cycle, and the modulation of cardiac function may contribute to the risk of cardiovascular events (Boudreau et al., 2012). Shift work, which can cause circadian rhythm disruptions, are additionally associated with a high rate of cardiovascular disease (Boggild and Knutsson, 1999).

Experimental research on animals has found that to activate wake from sleep the SCN during rest sends a signal to the heart via the autonomic nervous system as a response to light (Scheer et al., 2001). The heart is controlled by the SCN, but the autonomic nervous system is the main determiner of heart rate (Freeman, 2008). When resting, heart rate can indicate levels of fitness or the presence of illness, and during exercise it indicates level of fitness and the intensity of the exercise, with the maximum level being an indicator of fatigue (Robergs and Landwehr, 2002). Under submaximal exercise the heart rate of a healthy person increases linearly with the increase in oxygen uptake and exercise intensity. In fact, during progressive testing or a race, heart rate behaviour changes in a positive linear mode and shifts to two major transition points: point of inflection, where the body approaches the minimal lactate steady state and point of deflection where the body approaches the maximum lactate steady state. Maximum lactate steady state is defined as the highest steady state exercise level one can maintain while also maintaining an equilibrium between the elimination of blood lactate and the diffusion of lactate into the blood. The minimum lactate steady state is the minimum point on the lactate curve) (Buchheit et al., 2007). However, when the athlete nears the point of exhaustion, heart rate begins to level off and this is an indication of approaching the maximum value of

heart rate. However, it has been reported that endurance training decreases resting and submaximal heart rate (Perini et al., 2006), this decrease after endurance training could be due to a decrease in sympathetic activity to the athletes heart (Wilmore et al., 2001). In Wilmore et al. (2001) after a 20 week endurance training program in 507 healthy men and women with building up the intensity of the Wingate test from 55% of $\dot{V}O_2$max up till 75% of $\dot{V}O_2$max. The reduction in heart rate beat ranged between, low intensity 11.3 beats min^{-1} and at high intensity 0.6 beats min^{-1}, respectively. The author concluded that this reduction in heart rate beat during exercise were substantial and clinically important. In addition, it confirmed that the HR increases soon after the onset of a muscle contraction (Gladwell and Coote, 2002).

Heart rate has been shown to vary with an amplitude of 5 to 15% in 24 hours and an acrophase around 15:00 hs (Armstrong et al., 2011). Similar rhythm characteristics in stroke volume, blood flow, cardiac output and blood pressure are demonstrated (Jones et al., 2009; Atkinson, 2010). Refinetti, (2006) reported that heart rate and blood pressure are highly influenced by exogenous factors such as sleep, diet and exercise. Heart rate oscillates during the day and demonstrates an underlying endogenous circadian rhythm which is masked by activity, sleep and eating habits (Yoshizaki et al., 2013).

In a study conducted by Callard et al. (2001) heart rate was studied for a period of 24 hs in cyclists who were either continuously cycling (65% to 70% from maximal aerobic speed) or were at complete rest. In both conditions heart rate showed a significant diurnal variation, with a minimum value during the night (110 Beats $mins^{-1}$) and a maximum value in the early evening (140 Beats $mins^{-1}$). Moreover, Bessot et al. (2007) and Cruz et al. (2013) found no diurnal variation in heart rate in fifteen competitive endurance cyclists and eight runners, respectively. The cycling trial was carried out at two different times of the day, 06:00 hs and 18:00 hs, with RER showing a progressive liner increase with heart rate during the exercise. Where runners ran 3000m and 5000m along a measured running track (400m) in the fastest time at 3 different times of the day, morning (between 08:00 hs and 10:00 hs), afternoon (between 14:00 hs and 16:00 hs), and evening (between 18:00 hs and 20:00 hs). The study showed no difference in heart rate for each lap in both trials 3000m and 5000m during the three separate periods of the day. However, participants in

the morning showed higher physical demand in each lap compared to afternoon and evening. The author concluded that pacing strategy was not affected by time of day during these middle distance running trials. Participants demonstrated their best performance in the evening, in both distances, compared to the afternoon and the morning trials supporting previous findings of better evening performances (Atkinson et al., 2005).

Bilchick et al. (2006) defined heart rate variability as the physiological variation in the time interval between heartbeats in humans. Tsunoda et al. (2001) looked at the effect of light intensity and sleep stages on heart rate variability in healthy participants; where heart rate variability increased significantly during exposure to either bright light (10 000 lx) or extreme dark (< 0.01 lx). Furthermore, during exposure to a hot environment, heart rate increases when heat storage exceeds heat removal.

Human physiological parameters are associated with each other as demonstrated by Guo et al., (2012), who found elevated blood pressures were associated with high heart rate and this increases the risk of cardiovascular problems (prevalence of 30.5% among men and 28.5% among women in USA population). Blood pressure is the pressure exerted by circulating blood upon the walls of blood vessels, were cardiac output increases as a result of peripheral resistance (Klabunde, 2005). Systolic and diastolic blood pressure were reported to rise in the morning with a peak at noon and a nadir during sleep time (Jones et al., 2006). Moreover, Ingelsson et al. (2006) reported a link between blood pressure pattern and the risk of congestive heart failure; where it is reported that high blood pressure at night may lead to congestive heart failure. Jones et al. (2008) investigated the masking effect of nocturnal sleep on blood pressure post-exercise. After a 4 hour night sleep, participants cycled 30 minutes at different intensities (70% and 40% of $\dot{V}O_2max$) at 04:00, 06:00, 08:00, and 10:00 hs, on different days and after a 4 hour day sleep (nap) and at 16:00, 18:00, 20:00, and 22:00 hs. Mean arterial blood pressure (MAP physiologically is the pressure that is primarily regulated) was higher in the morning post-exercise compared to the evening. Miyai et al. (2002) stated that exaggerated blood pressure is linked to the increase of heart rate during rowing exercise, where this increase in blood pressure is associated with the development of hypertension. It is difficult to ascertain the diurnal blood pressure's response during exercise, as most of the existing

research has looked at pre and post-exercise without examining blood pressure during exercise.

The cardiovascular system and the respiratory system works to ensure cellular and organ functioning. The pulmonary system (respiratory system and ventilatory system) consists of specific respiratory organs and structures involved in the intake of oxygen and exchange of carbon dioxide between the human body and the environment (Maton et al., 2010). Nevertheless, there is a two quite different meanings of respiration, firstly in the utilisation of oxygen in the metabolism of organic molecules by cells and secondly, the organism exchange of the oxygen and carbon dioxide between cardiorespiratory system and the external environment. However, most of cells energy is obtained from a chemical reaction involving oxygen (Cotes at al., 2009).

The human body comprises of two lungs and each lung is divided into several lobes. In addition the lung is designed to provide an adequate distribution of inspired air and pulmonary blood flow. This lung design allows the exchange of oxygen and carbon dioxide between alveolar and the pulmonary capillary blood. Therefore, alveoli is the site of the gas exchange with the blood and the airways. This gas exchange or breathing cycles is called ventilation (Cotes at al., 2009). Therefore, ventilation is the process by which air moves into (inspiration, is the air movement into the alveoli during breathing) and out (expiration, the opposite direction of the air movement in the lung) of the lungs exchanging the gas across the alveolar-capillary membrane (Polak and Mroczka, 2006).

The path of this ventilation is called the respiratory tract (the pathway of the air from the nose to the lungs), the respiratory tract includes upper and lower respiratory system. The upper respiratory tract consists of the nostrils, nasal cavities, pharynx, epiglottis, and the larynx. Whereas, the lower respiratory tract consists of the trachea, bronchi, bronchioles, and the lungs (Cotes at al., 2009).

During the breathing process the nasal hair partially filters the air entering through the nostrils, afterward, this air will flow into the nasal cavity (breathing air consists of 78% nitrogen, 21% oxygen, and 1% other gases). The nasal cavity secretes mucous which

further filters the air (Elad et al., 2005). In addition, cilia (tiny hair) in the endothelial lining of the nasal cavity serve to transport foreign particles including dust which is trapped in mucous at the nasal cavity to the pharynx. After passing through the nasal cavity, the air flows down to the lower respiration tract where gas exchange actually takes place (Cotes at al., 2009).

During the respiration the right ventricle of the heart pumps the blood through the capillaries surrounding each alveolus. In addition, an extremely thin membrane separates blood capillary and the alveolar air from each other, across which oxygen and carbon dioxide diffuse (Polak and Mroczka, 2006). However, at rest in normal conditions, approximately 4 liters of the air enters and leaves the alveoli every minute in a healthy adult, while 5 liters of the blood flows through the pulmonary capillary (West, 2012). Furthermore, during exercise the rate of ventilation increases as a result of work load (Harms et al., 2000).

The alveoli oxygenation and the oxygen consumption in the cell create a partial pressure of oxygen (P_{O2}) gradient that produces net diffusion of oxygen from the alveoli to blood in the lungs and from the blood to cells in the rest of the body. Contrariwise, the carbon dioxide production by the cells and its elimination from the alveoli via expiration, create partial pressure of carbon dioxide (P_{CO2}) gradients that produce net diffusion of carbon dioxide from cells to blood in the rest of the body and from blood to alveoli in the lungs (Cotes et al., 2009).

Lung function testing procedure is widely used to assess and monitor lung and respiratory related diseases. These tests are used to establish baseline lung function, monitor effects of therapies, evaluate dyspnea, used to treat respiratory disease, detect pulmonary disease, evaluate respiratory impairment and other measurements (Ruppel, 2012). In addition, using computer controlled lung function equipment allows researchers to perform an array of tests to investigate lung function in a repeatable and standardised manner (Ruppel, 2012). Thus, some of the more common values that may be measured during lung volume testing include, Forced vital capacity (FVC) which is the maximum amount of air a person can expel from the lungs after a maximum inhalation, Forced expiratory

volume (FEV1) which is forced expired volume in one second, Forced expiratory flow (FEF) which is the average rate of flow during the middle half of the FVC test and Peak expiratory flow (PEF) which is the maximum speed of expiration (Pellegrino et al., 2005). However, a number of factors must be considered when testing lung volumes including: matching of patient populations; the same size and the age range of participants actually studied (Tafuro et al., 2014).

The parameters of lung function tend to have a relationship with lifestyle where physical activity can play a role in overall lung capacity (ShobhaRani Vedala et al., 2013). ShobhaRani et al. (2013) showed that athletes reported higher lung function parameters than sedentary population (FVC 88 versus 79.8, FEV1 86 versus 72 and PEF 93 versus 86.4). In addition, Adegoke and Argundade, (2002) suggested that respiratory function may increase as a response to chronic exercise which could be due to increased development of respiratory musculature incidental to physical training. In contrast Tasgın and Dönmez (2009) indicated that exercise had no effect on FVC, FEV1 after a 3 month training program performed in children aged 10-16 years. Atan at al. (2012) compared the lung capacity between different sports types (football, basketball, hand ball, volleyball and sedentary males), with the highest value in lung function parameters recorded in handball players.

Several studies have shown that physically intense and prolonged exercise causes significant stress to the respiratory system from the associated hyperventilation and increased airway exposure to toxins in inhaled air during daily training (Rundell et al., 2008). Chimenti et al. (2010) reported that endurance athletes including runners present a remarkable increase in inflammatory cell counts including, increases in bronchial biopsies, bronchio-alveolar lavage fluid or induced sputum compared to rest (increase in neutrophils and lymphocytes). Physiologically, the main indictors of pulmonary airway resistance are forced expiratory volume (FEV) and peak expiratory flow (PEF), and both are affected by the time of the day, falling to a minimum between 03:00 hs and 08:00 hs (Spengler and Shea, 2000). A diurnal rhythm in pulmonary function exists in both a healthy population and individuals with chronic diseases (Spengler and Shea, 2000). Spengler and Shea (2000) stated that the pulmonary airway variables in healthy

individuals occurred within the usual sleep time. This finding suggests that diurnal rhythms of pulmonary function are partly controlled by the endogenous circadian pacemaker.

Previous research suggested that asthmatic athletes or those that developed airway inflammation are advised not to perform intense training early in the morning for the reason that exercise causes cooling and drying of the lining of the air passages which can increase asthma symptoms (Smolensky and Alonso, 1997). Additionally, lung function according to circadian physiology will rise throughout the day and peak at 16:00 hs, and this coincides with the time of peak performance of patients with upper airway inflammation (Medarov et al., 2008). Medarov et al. (2008) undertook lung function testing for a large number (4756) of individuals. Tests were performed 9 times intervals within the same day (8:00 - 8:59 hs; 9:00 - 9:59 hs; 10:00 - 10:59 hs; 11:00 - 11:59 hs; 12:00 - 12:59 hs; 13:00 - 13:59 hs; 14:00 - 14:59 hs; 15:00 - 15:59 hs; and 16:00 -16:59 hs). Forced expiratory volume in 1 second (FEV1) between the 12:00–12:59 hs and 16:00–16:59 hs intervals was 17.6% (Difference between the lowest and the highest value). Mean lowest FEV1, FVC, PEF and FEV1/FVC values were recorded in the 12:00 - 12:59 hs interval. Mean highest values were recorded in the 15:00 - 15:59 hs and 16:00 - 16:59 pm intervals. In addition, two additional smaller peaks were observed at 08:00 - 08:59 hs and 11:00 - 11:59 hs. This rhythmicity has a direct effect on the best times to engage in daily activities. The author showed pulmonary airway function, as determined by spirometery, to have an independent circadian rhythm that may govern the daily energy intake for the human body's exertion throughout the daytime. Moreover, Medarov et al. (2008) suggested for patients on bronchodilators to take their medication at midday (where FEV1/FVC ratio is low), when an extra boost is needed, rather than later in the day when lung function peaks. Kelly et al. (2004) reported that for the airway inflamed population, a correlation between lung function variations and inflammatory changes during a 24 hour period exist. Cheng et al. (2003) found that a healthy active population has higher lung function measurements than sedentary people.

The factors that determine the magnitude of variation in pulmonary function are still under debate. There is growing evidence that circadian rhythms cause dangerous changes

in the bronchial diameter of unhealthy populations suffering with chronic airway diseases, in contrast little change occurs in the healthy population. Therefore, there are numerous immunological and physiological variables that could create diurnal rhythms in the severity of airway diseases. Knowing the circadian rhythm of these variables in both healthy and unhealthy populations can allow for greater understanding of risk factors associated with airway diseases in athletes engaging in prolonged exercise. This is important as there is a growing body of evidence showing the increased development of upper respiratory tract infection in athletes after intense or prolonged exercise (Walsh et al., 2011). This topic will be discussed in more detail later in Section 2.5.

2.3 Physiological circadian rhythm in humans

2.3.1 Body temperature

Core body temperature in humans varies during day time by just 1.0ºC, regulated at 37ºC ±1ºC. The thermoregulatory centre in the hypothalamus plays a very active role in keeping core body temperature in the normal range. There are exogenous (*climatic*) and endogenous (*metabolic*) heat source factors that may influence core body temperature such as: heavy exercise, illness, hot/cold environment, ambient temperature, humidity, air movement, and radiant heat from the sun, as well as warm surfaces that all contribute to climatic heat stress (Lim et al., 2008). The human body temperature is comprised of core and shell temperatures. The core temperature refers to the temperatures of the internal body organs and cavities including abdominal, thoracic and cranial cavities, whereas the shell temperature refers to the temperatures of the skin, subcutaneous tissue and muscles (Cossins, 2000). Core temperature is regulated and controlled by the hypothalamus (approximately 36.8°C during rest); whereas shell temperature is influenced more by environmental conditions and skin blood flow (Cossins, 2000). Core body temperature is well-documented and has been under investigation since before the 20th century. An early study by Gierse (1842 cited in Krauchi, 2002), measured core body temperature orally and reported a difference of 0.9°C with maximum temperature reported in the early evening and the lowest in the early morning. In studies conducted by Waterhouse et al. (2004) and Waterhouse et al. (2005) the circadian rhythm of core body temperature was shown to be controlled by the changes in body heat production and

heat loss. They concluded that heat production undergoes a circadian rhythm which is phase advanced by 1.2 hours with respect to the circadian rhythm of heat loss, and this peak of temperature occurs when heat production surpasses heat loss. Heat production under resting conditions depends mainly on internal organs' metabolic activity. Moreover, these internal organs produce 70% of the entire resting metabolic rate of the human body (Krauchi, 2002) with heat transported by blood from the core to the skin.

Environmental conditions such as heat, cold, air movement, and humidity are important determinants of heat loss. Heat exchange with the environment occurs often by means of conduction, convection, radiation and evaporation. During exercise the accumulation of heat stress in the body starts when the heat loss mechanisms are unable to cope with metabolic heat production, this imbalance leads to an increase in core body temperature (Lim et al., 2008). During exercise, an increase of 10 to 20 fold in metabolic heat production has been recorded with less than 30% of the heat generated being converted to mechanical energy, and the remaining 70% transported to the skin for dissipation to the environment (Lim et al., 2008). In core body temperature regulation, it is essential that the body transfers internal heat to the external environment. However, the mechanism of losing heat will apply once the heat reaches the skin and is transferred to the external environment. Krauchi (2002) attributes the circadian rhythm of core body temperature to fluctuations in heat loss mechanisms rather than heat production.

Atkinson and Davenne (2007) reported that the two major exogenous factors that influence core body temperature are sleep and exercise. Mongrain et al. (2004) demonstrated the relationship between core body temperature and performance while controlling for circadian rhythm and hours awake. Both groups reported that a higher core body temperature represents an increase in physiological arousal that enhances human performance. Wesensten et al. (2002) provided evidence that core body temperature can be affected by pharmacological agents (e.g. caffeine, modafinil). Additionally, bright light exposure has also been found to influence core body temperature: for example, exposure to bright light in the evening tends to phase delay the temperature rhythm by several hours (Kubota et al., 1998).

Sport performance is markedly affected by changes in core body temperature (Hammouda et al., 2012). The effect of a raised core body temperature through exercise is, however, dependent on the type, duration and intensity of exercise performed (Nybo and Nielsen, 2001a). Prolonged maximal exercise performance is reduced as a result of core body temperature increases (Gregson et al., 2002), whilst on the other hand, an increase in core body temperature before a long-duration exercise can decrease performance by altering the heat storage capacity. In contrast, sprint performance is enhanced when muscle temperature is elevated (Ball et al. 1999). An elevation of 0.6°C in core body temperature in the afternoon compared to the morning resulted in significantly better performance in a Wingate test (Hammouda et al., 2011). In Faulkner et al. (2012) external heating of the quadriceps and hamstrings (muscle temperature) during periods of inactivity between 30 seconds cycling trial leads to a significant and relevant improvement in peak power output (by 9.6% and 9.1%, respectively). This result may have important practical implications for sprint performance, were the athletes may experience competition delays following the completion of a warm-up.

In the study of El Helou et al. (2012) heat was reported to have a significant effect on marathon runners' performance. The study analysed the performance of 1,791,972 participants from six major marathons worldwide (Paris, London, Berlin, Boston, Chicago and New York) between the years 2001 to 2010. In addition, several studies supported the presence of supremacy in exercise performance in cool conditions compared to hot conditions (Tatterson et al., 2000). Nybo and Nielsen (2001b) linked this impaired performance to central fatigue, in which motor-unit recruitment fails after the core body temperature reaches approximately 40°C (due to the hyperventilation cerebral blood flow is reduced by approximately 20% during exercise with hyperthermia). This suggests the inability of the brain to recruit motor units to allow muscle contraction to continue at the required work rate. Indeed, in long-duration exercises such as marathons, an increase in core body temperature is the main factor in decreasing performance as the heat storage capacity is altered (Gonzales-Alonso et al., 1999). In contrast, pre-cooling increased the running time to exhaustion due to the capacity for increased heat storage (Gonzales-Alonso et al., 1999). Moreover, Gonzales-Alonso et al. (1999) stated that time to exhaustion during cycle ergometer exercise at 60% of maximal $\dot{V}O_2$ uptake at 40°C is

directly related to cyclists' heat storage. There was a correlation between the increase in heat storage and the rate of fatigue in trained cyclists, and in addition, the increase in core body temperature (2–3°C) and skin blood flow (4-fold) caused significant reductions in cardiac output and a larger decline in stroke volume. Heat storage varies depending on the time of the day and this together with core temperature circadian variation, will modify the capacity of heat storage and the exercise ability during prolonged exercise. Furthermore, heart rate increase paralleled with the increase in core body temperature (36 to 40°C) and with reductions in stroke volume (Gonzales-Alonso et al., 1999).

Aldemir et al. (2000), found that core body temperature increased significantly in the morning compared to the evening, although heat storage was greater in the morning (08:00 hs in heat gain mode), than in the late afternoon (18:00 hs in heat loss mode). The mechanism behind reductions in performance due to high core temperature is still unclear and the effects of body temperature on heat storage or loads fluctuate between intensity short-duration, and long duration intensity exercise. However, an increase in muscle temperature increases athletes performance due to a number of muscle mechanism changes, such as reduction in muscle joint and muscle stiffness, increase in the rate of nerve impulse transmission, and skeletal muscle contractile properties. During prolonged exercise, heat production increases as a result of an increase in core body temperature. Indeed, skeletal muscle temperature rises because of exercise, and this continual rise in muscle temperature decreases performance instead of benefiting performance.

Morrison et al. (2004) elevated core body temperature using a water-perfused jacket from 37.4 to 39.4°C, measured by rectal probe. Higher systemic concentrations of serum prolactin, which is a marker of central fatigue, were observed compared to exercise without thermal stress (37.4°C using a liquid conditioning garment). Changes in electroencephalogram could be another factor of central fatigue and reductions in the blood supply to critical parts of the brain indicating a reduction in arousal, alertness and motivation to exercise (Nybo and Nielsen, 2001b). The relationship between electroencephalogram and circadian rhythm physiology will be discussed later in this review in Section 2.6.7.

2.3.2 Melatonin

Melatonin is a hormone that is produced mainly at night by the pineal gland in the mammal's brain. The melatonin secretion cycle is generated by the suprachiasmatic nuclei and relies almost entirely on the night and day alternation, therefore maintaining the body's circadian rhythm and regulating other hormones (Bhatti et al., 2014). Maximum melatonin plasma levels occur around 03:00 to 04:00 hs under normal environmental conditions (the nocturnal peak can be 30 times higher than day time levels) (Atkinson et al., 2003). Additionally, melatonin production can be suppressed or entrained according to the time and length of light exposure (Arendt, 2000).

Melatonin synchronizes endogenous cycles (biological), predominantly core body temperature and sleep/wake cycles. A study suggested that melatonin levels vary with age group: with children reporting the highest nocturnal levels of melatonin and these levels dropping with age (Cornelissen et al., 2000). Melatonin circadian rhythm production is inversely related to core body temperature circadian rhythm, with the downturn of the melatonin curve corresponding with an upturn of core body temperature and vice-versa (Cajochen et al., 2005). Additionally, a study reported a strong antioxidant effect of melatonin. Kim et al. (2012) suggests that melatonin may help to prevent and strengthen the immune system. Exogenous melatonin can be used to treat disorders of biological rhythms (shift workers, jet leg), with melatonin hypothesised to decrease the symptoms of jet lag in athletes (Manfredini et al., 2000). Buxton et al. (1997) found that melatonin secretion was low with reduced activity (40 to 60% for a 3h duration from maximum $\dot{V}O_2max$), but increased more during intense exercise (workload of 75% $\dot{V}O_2max$ for a duration of 1h). In addition to the effects on sport performance, melatonin may have sleep promoting properties (jet leg in athletes). It has been found to induce changes associated with sleep: to lower core body temperature and induce sedation (Stone et al., 2000).

Several studies have instigated the melatonin secretion response to exercise. However, it is still under debate whether melatonin concentration changes in athletes (increase, decrease or remain stable) by exercise (a single bout). Nevertheless, higher levels of melatonin have been reported in high intensity exercise studies compared to low

intensities (Buxton et al., 2003), with age and fitness level of the participants (Barriga et al., 2000), lighting conditions and the time of day the exercise is performed also affecting melatonin concentration levels (Barger et al., 2004). In Lucia el al. (2001) nine professional cyclists who participated in a three week tour race (Vuelta a España 1999). Morning urinary levels of 6-sulphatoxymelatonin (aMT6s) (80 - 90% of the melatonin is secreted as 6-Sulfatoxymelatonin in the urine) were collected, mean urinary aMT6s levels increased significantly during the day after each stage (1091 versus 683 $ng.ml^{-1}$ at the end of the first week; 955 versus 473 $ng{\cdot}ml^{-1}$ at the end of the second week and 647 versus 337 $ng{\cdot}ml^{-1}$ at the end of the 3 week).

Barriga et al. (2000) measured salivary melatonin levels in both sedentary and trained boys and girls, aged between 14 and 15 years before and after daytime physical activity. No significant differences between pre- and post-exercise in melatonin concentrations were recorded in both boys groups. Whereas, both of the girls groups showed an exercise-induced increase in melatonin, were the change was higher in the trained girls compared to the sedentary group. Furthermore, in nocturnal animal studies it was found that an increase in melatonin level occurred during darkness when these animals increased their activity (Kennaway et al., 2002).

2.4 Circadian rhythmicity of the immune system

The human immune system is a complex defence system that consists of many different types of cells that protect the body against health disorders by identifying and killing pathogens and tumour cells (Abbas et al., 2012). Innate immunity (also known as the non-specific immune system) is a subsystem of the immune system and is the primary defence against infectious pathogens and is involved in the repair of damaged tissue (Walsh et al., 2011). In terms of pathogen recognition, innate immunity has no memory function and is only able to deal with pathogens in a non-specific, generic way. Cells of innate immunity include eosinophils, neutrophils, natural killer cells and monocytes (Walsh et al., 2011). Neutrophils are the first cells to migrate to sites of infection and induce the local state of inflammation. Monocytes and neutrophils are primarily responsible for capture, engulfment, and breakdown of microorganisms (phagocytosis). In addition,

neutrophil cells account for 50 to 60% of the total white blood cells (Nierhaus et al., 2013). The increase in percentage and numbers of neutrophils is a good marker and indicator of increased levels of inflammation.

Another important subsystem is adaptive immunity (also known as the acquired immune system) which combats infections by destroying invading micro-organisms. Unlike innate immunity, adaptive immunity is able to retain the memory of a pathogen once it has been exposed to it, which enables a faster response if exposed to the same pathogen. Cells of the adaptive immune system include T cells, B cells and basophils (Abbas et al., 2012). Furthermore, T cells and B cells are a subset of lymphocyte cells; T cells are responsible for cellular direction of the immune response, whereas B cells are responsible for humoral immunity by producing antibodies.

The effects of exercise on immune function are well documented (Nieman, 2006; Whitham et al., 2006; Nieman et al., 2011). The relationship between exercise intensity, immunity, and infection was studied for the first time early in the 20th century. Larrabee (1902 cited in Nieman, 2007) showed a large increase in blood neutrophils among four athletes who ran the 1901 Boston Marathon and reported changes in white blood cell differential counts. Ostrowski et al. (1999) showed that running a full marathon increases circulating white blood cell and cytokines plasma levels (pro- and anti-inflammatory). Horn et al. (2010) stated that athletes engaging in endurance sport, at rest present a normal level of blood circulating neutrophil numbers. However, studies conducted by Horn et al. (2010) showed lower neutrophil counts in trained distance cyclists and elite triathlon athletes (2.8 and $2.9\times10^{9}.l^{-1}$, respectively). Nevertheless, the number of circulating neutrophils can increase during or after exercise and depends on the intensity and duration of exercise (Shaukat et al., 2003). This increase is due to the detachment of these cells from the vascular endothelium and has been recorded in both trained and untrained individuals.

Exercise increases the concentration of lymphocytes in the blood; however, lymphocytosis becomes established during a variety of human activities including walking (Nieman et al., 2005), running (Shaukat et al., 2003) cycling (Tauler et al., 2006),

and rowing (Morici et al., 2005). This increase in lymphocyte counts is related to both the duration and the intensity of exercise (Reihmane et al., 2013). In contrast, the number of circulating lymphocytes decreases below resting value for several hours after exercise the classic bi-phasic response (Simpson et al., 2006 and Figure 2.3). Moreover, in Simpson et al. (2006) and Simpson et al. (2007a) the number of circulating lymphocyte cells fall below the resting value during the early stages of recovery, before steadily returning to resting values. In Simpson et al. (2007a) eight male runners performed an intensive treadmill-running protocol at 80% from their $\dot{V}O_2$max until exhaustion. Blood lymphocyte counts were measured pre-, post- and 1h post-exercise. Total lymphocytes post-trial was higher by 81% compared to pre- trial, whereas 1h post- trial 77% below the pre-trial counts (pre-exercise 1.6±05, post-exercise 2.9±0.9, 1h post-exercise 0.9±0.4, value are mean ± SD in $\times 10^9.l^{-1}$).

In addition, Ronsen et al. (2001) reported that the second daily maximal exercise was associated with higher concentrations of lymphocytes (1-fold higher). Moreover, after a second bout of exercise it seems the immune system is further suppressed. However, the recovery between exercise workload sessions is very important to maintain the immune system.

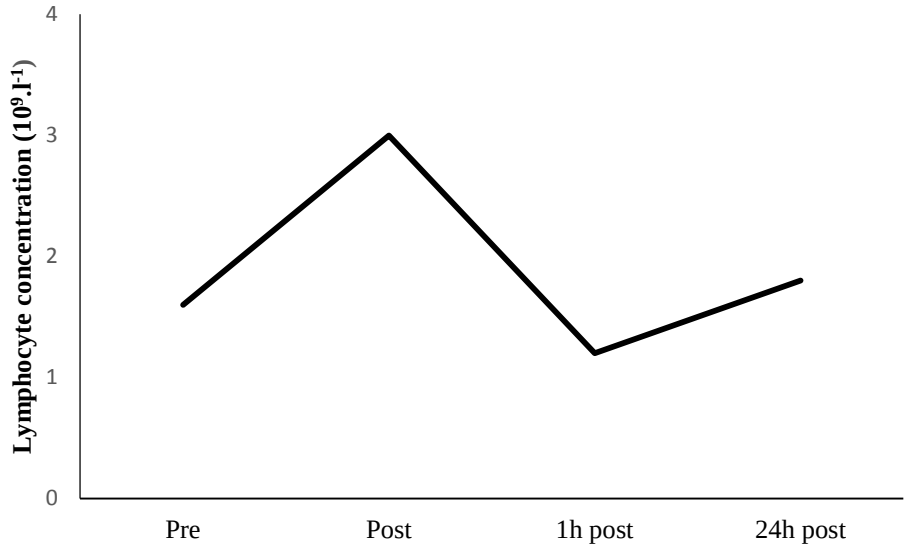

Figure 2.3: Lymphocyte bi-phasic response to exercise, adapted from Simpson et al. (2006), page 113.

White blood cell counts consist of 1% to 3% of monocytes in the body, depending on the individual level of health. These cells come from the bone marrow; monocytes migrate to the site of infection in approximately one to three days. Monocytes act in the same way as neutrophils, in that they are the first line of defence to eliminate infections, and are involved in muscle tissue inflammation caused by exercise (Peake et al., 2005a). Acute exercise increases monocytes circulating in the blood; they are transformed into macrophages when they are moved to injured or damaged tissue (Lu et al., 2011). Macrophages are essential for muscle repair and perform several functions in the damaged tissue (Chazaud et al., 2003).

Several studies have reported that monocytes increase during exercise; an early peak immediately post-exercise with a sustained increase up to two hours from post-exercise (Ramel et al., 2003; Mayhew et al., 2005; Morici et al., 2005). Such as in Ramel et al. (2003) monocytes recorded after 2 hours post- exercise 84% and 37% higher in non-resistance trained participants and resistance trained participants, respectively, compared to pre-exercise (878±345 in non-resistance trained participants and 571±213 in resistance trained participants, value are mean ±SD in $\times 10^{3}.ml^{-1}$). Most studies regarding the impact of exercise on circulating monocytes are involved in resistance exercise where the stimulus duration is short and lasts only a few minutes. The studies that looked at the effect of prolonged exercise on circulating monocytes are limited and more studies are needed to address this gap in knowledge.

During infection, inflammation or other foreign substance invasion, the body's lymphocytes and neutrophils release substances to attract eosinophils and then release toxic substances to kill the invader. Eosinophil numbers rise in blood as a reaction to allergy and in parasitic diseases (MacKenzie et al., 2001). Moreover, eosinophilia plays an important role in asthmatic airway inflammation or allergy (Jacobsen et al., 2008). A link between the increase in eosinophil count in blood and the presence of an allergic reaction has been found, but the increase in eosinophil count during exercise is less understood (Setyawan, 2005). It has been reported that exercise enhances athletes' resistance to upper respiratory tract infection and the reduction of allergic recurrence (Moreira et al., 2009). In Setyawan (2005) blood eosinophil was measured in eighty two

males divided into three groups; the study showed that intensity of exercise increased eosinophil concentration in athletes' blood (control group, 0.28±0.2, group 1, 0.31±0.32, group 2, 0.35±0.23, group 3, 0.43±0.31. value mean + SD in $\times 10^3.ml^{-1}$).

Most of the literature reviewed relating to intense exercise and the immune system agrees that immune function changes dramatically after prolonged and intensive exercise. During this altered immunity "open window", which may last several hours, the risk of bacteria and viruses gaining a foothold may increase (Kakanis et al., 2010). It has been reported that circadian rhythms exists in the human immune system (Haus and Smolensky, 1999; Dimitriou et al., 2002; Miles et al., 2008). Haus and Smolensky (1999) reported that circulation of white blood cells presents a high-amplitude circadian rhythm: being lower in the morning and higher in the afternoon. In addition, circadian rhythmicity was reported in patients with infection (Wang et al., 2014), rheumatoid arthritis (Cutolo et al., 2005), and asthma (Merikanto et al., 2014). However, underlying mechanisms of circulating blood cells and circadian changes in athletes have not been fully understood. It is unknown how the circadian rhythm system and the immune system are communicating. It could be that circadian controlled hormonal factors by the autonomic nervous system may regulate gene expression and protein activity (Mendez-Ferrer et al., 2008). Another possibility is that immune cell local clocks are directly controlled by cellular immune functions (Keller et al., 2009).

Circadian rhythm of immune function is an important factor for the regulation of cellular homeostasis being responsible for growth and aging (Reppert et al., 2002). Any change in clock genes can effect human sleep and lead to sleep disorders (Toh et al., 2001). Monocytes and neutrophil levels fall during the day, whereas the number of lymphocytes peak during the night. Eosinophil counts show a considerable diurnal variation which can be as much as 100% (Rothenberg, 1998), where the lowest counts are found in the late morning (10:00 hs to 12:00 hs) and the highest counts during the night (00:00 hs to 04:00 hs). Rothenberg linked the morning eosinophil lowest values to the same time at which endogenous steroids are the lowest. Diurnal variations in monocyte subsets were assessed in sixteen healthy volunteers by Shantsila et al. (2012): participants undertook the Bruce protocol and continued until exhaustion (06:00 hs, 12:00 hs, 00:00 hs and 18:00 hs).

General monocyte counts showed no diurnal variation during the day (457 cells/ul at 06:00 hs, 463 cells/ul at 12:00 hs, 458 cells/ul at 18:00 hs and 439 cells/ul at 00:00 hs); whereas, monocyte subsets presented a significant diurnal variation (34 cells/ul at 06:00 hs, 34 cells/ul at 12:00 hs, 51 cells/ul at 18:00 hs and 39 cells/ul at 00:00 hs).

2.5 The impact of environmental temperature on the immune system

2.5.1 Immune system response to exercise in a hot environment

Several studies suggested that moderate exercise may positively enhance immune function above inactive levels, while high intensity exercise may impair immune function (Mooren et al., 2012; Reihmane et al., 2013).

It is well known that heat stress causes greater physiological demands on the human body than a thermo-neutral environment. Several of these exacerbated physiological demands may possibly be responsible for eliciting a larger number of white blood cells when exposed to the heat (Mitchell et al., 2002; Niess et al., 2003: Starkie et al., 2005). A rise of at least 1.2°C in core body temperature will increase circulating white blood cells and hormone levels after exercise ($8 \times 10^{9}.l^{-1}$ after 3hs for neutrophils and 600 $nmol \cdot l^{-1}$ for cortisol) (Niess et al., 2003). Scientists report that exercise in the heat leads to higher circulating levels of stress hormones and increased cardiac output, both of which are potential mediating factors for neutrophilia (Shephard, 1998). Limited studies have been conducted in relation to the effect of exercise in a warm environment on immune function and exercise rhythmicity.

Neutrophil response to exercise in heat is not clear, some studies suggest there is little or no difference in neutrophil counts during exposure to heat following exercise compared to a thermo neutral environment (McFarlin and Mitchell, 2003); while others suggest that exposure to heat during exercise causes a significant increase in neutrophil circulating numbers compared to exercise at neutral conditions (Mitchell et al., 2002; Niess et al., 2003). Niess et al. (2003) found that neutrophil counts were considerably higher in the hot environment only at 2 and 3 hours after 60 minutes of treadmill run (7 highly trained runners, 60 minutes treadmill run at 28°C and 18°C, neutrophils counts were, ($8 \times 10^{9}.l^{-1}$)

after 3hs post-exercise compared to ($2.5 \times 10^9.l^{-1}$) at post-exercise. In Mitchell et al. (2002) 10 trained cyclist, cycling 75 minutes at 22°C and 30% relative humidity, and again at 38°C and 45% relative humidity. In addition, in Starkie et al. (2005) seven male cyclists, cycled for 90 minutes, at 15°C and 35°C, ($8.1 \pm 1.7 \times 10^9.l^{-1}$) in hot conditions compared to ($7.3 \pm 1.3 \times 10^9.l^{-1}$) in cooler conditions.

Contrasting the above-mentioned studies McFarlin and Mitchell (2003) investigated 10 male participants who completed two 60 minute cycling trials at an intensity of 60% $\dot{V}O_2$max in two different environmental conditions; a hot condition at 38°C with 45% relative humidity and a cold condition at 8°C with 50% relative humidity. They reported that neutrophil counts were not significantly different between the hot or cold environmental conditions ($4.5 \times 10^9.l^{-1}$ in hot conditions compared to $4 \times 10^9.l^{-1}$ in cold conditions).

Exercise in the heat appears to exacerbate the athlete's immune system response by rising circulating neutrophil counts. The question then arises, is this increase in circulating neutrophils in response to the heat a protective or harmful response and will it damage or repair healthy host tissue? Clinically, individuals with chronic inflammatory diseases exercising in a hot environment (for example: rheumatoid arthritis) may need to employ caution because the increased neutrophils after exercise might cause tissue damage and prolong the tissue repair process (Krüger et al., 2014). Coaches and athletes have to be aware of when individuals with chronic inflammation or disease are engaged in physical activity and exposed to heat. This may lead to altering the exercisers training program to allow the body a longer recovery period giving tissue time to repair, especially if that is an alien environment to their normal training environment.

It has been reported that exercise in a hot environment increases the number of circulating lymphocytes. The usual exercise-induced response for circulating lymphocytes is an elevation followed by either a return to pre- exercise or a slight reduction below the pre-exercise value after two hours of recovery (Bi-phasic). In Mitchell et al. (2002) lymphocyte counts increased post-exercise in the heat and decreased after 2 hs post-exercise under both conditions (pre-exercise 2 and 1.99 cells$\times 10^{6}.l^{-1}$, post-exercise 2.5

and 2.4 cells×10^{6}.l^{-1} and 2h-post-exercise 1.5 and 1.5 cells×10^{6}.l^{-1}) respectively. Furthermore, McFarlin and Mitchel (2003) found that lymphocyte counts were higher by 24% post-exercise in the heat compared to the same exercise in the cold, and either returned to the pre- exercise value or showed a slight reduction below baseline at 2 hs post- exercise (in hot environment, pre-exercise 2.1 cells×10^{9}.l^{-1}, post-exercise, 3.5 cells×10^{9}.l^{-1} and 2h-post-exericse, 2.1 cells×10^{9}.l^{-1}, in cold environment, pre-exercise 2.6 cells×10^{9}.l^{-1}, post-exercise, 2.7 cells×10^{9}.l^{-1} and 2h-post-exericse, 2.6 cells×10^{9}.l^{-1}). In contrast, lymphocyte counts in the cold environment were higher than the hot environment after 2 hs post-exercise (2.6 cells×10^{9}.l^{-1} in cold environment versus 2.1 cells×10^{9}.l^{-1} in hot environment). Romeo et al. (2008) stated that no difference was observed in monocyte counts after testing 22 males in environmental conditions of 35°C for a duration of 60 minutes run at 60% $\dot{V}O_2$max (0.30±0.12 ×10^{9}.l^{-1} at pre-exercise compared to 0.29±0.12 ×10^{9}.l^{-1} at post-exercise). One of the major limitations in this study was that only a single bout of exercise was used without including a group control. Whereas, Fehrenbach at al. (2001) showed a maximum increase observed after 24 hs and a slight decrease after 48 hs from post-exercise, where the study tested two groups at two different environment temperatures (18°C and 28°C), the participants performed a 60 minute run. There is a limitation to research investigating the effect of eosinophils in sport performance in general and in particular during prolonged exercise. Romeo et al. (2008) showed no significant difference in basophil counts after exercise compared to pre-exercise in a hot condition (0.03±0.02 ×10^{9}.l^{-1} at pre-exercise compared to 0.02±0.01 ×10^{9}.l^{-1} at post exercise).

2.5.2 Immune system response to exercise in a cold environment

As discussed previously, white blood cells respond biphasicaly to exercise in both cold and warm environments (Mitchell et al., 2002; Niess et al., 2003). This response is characterised by an initial increase during exercise, followed by a secondary larger increase two hours post-exercise. Several studies have implicated immune system suppression after strenuous exercise to be responsible for the increased incidence of URTI in athletes in different types of sport (Moreira et al., 2009). Studies with athletes and army personnel showed that infection and illness peak mostly at winter time when environmental temperatures are lowest (Whitham et al., 2006). McFarlin and Mitchell

(2003) had 10 participants perform 60 minutes of cycling at 60% of their $\dot{V}O_2$max in two conditions: hot (38°C, 50% relative humidity) and cold (8°C, 50% relative humidity). The authors reported no significant difference between neutrophil counts, but lymphocyte counts were lower by 24% post-exercise in the cold environment.

Beachey (2012) found exposure to a cold environment can damage the physical barriers to pathogens, increase mucus viscosity, and decrease the action of cilia in the upper respiratory system. Therefore, this decrease in mucosal immune function in athletes during the winter period might explain the increases in URTI via a decrease in the effect of the protective mucosal surfaces' antibodies. Hence, the body increases hormone release during exposure to cold air, leading to an increase in circulating white cells, and a reduction in the production of an inflammatory mediator (Brenner et al., 1999). However, exercising at a specific time of day may be able to reduce the risk of infections; which could lead coaches and athletes to better understand at which time of day exercise can be performed with less risk of infections.

2.6 Airway inflammation and sport performance

Inflammation is the body's response to perturbations of homeostasis, which include infection, trauma, and hypersensitivity (Pillarisetti et al., 2011; Ramlackhansingh et al., 2011; Humes et al., 2012). However, inflammation is a complex possess and involves a variety of mechanisms to defend against pathogens and repair damaged tissue (Corradi et al., 2002). This process is to prevent the nearby tissues from damaging agents, dispose of cells and pathogens and finally to set repair process. Inflammation may occur as a result of external injury, chemical or radiation response, bacteria, viruses and disease, such as bronchitis, where the inflammation occurs in the bronchi. In addition the four cardinal signs of inflammation are redness, heat, swelling and pain.

The inflammatory process begins with the body's release of inflammatory chemicals including cytokine which plays an important role in the development of an inflammatory process (inflammatory mediators) (Miles et al., 2008). Therefore, an increase in inflammatory cells in the blood stream, such as neutrophils, macrophages, Interleukin-6

(IL-6), Interleukin-8 (IL-8), and prostaglandins will occur (Jankord et al., 2004; Pedersen, 2007; Gomes et al., 2011). In addition to the above cited process, the body releases hormones bradykinin and histamine that cause blood vessels in the injured tissue to dilate, which allow more blood supplies to reach the injured tissue (more defense cells can enter the affected tissue). Thus, the immune system releases more cells along with the blood to the injured tissue, to help with the healing process (Neveu et al., 2010). Neutrophils and lymphocytes are some of the immune system cells that are released along with blood to the injured tissue (MacKenzie et al., 2001).

Immune system cells are classified as innate (non-specific) and adaptive cells, where innate cells are the primary defence against infectious pathogens and are involved in the repair of damaged tissue (Krüger et al., 2014). Cells of innate immunity include eosinophils, neutrophils, natural killer cells and monocytes (Walsh et al., 2011). Therefore, neutrophils are the first cells to migrate to sites of infection and induce the local state of inflammation. Monocytes and neutrophils are primarily responsible for the phagocytosis process. In addition, the increase in circulating neutrophil numbers is a good marker and indicator of increased levels of inflammation (MacIntyre et al., 2001). Adaptive immunity cells are able to retain the memory of a pathogen once it has been exposed to it, which enables a faster response if exposed to the same pathogen. These cells include T cells, B cells and basophils. Where, T cells and B cells are a subset of lymphocyte cells; T cells are responsible for cellular direction of the immune response, whereas B cells are responsible for humoral immunity by producing antibodies (Janeway et al., 2001).

The upper and lower airway can be classified as a major site of pathogen entry into the human body and therefore require effective and rapid innate response to prevent pathogens from establishing infection and to minimize their spread in the rest of the body. Lung inflammation is usually caused by exposure to pollutants (Gomes et al., 2010), toxins, irritants (Rundell et al., 2008) and allergens or pathogens (Bauer et al., 2012). Furthermore, the airway epithelium is the first site of contact with inhaled agents. Hence, the epithelial cells secrete a variety of substances such as lactoferrin, defensins, lysozyme, nitric oxide, and mucins, which non-specifically protect the respiratory tract from

infectious pathogens. The epithelial cells also produce a number of mediators such as cytokines and reactive oxygen radicals to recruit inflammatory cells onto the site of inflammation (Gleeson et al., 2013).

The dendritic cells and macrophages are the first defence line in recognizing various pathogens during lung inflammation. Macrophages were found in the airways and alveoli or migrate into the lung microvasculature (Lodoen et al., 2006). However, the main source of cytokines, chemokines, and other inflammatory mediators that suppress the immune response is macrophage. Following an inflammation, epithelial cells and macrophages secrete cytokines and chemokines, promoting neutrophil accumulation and local inflammation (Bender et al., 2005). In addition, during pulmonary infection neutrophils provide second-line of defence and migrate out of the pulmonary capillaries and into the air spaces. After phagocytosis process, neutrophils kill ingested pathogen with reactive oxygen species, antimicrobial proteins and degradative enzymes (elastase) (Mizgerd, 2002).

Pillarisetti et al. (2011) reported that pulmonary inflammation is associated with lower lung function and pulmonary infection is associated with a greater rate of decline in lung function. Haverkamp et al. (2007) studied 19 asthmatic participants before and after 6 weeks' treatment with an inhaled corticosteroid or placebo. The participant group treated with inhaled corticosteroid had decreased exercise-induced bronchospasm, improved resting pulmonary function and decreased post-exercise sputum histamine. The author linked this improvement in lung function performance due to increase in alveolar ventilation and improvement in pulmonary gas exchange efficiency.

There is no doubt that upper respiratory tract infection or airway inflammation is associated with exercise, predominantly in elite athletes (Bougault et al., 2009). Airway inflammation is known as an irritation in the human airways and is the response of the immune system to a perceived threat and causes symptoms such as swelling. It has been reported that elite athletes present an increased risk of asthma including those who take part in endurance sports, such as running, cycling and swimming (Couto et al., 2013). Studies conducted by Voy (1986) and Goubault et al. (2001) showed that at the 1984 Los

Angeles Olympic Games, 41 of 67 athletes with proven exercise induced asthma won medals in a wide range of sports including basketball, swimming, rowing and athletics. Weiler et al. (2000) additionally studied the prevalence of asthma among American athletes participating in the 1996 Atlanta Olympic Games. According to the study of 699 athletes, 15.3% reported a diagnosis of asthma, 13.9% had used an asthma medication in the past, and 10.4% were receiving asthma treatment during their event at the Games. In addition 5.6% from the athletes that took part in the Olympic Games 2000 in Sydney used inhaler β2-agonists (Fitch et al., 2008) and 813 applications made use of inhaled β2-agonists, and 781 of these were approved in Beijing Olympic Games (World Allergy Organisation 2009).

Study results show that the prevalence of asthma is higher in athletes than in the general population. Moreover, airway inflammation prevalence is greater among elite athletes than in the general population (Bougault et al., 2009), and is higher among certain groups of athletes such as distance runners and cyclists. The reason for this inflammation is unknown, but potentially can be caused by exposure to different environmental conditions such as cold dry air, airborne allergens or environmental irritants (Martin et al., 2012). This may be due to frequent exposure to adverse environmental conditions during daily training such as cold or hot environments (Bougault et al., 2009). The effect of exercise on the immune system can be described in three main theories: the J-curve theory (Nieman, 1994); the "open window" theory (Kakanis et al., 2010); and the S-curve theory (Malm, 2006). The J-curve model (Figure 2.4) is designed for a recreational population, and when elite athletes are included the curve is more S-shaped (Figure 2.5). The S-shape hypothesis predicts that low and high load intensity increases the infection odds ratio, while moderate and elite exercise load intensities decreases the infection odds ratio. In recent decades, the technology development for rapid DNA sequencing and genotyping has permitted the discovery of some of the elite athletes' genetic variations that contribute to athletic performance (MacArthur and North 2005). For example genetic influences have been identified in anaerobic performance and explosive power (skeletal muscle strength) (Calvo et al., 2002). However, this decrease of the rate of infections in elite athletes compared to non-elite athletes could be down to the genetic superiority of elite athletes. Another factor that makes elite athlete's immune system able to withstand

infections even during severe physiological and psychological stress are environment factors. During daily training athletes learn to deal with different environment conditions. A limitation of this hypothesis (genetic variations that contribute to athletic performance) is that a larger number of participants are needed to adequately verify results. Access to large numbers of elite athletes is not easy, and coached athletes need to have an individual assessment.

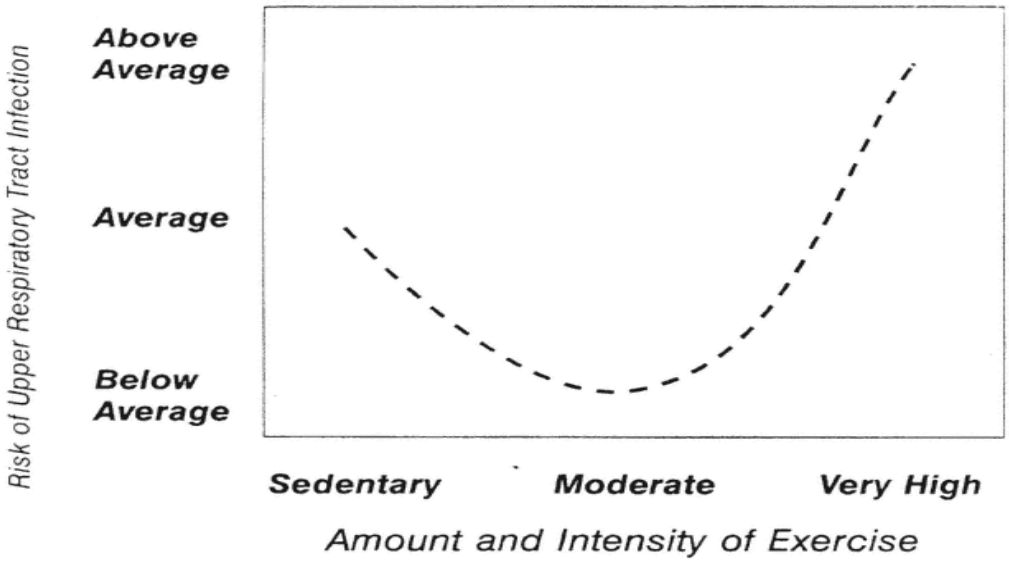

Figure 2.4: The 'J' shaped model, the relationship between exercise intensity and the risk of upper respiratory tract infection. This model sited by Nieman (1994) suggested that prolonged high intensity exercise led to increased risk of upper respiratory tract infection, whereas, moderate aerobic exercise reduces the risk of infection.

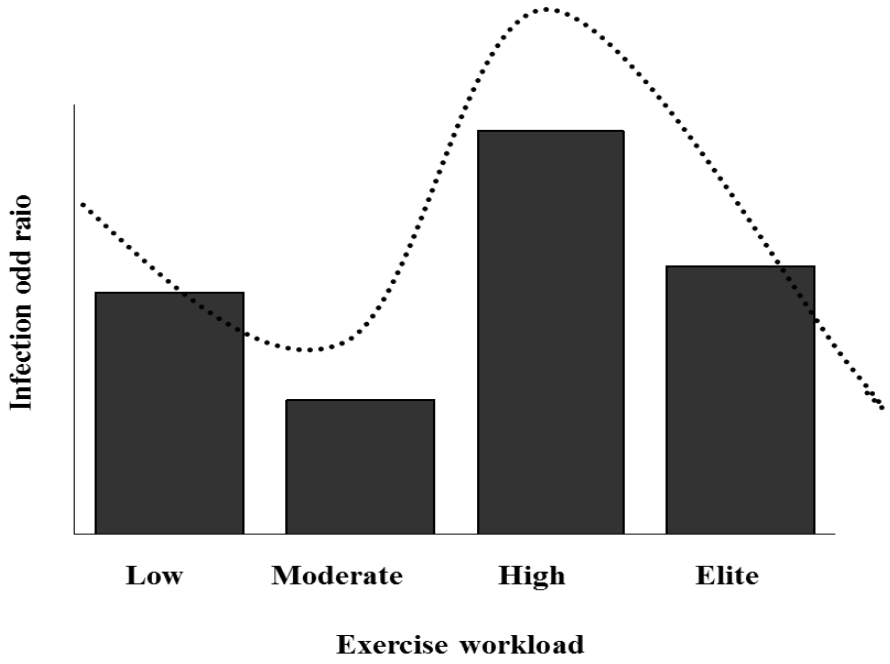

Figure 2.5: The 'S' shaped model, the relationship between exercise intensity and the risk of upper respiratory tract infection in different categories. This model sited by Malm (2005) predicts that low and maximum load intensity increases the infection odds ratio, while moderate and high exercise decreases the infection odds ratio. However, to become an elite athlete, need to possess an immune system which is able to withstand infections even during severe physiological and psychological stress.

The J-curve is a model designed by Nieman (1994) that shows the relationship between exercise and the risk of upper respiratory tract infection. In elite athletes, upper respiratory tract infections are the most common problem reported to the sports clinic (Engebretsen et al., 2010). The debate of the causes of upper respiratory tract infection remains unclear, however, there have been a number of hypotheses put forward by scientists, and include causes due to an infection, or due to other inflammatory stimuli associated with exercise (Martin et al., 2012). The lack of pathogen identification in studies investigating upper respiratory tract infection and exercise mean that the causes remain unclear. However, moderate exercise and balanced training leads to better performance and better immune system surveillance, but strenuous exercise suppresses the immune system function briefly. This suppression of the immune system can provide an "open window" period in which the immune system is weakened for 3 to 72 hours after prolonged strenuous exercise (Figure 2.6) (Kakanis et al., 2010).

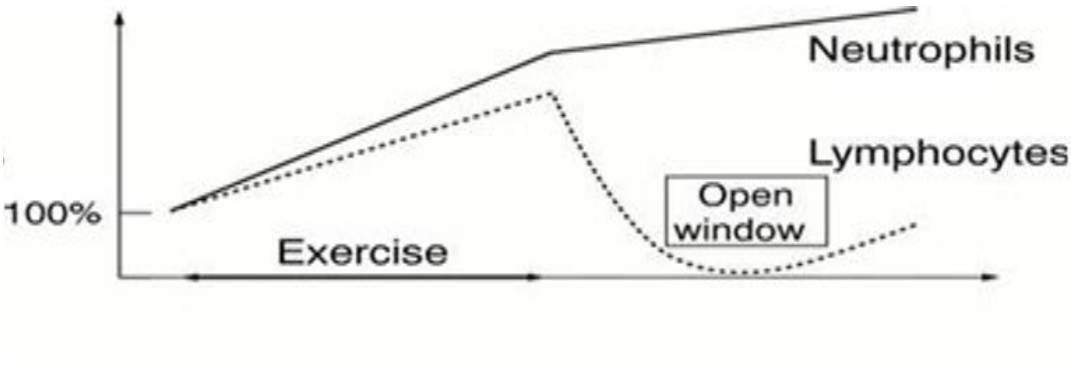

Figure 2.6: Immune system open window to infection (Pedersen and Toft 2000).

Mochida et al. (2007) showed that neutrophils' phagocytic function is impaired after strenuous exercise. Immunoglobulin A (IgA) in saliva takes up to 18 hours, after intense exercise, to return to baseline level in triathletes after undertaking a race (increased from 204 ug/ml to 369 ug/ml) (Steerenberg et al., 1997). Langdeau and Boulet (2001) reported that athletes performing in winter sports had airway hyper responsiveness compared to the general population (the athletes had a 49% prevalence of hyper responsiveness). In contrast, Ekblom et al. (2006) did not support the theory of an "open window" in recreational runners, and suggested the rate of infections in athletes can be caused by work loaded exercise too soon after an infection.

The concept of moderate exercise protecting against upper respiratory tract infection is not well understood and remains a topic of debate between scientists. Nonetheless, this type of exercise appears to positively boost the immune system through increasing natural killers and neutrophils by acquired immunity and increasing antibodies (Murphy et al., 2004). This boost leads to an improvement in immune system function against pathogens for up to 3 hours, and if moderate exercise is regular, can reduce the risk of upper respiratory tract infection by 18% to 67% in both genders of all ages (Matthews et al., 2002; Nieman, 2011). Psychological factors may also positively contribute to reduced risk of upper respiratory tract infection during moderate exercise, through the reduction of emotional stress. A healthy lifestyle combined with a good diet and regular sleep pattern also plays an important role in reducing the risk of upper respiratory tract infection (Wolvers et al., 2006; Cohen et al., 2009).

Foster (1998) reported that a high percentage of sicknesses can occur when elite athletes train above lactate threshold levels, and is mostly related to the intensity and quality of training. These findings are supported by studies in the animal model were it has been identified that one or two periods of exhaustive exercise leads to infections and to a higher fatality rate in animals (Davis et al., 2007). Thirty-two elite and 31 recreationally competitive cyclists and triathletes, and 20 sedentary controls took part in Spence et al. (2007) study (age range 18-34 year). Nasopharyngeal and throat swabs were used to determine URTI. The rate ratios for illness were higher in both the control participants (1.93, 95% CI: 0.72-5.18) and elite athletes (4.50, 1.91-10.59) than in the recreationally competitive athletes. The results confirm a higher rate of upper respiratory tract infection among elite athletes than recreationally competitive athletes. However, Nieman (2005) reported that 43% of runners experience fewer colds than their healthy inactive control population.

Moderate exercise has been shown to lower the risk of upper respiratory tract infection compared to both sedentary behaviour and high intensity exercise in healthy adults (Nieman et al., 2011). Several studies have reported that the risk of upper respiratory tract infection is elevated during periods of heavy training and in the period of up to two weeks post-endurance races, such as in marathon and cross country skiing (Nieman, 2007). The direct evidence that athletes engaged in intense prolonged training are more susceptible to upper respiratory tract infection is reported from athletes and coaches (Powers and Howley, 2012). For instance, Nieman (1998) reported that the Olympian marathon runner Alberto Salazar caught 12 colds in 12 months prior to the 1984 Olympic marathon. Furthermore, there is a significant link between the severity and length of viral infections and the development of chronic fatigue, which can be an additional threat to the athlete.

Table 2.2: The effect of strenuous exercise on selected immune system and physiological parameters

Parameters	During exercise	Post-exercise (1h up to 24 hs)
Neutrophil	↑	↑
Monocyte	-	↑
Lymphocyte	↑	↓
Eosinophil	-	↓
Basophil	-	-
IL-1	↑	↑
IL-6	↑	↑
IL-10	↑	↑
TNF-α	↑	↑
Cortisol	↑	↑
HSP70	↑	↑
CC16	*	↑
Heart rate	↑	↓
Core body temperature	↑	↓

↑ Increase; ↓ decrease; - no changes; * not been investigated ; TNF-α, tumour necrosis factor-α; IL interleukin; HSP70 heat shock protein 70; CC16 Clara cells 16; heart rate Heart rate; core body temperature.

To the best of our knowledge there are few studies that have investigated the effect of the time of the day and training on upper respiratory tract infection and sports performance. The combination of upper respiratory tract infection and exercise may lead to chronic stress. This, affects and masks the endogenous circadian rhythms in athletes and significantly elevates stress hormone concentration for prolonged periods (open window), suppresses the immune system and increases susceptibility to infection (Dimitriou et al., 2002). In Spengler and Shea (2000) the circadian minima of lung function variables including forced expiratory volume in 1 seconds, (FEV1), forced expiratory volume (FEV) and peak expiratory flow (PEF) in 10 healthy adults occurred within the usual sleep period. Panzer et al. (2003) work supports previous studies and showed that airway function in asthma increases symptoms and decreases lung function during the nocturnal period and the early morning. This change is associated with increased diurnal variations in peak expiratory flow rate (PEFR) (Wijnhoven et al., 2001). Spengler and Shea (2000)

additionally reported the amplitude of variation to be between 3 - 4% of the Mesor value, an amplitude which is within the normal range of healthy individuals. Moreover, previous studies noticed that changes in PEFR are greater in patients with severe asthma compared to those with mild airway inflammation. Kraft et al. (1998) related PEFR variability to diurnal changes of endogenous hormones such as cortisol. Furthermore, intensity, workload (of exercise) and environmental conditions have been identified as the principal key of airway epithelial injury during exercise (Morici et al., 2004; Chimenti et al., 2009).

A limited number of studies reported upper respiratory tract infection in sport performance when the outcomes were based on questionable resulting conclusions. Human participants were too dissimilar to confidently compare with one another. The type of sport, fitness of participants, environmental conditions, stress levels and condition will make any generalisation open to question. Evidence supporting clinical upper respiratory tract infection in sport performance is limited and future studies should address this. It is known that the concentration of many cytokines increase dramatically in response to stressful exercise such as with IL-6 (Sugama et al., 2012). These facts indicate that IL-6 may represent an important marker as a mediator in the development of an inflammatory process, and represents an important link between muscle contraction and exercise-related metabolic changes.

2.6.1 Interleukin 6 (IL-6)

Cytokines are soluble glycoproteins produced by several cell types, including immune cells, endothelial cells, myocytes, and adipocytes. The human body's production of cytokines by white blood cells and other body cells facilitates intercellular signalling during the activation of innate and specific immunity (Theze, 1999). These regulate a wide array of pathophysiological and physiological processes, including the initiation and coordination of the immune system, inflammatory responses in human health and disease (Parsons et al., 2005). In a healthy population plasma cytokine levels at rest are kept very low (Fernandez-Real et al., 2001). However, the concept of IL-6 being either bad or good with regards to metabolism has recently been debated in a counterpoint discussion (Pedersen et al., 2007).

This cytokine is also involved in neutrophil activation and is released from several cell types as a response to an inflammatory stimulus (Takahashi et al., 2001). Together with IL-1 and TNF-α stimulate inflammatory response (Steensberg et al., 2000). Furthermore, IL-6 is a good cytokine biomarker during immunological investigations of responses to exercise (Steensberg et al., 2000; Thompson et al., 2010). Oberholzer et al. (2000); Moldoveanu et al. (2001) and Sugama et al. (2012) suggested that strenuous exercise elicits a cascade of cytokine secretion in athletes' bodies and dramatically increases the concentration of many cytokines in response to stressful exercise. Peake et al. (2008) reported that intense exercise increased cytokine production and entailed increases in both pro-inflammatory, such as IL-6 and TNFα, and anti-inflammatories, such as IL-1ra, IL-10. Several studies show an increase in IL-6 over 100 times resting values post- resistance exercise, post-moderate intensity exercise (in plasma, at pre marathon, 1.27±1.19 $pg{\cdot}ml^{-1}$ in urine 2.86±6.91 $pg{\cdot}ml^{-1}$ and at post-marathon, in plasma 101.40±50.34 $pg{\cdot}ml^{-1}$, in urine 23.60±19.94 $pg{\cdot}ml^{-1}$) and post-marathon races (Suzuki et al., 2003). This rise in IL-6 may last for up several hours after the end of the exercise (Kasprowicz et al., 2013).

The amount that IL-6 increases is correlated to exercise intensity, duration, muscle mass and power involved in the mechanical work. Moreover, it has been suggested that the release of IL-6 in exercise is related to the occurrence of muscle damage (Sugama et al., 2012). Exercise immunologists have used various protocols to investigate cytokine responses to muscle damage. These protocols include downhill running, eccentric exercise of the leg or arm muscles, and resistance exercise (see Table 2.3).

Table 2.3: Summary of IL-6 responses to different types of exercise

Reference	Type of exercise	Post- exercise	More than 1h post-exercise
Peake el al. (2005) Smith et al. (2007)	Downhill running	↑IL-6	↓50% IL-6 (after 12h compare to post- exercise value)
Bruusgaard et al. (1997)	30 minutes concentric exercise and 30 minutes eccentric exercise and cycling.	↑IL-6	↑IL-6 after 24h
Toft at al. (2002)	60 min of eccentric lower limb exercise.	↑IL-6	↑IL-6 peaked after 4h
Paulsen et al. (2005)	Eccentric & quadriceps	↔ IL-6	↑IL-6 increase after 6h.
Willoughby et al. (2003)	Knee extensors	↔ IL-6	↑IL-6 increase after 6h.
Peake et al. (2006)	Elbow flexor	↔ IL-6	↑IL-6 increase after 3h.
Uchida et al. (2009)	Bench press exercise	↔ IL-6	Not measured
Kasprowicz et al. (2013)	Running a 100-km ultra-marathon	_	↑IL-6 peak at 75 km and remain higher to pre- value after 14h post- run.
Robson-Ansley et al. (2008)	Cycling a total distance of 468 km over 6 days	↑IL-6	↑IL-6 was elevated immediately post-exercise on Day 1 but was unchanged at rest for the duration of the event
Wright et al. (2012)	Cycling 15 minutes and 46°C, 10% relative humidity and 33°C, 60% relative humidity	↑IL-6 both higher. Hot and dry was much higher than hot and humid	Not measured
Patterson et al. (2008)	Cycling 60 minutes at 0°C and 20°C.	↑IL-6 both higher	↑IL-6 higher after 1h at 20°C. At 0°C was lower.

During exercise, muscle contractions release IL6 where it is primarily regulated by an altered intramuscular milieu (Mckay et al., 2009). Accordingly, an imbalance in glucose availability will occur and this is due to a combination of changes in calcium homeostasis and increased formation of reactive oxygen species, however, these changes are associated with exercise known to regulate IL-6 levels. Furthermore, IL-6 in humans is linked to an increase in lipolysis, fat oxidation and insulin-mediated glucose disposal (Carey et al., 2006). IL-6 appears to play an important role in some modelling effects (Sugama et al., 2012). Finally, IL-6 facilitates an anti-inflammatory environment and this could affect some of its biological effects via inhibition of the pro-inflammatory TNF-a (Pedersen et al., 2007).

Diurnal variation in IL-6

Vgontzas et al. (2003) reported an IL-6 circadian rhythm in healthy active individuals, which peaked during sleep, between 01:00 hs and 05:00 hs and the nadir was reported during morning hours between 08:00 hs and 10:00 hs (Figure 2.7). Vgontzas et al. (2003) compared IL-6 in two groups (young and elderly) and observed a biphasic circadian pattern of IL-6 secretion with two nadirs at approximately 09:00 hs-10:00 hs and at 21:00 hs, and two zeniths at approximately 05:00 hs and 03:00 hs for both groups. The authors suggested the elevated circulating early morning IL-6 and cortisol in healthy adults are due to sleep disruption effects (slept poorly at night or awakening from sleep). IL-6 and cortisol mean concentrations after 24 hours were significantly higher in the elderly group compared to the young group, with IL-6 leading cortisol.

Miles et al. (2008) observed diurnal variation in IL-6, with a decrease at 12:00 hs, 16:00 hs and 20:00 hs compared to 07:00 hs during high-force eccentric resistance exercise using the elbow flexor muscles (1.5 $pg{\cdot}ml^{-1}$, 1.4 $pg{\cdot}ml^{-1}$ and 2 $pg{\cdot}ml^{-1}$ compared to 2.6 $pg{\cdot}ml^{-1}$). Furthermore, a peak in plasma IL-6 concentration was reported after 8 hs post-exercise (2.6 $pg{\cdot}ml^{-1}$). In DeRijk et al. (1997) participants performed a run at a speed of 7 $mile.hr^{-1}$ with an increasing incline of 2.5% every 2 minutes, the plasma concentrations of IL-6 were not statistically different between morning and evening. This suggests that both intensity and duration of exercise have an effect on the IL-6 circadian rhythm. This may be due to the resistance of IL-6 production to cortisol suppression, in contrast with

other cytokine, such as TNFa (very sensitive). Furthermore, early morning eccentric exercise is associated with high IL-6 concentration (Miles et al., 2008). Most studies of circadian rhythmicity in IL-6 are linked to the clinical conditions in which they have been observed, whereas studies investigating the effect of exercise and circadian rhythm on IL-6 are limited.

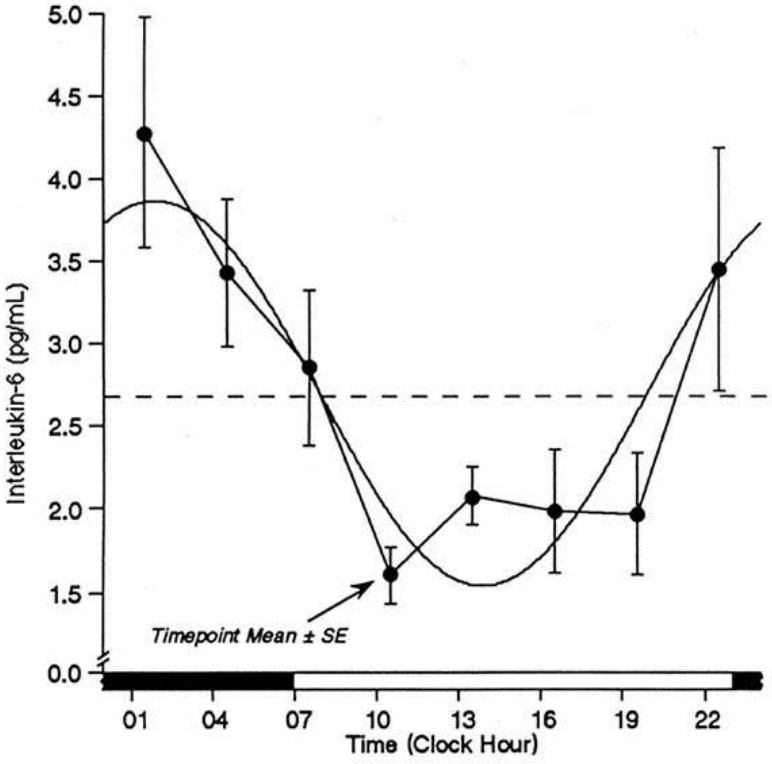

Figure 2.7: Circadian rhythm in serum IL-6 in 11 males between 46 to 72 years old. Samples were taken every 3 hours for a period of 24 hours (Sothern et al., 1995, page 1032).

Heat impact on IL-6 during exercise

Cosio-Lima et al. (2011) studied the effect of exercising in heat on IL-6 in six elite male cyclists, the cyclists biked for 2.5 hs at 60% from maximum power capacity or 75% from their $\dot{V}O_2max$. The cyclists were asked to cycle at two different temperatures set at 15°C with 40% relative humidity and at 35°C with 40% relative humidity. Plasma IL-6 levels were significantly higher post-exercise in both neutral and in hot conditions (3.4 ± 0.9 $pg \cdot ml^{-1}$ in neutral condition and 1.7 ± 0.2 $pg \cdot ml^{-1}$ in hot condition at post-exercise compared to 0.1 ± 0.0 $pg \cdot ml^{-1}$ in neutral condition and 0.3 ± 0.1 $pg \cdot ml^{-1}$ in hot condition

at pre-exercise). The author stated that IL-6 increased at post-exercise, where heat exposure tends to stimulate the release of IL-6. Moreover, Brenner et al. (1999b) reported that slight increases of plasma IL-6 measured after 60 min of cycling at 35°C vanished when the same exercise procedure was performed at 18°C. A study conducted by Selkirk et al. (2008) in which participating participants were divided into two groups (12 trained participants and 11 untrained participants) with an experimental test of walking at 4.5 km. hr^{-1} with 2% elevation in a climatic chamber (40°C and 30% relative humidity), wearing protective clothing until exhaustion (army personal study). IL-6 showed a consistent increase throughout the heat stress trial in both groups (started from below 3 $pg{\cdot}ml^{-1}$ at rest and increased up to 10 $pg{\cdot}ml^{-1}$ at post-exercise). Moreover, Starkie et al. (2005) investigated the IL-6 response in 7 endurance male cyclists for duration of 90 minutes at 70% from their $\dot{V}O_2max$ at 15°C and 35°C, IL-6 was higher at post-exercise in hotter conditions compared to the control (at 15°C 0.3 $pg{\cdot}ml^{-1}$ at rest increased to 0.7 $pg{\cdot}ml^{-1}$ at post-exercise and at 35°C at rest 0.3 $pg{\cdot}ml^{-1}$ increased to 3.5 $pg{\cdot}ml^{-1}$ at post exercise).

Peake et al. (2008) exposed well trained males to environmental temperatures of 18°C and 32°C during two different exercise trials. Each trial consisted of 90 minutes of cycling at 60% $\dot{V}O_2max$ and was directly followed by a 16.1 km time cycling trial; IL-6 was higher post- exercise at 32°C compared to 18°C (7.1 $pg{\cdot}ml^{-1}$ at 32°C compared to 6.1 $pg{\cdot}ml^{-1}$ at 18°C). Cosio-Lima et al. (2011) stated that IL-6 can serve as a glucoregulatory hormone in cyclists, which are highly reliant on carbohydrates during the 2.5 h exercise bout. On the other hand, the evidence that could make IL-6 anti-inflammatory is due to the fact that IL-6 levels paralleled the level at post-exercise with cortisol. However, it is not clear if IL-6 played an anti-inflammatory role or acted as a glucoregulatory hormone during exposure to heat (Cosio-Lima et al., 2011). As IL-6 functions as a glucoregulatory hormone and when muscle glycogen levels fall during exercise contracting skeletal muscles are responsible for the release of IL-6. Furthermore, Lim et al. (2009) investigated 18 male runners exercising in the heat (35°C and 40% relative humidity), where they performed 14 days of normal training or 14 days of 20% intensity load (2 equal groups). The study found that the increase in IL-6 levels responded as an anti-inflammatory cytokine in athletes exposed to tolerable heat values.

2.6.2 Cortisol

When the human body is exposed to stress, the adrenal gland releases cortisol. Physical exercise forces the body to deviate temporarily from its natural set-point and causes a temporary stress on the body. However, studies have shown that regular exercise allows the body to respond better to stress over time, resulting in less cortisol being released (Hill et al., 2008).

Brenner et al. (1998) stated that cortisol values generally increase when exposed to a stressor, and that the cortisol response to exercise is modulated by the time of day. It has been shown that cortisol has a positive response to exercise, with cortisol release increasing post-exercise, even for moderate intesity exercise (Fatouros et al., 2010). Ramel et al. (2003) showed 60 minutes post-resistance exercise cortisol either returns to, or falls below, pre-exercise concentrations (1146+317 $nmo{\cdot}l^{-1}$ at rest, 843+443 $nmo{\cdot}l^{-1}$ at post-exercise and 632+612 $nmo{\cdot}l^{-1}$ 2h-post-exericse). In contrast, neutrophils (4.34+1.50 versus 2.45+0.93 cells × $10^{6}{\cdot}ml^{-1}$) and monocytes (480+131 versus 416+141 cells $\times 10^{3}{\cdot}ml^{-1}$) showed an increase in concentrations post- exercise, which may be due to a relationship between changes in cortisol and white blood cell subsets post- exercise (Ramel et al., 2003). Nevertheless, the release of cortisol is due to physical and/or psychological stress and one of the main roles of cortisol during exercise is maintaining blood glucose levels by increasing amino acid and lipid mobilisation and this occurs by acting upon skeletal muscle and adipose tissue to increase amino acid and lipid mobilization (Galbo, 2001; Wolfe, 2001).

Niess et al. (2003) tested seven trained non-acclimatised runners who performed a 60 minutes run at 28°C and 50% humidity at 75% $\dot{V}O_2max$. The study found that plasma cortisol was only elevated by 0.5 nmol/L after 3 hours post-exercise in the heat when compared to the same exercises performed at 15°C and 50% humidity. However, these contrary study findings have been criticised in the literature for a number of reasons. Cosio-Lima et al. (2011) mentioned that there was a lack of low plasma cortisol levels which could be attributed to the short duration of the trial. We can speculate that 60 minutes of exercise is enough time to stress the body enhancing the athletes' cortisol levels and the lack of low plasma cortisol could be due to the high fitness levels of the

participants and not the environmental conditions. The fitness level of the athletes that took part in the Neiss at al. (2003) study could also have resulted in quicker recovery time, found in physically fit participants compared to untrained participants. The results may also have been skewed as a result of the small size of the study group. Furthermore, earlier studies suggested that cortisol concentrations increase only when exercise intensity exceeds 60% of $\dot{V}O_2max$ (Hill et al., 2008).

Cortisol circadian rhythmicity

The circadian rhythm of cortisol has a nadir value first occurring at about 00:00 hs, then starts to rise approximately 2–3 hours after sleep onset and continuing to rise until early morning, with the peak cortisol value recorded at approximately 09:00 hs, followed by a steady decline as the day continues (Debono et al. 2009, Figure 2.8). Kanaley et al. (2001) tested ten healthy moderately trained males at a constant velocity on the treadmill for 30 minutes on three separate days, at 07:00 hs, 19:00 hs and 00:00 hs. Peak cortisol concentrations were highest at 07:00 hs, followed by 00:00 hs, then 19:00 hs. In contrast, cortisol concentrations over time showed the maximal increase in comparison to control conditions at 00:00 hs, followed by 07:00 hs, then 19:00 hs.

In Dimitriou et al. (2002), 14 competitive swimmers performed two swims at two different times of the day 06:00 hs and 18:00 hs on two separate days. Morning cortisol levels at resting and post- swim were higher by 62% and 12%, respectively, than the evening level. The results of Dimitriou et al. (2002) study suggest that the optimal time for swimmers training should be in the evening were the immunosuppressive effect is low.

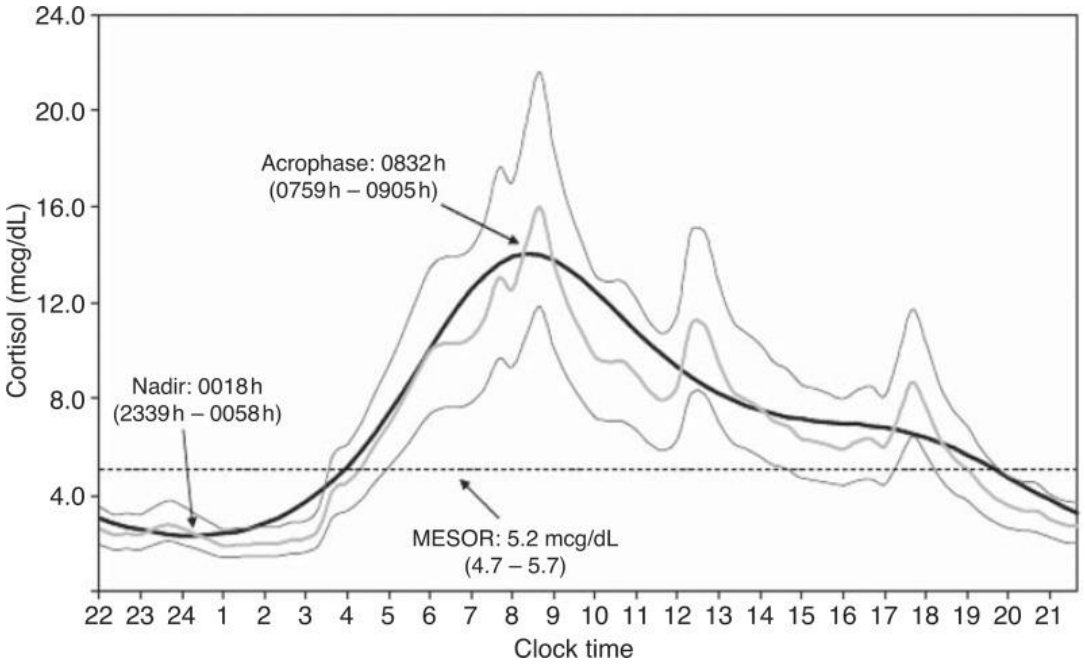

Figure 2.8: Circadian rhythm. A graphic depiction of cortisol values over a 24-hour period (Debono et al. 2009: page 1551).

Heat impact on cortisol during exercise

It is well documented that the combination of exercise and heat stress elevates both white blood cells and hormonal responses (Niess et al., 2003). Cosio-Lima et al. (2011) showed an increase post-exercise in both the thermo-neutral and hot environment in plasma cortisol concentration (730.4 ± 52.0 nmol·l^{-1} at rest and 1213.7 ± 32.7 nmol·l^{-1} at post-exercise in neutral condition, in hot condition 791.4 ± 121.5 nmol·l^{-1} at rest and 1109.1 ± 69.7 nmol·l^{-1} at post-exercise). Plasma cortisol was elevated after 3 hs post-exercise by only 0.5 nmol/L in the heat compared to the same exercise in a thermo-neutral environment. Brenner et al. (1998) tested the effect of repeated heat exposure and exercise on cortisol in 11 healthy male participants at a temperature of 23°C or 40°C with 30% relative humidity in a climatic chamber. Participants performed either cycling for 30 minutes at 50% $\dot{V}O_2$max, separated by a 45-min recovery interval or remained seated for 3 hs. The result showed that under thermo-neutral conditions there was no significant change to the concentrations of cortisol, with an increase observed during exposure to the heat. Niess et al. (2003) conducted a study where seven endurance-trained male athletes performed a 60 minute run on the treadmill (75% $\dot{V}O_2$max) in two different

environmental conditions (18°C with relative humidity 50% and 28°C with relative humidity 50%) with a week's interval between the runs. The results showed that additional elevated ambient temperature caused more stress on athletes and increased cortisol levels with no changes in cortisol concentration observed during the thermo-neutral condition (500 $nmol \cdot l^{-1}$ at 28°C and 390 $nmol \cdot l^{-1}$ at 18°C).

2.6.3 Heat shock proteins (HSP)

HSP are a protein group which increase when human cells are exposed to elevated temperatures or to other stress types (Guisbert and Morimoto, 2013, review). These stress conditions include: exercise, inflammation, infection, exposure of the cell to toxins (i.e. ethanol, ultraviolet light), starvation, and hypoxia or air deprivation. Generally HSP can be treated as part of the stress response (Sandstrom et al., 2008). However, HSP can also occur under non-stressful conditions as a result of simply "monitoring" the cells proteins and by carrying old proteins to the cells "recycling bin" (proteasome), or helping newly synthesised proteins to be activated (Njemini et al., 2011).

These activities are part of a cell's own repair system, called the "cellular stress response" or the "HSP response". HSP's, especially HSP70, are involved in binding antigens and presenting them to the immune system with HSP70 being the most highly conserved, and considered to be the most stress inducible of all of the HSPs (Theriault et al., 2005). HSP70 is involved in the folding of unfolded proteins. Extreme environmental stresses, such as high temperatures denature proteins from their proper structures and can cause them to unfold. These denatured, or unfolded proteins, may eventually kill the cell, so HSP70 is induced rapidly at high concentrations to re-fold and activate the denatured proteins (Saibil, 2013). Heat acclimation reduces physiological strain, improves the ability to exercise in a hot environment, and reduces the incidence of heat illness and stroke (Kuennen et al., 2011). HSP70 is present in cells under normal conditions, but when exposed to a sudden temperature or other stress parameter, HSP concentrations increase (Sandstrom et al., 2008). Likewise, HSP70 increases as a result of exercise (Fehrenbach et al., 2000). Studies have shown that the regulation of HSP70 during different exercises in neutral or hot conditions affects the cellular adaptation to heat acclimation (Maloyan et al., 1999).

A study conducted by Sandstrom et al. (2008), examined the changes in serum HSP70 during a 15-day heat exercise acclimation protocol on a male ultra-marathon runner. Serum HSP70 at pre- acclimatisation concentrations increased over the 15 days (at rest, day 1, 108,8 ng/ml, day 15, 145.5 ng/ml and at post-exercise, day 1, 168 ng/ml, day 15, 173.5 ng/ml), and this increase during exercise was inversely correlated to the resting serum HSP70 concentration, a finding which supports other studies (Liu et al., 1999). Liu et al. (1999) tested highly trained rowers during a four week long specially designed training program, where the intensity increased progressively up to the end of the third week. Highly trained rowers were divided into A and B groups. Group A performed a high intensity workload during training phase one, whereas group B performed a high intensity workload during training phase two. Both training intensity and volume were reduced in the final phase. The HSP70 was high as a result of exercise intensity in both groups, and decreased in both groups as a result of the decreased training intensity. HSP70 increased 5 fold during exercise compared to pre-training concentrations and peaked during week three. This could be due to the reduction in volume and intensity of the training between the last week of training, causing a time-delay between HSP70 transition and accumulation. Due to the exercise adaptation, HSP70 protein may play a role of protein synthesis and folding. Simultaneously, Liu et al. (1999) observed a significant increase of actin and myosin (muscle contractile proteins); therefore, that synthesis of new contractile proteins may be expected to occur after exercise.

Fehrenbach et al. (2000) reported an increase in HSP70 post-half marathon at 3hs and remained elevated after 24hs. The study involved twelve trained athletes and twelve untrained athletes; at rest, HSP70 in trained participants was significantly lower compared to the untrained participants. This low concentration of HSP70 seems to be a result of adaptation mechanisms to regular endurance training, with strenuous exercise leading to an increase in blood HSP post-run, indicating a protective function of HSP in athletes' white blood cells to maintain function after intense workload. In contrast, Shastry et al. (2002) found no increase in HSP70 at 15 or 24hs post-exercise. This study involved eleven moderately trained males and females, who performed a 60 minute run at 70% $\dot{V}O_2$max. This contradiction could be due to the different cellular locations investigated

in both studies; Fehrenbach et al. (2000) measured HSP70 in monocytes, whereas Shastry et al. (2002) measured HSP70 in total white blood cell.

Only a few studies have investigated HSP and particularly HSP70 circadian rhythmicity, with even fewer investigating the relationship between HSP70, circadian rhythmicity and sports performance (Rensing and Monnerjahn, 1996; Sandstroem et al., 2009). Sandstroem et al. (2009) investigated monocyte-expressed HSP70 for a period of 24 hs and the data was collected every 4 hs in 17 healthy male participants. At rest, HSP70 shows a diurnal variation which strongly correlates with core body temperature, with a peak at 09:00 hs and 21:00 hs, and a nadir at 05:00 hs.

The effect of heat on HSP70

The role of HSP during heat stress and exercise has been investigated intensively (Fehrenbach et al., 2001; Walsh et al., 2001; Fehrenbach et al., 2005). The combination of heat and exercise significantly accelerates the synthesis of HSP70 (Walsh et al., 2001; Lovell et al., 2007). HSP70 consists of both a cellular and systemic protective role (Hunter-Lavin et al., 2004).

Sandström et al. (2008) investigated the effect of exercise and heat on ultra-marathon runners during 15 consecutive days (Marathon des Sable), the combination of heat and exercise caused an initial increase in HSP70 concentration, with the resting level of HSP70 by day 15 remaining higher than the resting level at the start of the protocol. Increased HSP70 concentration in the circulating plasma and from lymphocytes may play a part in the fatigue sensation of athletes, and act as a danger indicator (signal) from the immune system (Heck et al., 2011).

Daryanoosh et al. (2014) investigated the effect of 2 different climatic conditions (a neutral condition of 24±2°C and a hot condition of 38±2°C) for the HSP70 response in 30 participants; 15 participants were healthy athletes (climbers) and 15 were non-active healthy individuals. Bike tests were performed, beginning with 25 watts and increased by 25 watts every minute until exhaustion. HSP70 did not change in the non-active

healthy population in both conditions, whereas, HSP70 was higher by 43% in the hot condition compared to the neutral condition in athletes.

2.6.4 Clara cells protein

Clara cell protein (CC16, CC10, or CCSP) is the main constituent of secretory granules of clara cells (Arsalane et al., 2000), found in the pulmonary airways predominantly in the respiratory bronchioles and in the terminal bronchioles. They are one of the main secretory proteins of the lung (McAuley and Matthay, 2009) and work to protect the respiratory system against toxic inhaled agents, repair damaged epithelium, detoxify xenobiotics and secrete proteins with important biological activities, such as surfactant-associated proteins and leukocyte-protease inhibitors (Braido et al., 2007). Robin et al. (2002) presents CC16 as having a high content of xenobiotic metabolizing enzymes which protect the respiratory system from inflammation caused by oxidative stress, allergen and environment conditions (Broeckaert and Bernard, 2000). Irander et al. (2011) stated that CC16 can also be produced by the nasal mucosal epithelial cells. Clara cells have been detected in other body organs such as the male swimmers urogenital tract (Romberg et al., 2011) and in the endometrium, lung and kidney (Broeckaert et al., 2000a). The levels of CC16 found in other organs are on average 20 times lower than in the pulmonary airways (Broeckaert et al., 2000b). Blood also contains CC16 derived from the airways (Braido et al., 2007). The serum level of CC16 in healthy populations are on average 10 to 15 ug·l (Helleday et al., 2006), no values of serum level CC16 are available for an athlete population. St Helen et al., (2013) found that the concentration of this protein in the blood increases as a result of pulmonary inflammation and increases the permeability of the lung epithelial barrier.

CC16 acute lung injury

CC16 plays an important role in protecting the respiratory tract against inflammation and oxidative stress, and it is this characteristic that makes it suitable as a biomarker of lung inflammation and injury (Tufvesson et al., 2013). Exposure to toxicants or extreme environments has been shown to increase/decrease CC16 levels in humans. A study conducted by Bernard et al. (1997) found an increase in serum CC16 in fire fighters after

only 20 minutes of smoke inhalation, this was significantly higher after one hour of exposure (328% higher), and ten days later serum CC16 returned to normal levels. Serum CC16 can also be used to assess the chronic air pollution effects on the respiratory epithelium (Gomes et al., 2011). Robin et al. (2002) showed that the concentration of CC16 in serum is significantly decreased in tobacco smokers compared to non-smokers, with an average decrease of 15% of the protein concentration for each 10 cigarette packets per year. This effect, which has been a result of a reduction of CC16 in lung lavage due to the loss of clara cells.

Several studies have investigated CC16 as a potential biomarker of lung injury in various diseases, such as asthma (Laing et al., 2000), environmental lung injury (Blomberg et al., 2003) and chronic tobacco use (Van Miert et al., 2009). As a result of exercise, serum CC16 peaked post-swim in well trained swimmers (Carbonnelle et al., 2002). Furthermore, Kurowski et al., (2014) showed that a link exists between the degree of training intensity and health status of swimmers where the combination of high training intensity and health status (e.g. asthma) lead to an increase in CC16 leakiness in the airway to the blood stream post- swim. The main finding of Carbonnelle et al. (2002) was that well trained swimmers showed a peak of serum CC16 post-exercise from two different types of pools (chlorinated pool and copper/silver pool). No significant difference was found between the serum levels of CC16 in both pools. This increase in airway permeability reported in both pools in swimmers in Carbonnelle et al. (2002) is probably caused by the mechanical stress on the epithelial barrier caused by hyperventilation during swimming at high intensity. Tufvesson et al. (2013) looked at plasma CC16 after a treadmill run, where CC16 was higher from one minute post-trial and remained higher until 60 minutes post-trial (increased from 8 $ug \cdot l^{-1}$ at rest to 16 $ug \cdot l^{-1}$ at minutes 60). There are limited studies that have looked at CC16 as a marker for upper airway inflammation and circadian rhythm on sports performance.

CC16 circadian rhythmicity

To date no studies have looked at the relationship between CC16, circadian rhythmicity and sports performance. However, a limited number of studies look at the diurnal variation in CC16 within a healthy population (Helleday et al., 2006; Andersson et al.,

2007). Andersson et al. (2007) finding was in agreement with Helleday et al. (2006) that showed a decrease in serum CC16 concentration during the day time.

Helleday et al. (2006) investigated diurnal variation in CC16 in eighteen healthy non-smoking participants, over a 15 hour period and repeated this twice within a 3 to 4 week time period. The result demonstrated a pronounced diurnal variation in the serum concentrations of CC16 with significant drops in the CC16 levels between 11.30 hs and 22:00 hs after exposure to filtered air (drop by 1.89 $ug \cdot ml^{-1}$ at 11:30 hs, 2.15 $ug \cdot ml^{-1}$ at 14:30 hs, 2.16 $ug \cdot ml^{-1}$ at 18:00 hs and 1.33 $ug \cdot ml^{-1}$ at 22:00hs). Furthermore, in Andersson et al. (2007) repeated blood sampling was performed in 13 healthy participants, and a total of four blood samples were taken: morning, noon, afternoon and the next morning. The level of serum CC16 decreased over the day, where the mean serum CC16 decreased by 12% at noon and 17% in the third sample of the afternoon; the time between morning, noon and afternoon measurements was 6 and 9 hours respectively. By contrast, Andersson et al. (2007) found that urine CC16 concentration increased over the day; this increase in concentration could possibly be the reason for the decrease in serum CC16 concentration. Moreover, serum CC16 during the morning showed relatively stable serum CC16 levels on different days within participants; which could be a good advantage when using serum CC16 as a marker in epidemiological or experimental research.

2.6.5 The impact of heat on CC16

In Bolger et al. (2011) 21 male athletes performed on separate days two 8 minute treadmill runs at 80% $\dot{V}O_2max$ in cold (4°C with 37% relative humidity) and warm humid (25°C, with relative humidity 94%) conditions. The result showed a lower CC16 concentration after exercising in the warm condition compared to the cold condition (51 $ug \cdot l^{-1}$ in cold environment compared to 19 $ug \cdot l^{-1}$ in hot environment). Moreover, the study outcome stated that inhalation of dry cold air increased the risk of epithelium injury in humans, whereas, it remains constant with warm humid air. There are a limited number of studies looking directly at the impact of heat stress on CC16. Many have linked their research to heat and ozone pollution impact on CC16, such as in Gomes et al. (2011) where a hot, humid and ozone-polluted environment elicits early epithelial damage post- 8 km treadmill run.

2.6.6 The effect of exercising in cold and hot/humid environments on airway inflammation

Exercise in heat and heat acclimatisation

Athlete's exposure to heat can effect negatively human cardiovascular system (Trinity et al., 2010), core body temperature regulation (Trinity et al., 2010), body fluid balance (Cheuvront et al., 2003) and the athletes ability to maintain sufficient cardiac output and blood pressure to thermo-regulate heat in the body and sustain muscle force generation.

During exercise core body temperature rises when heat production exceeds the body's heat loss (Tatterson et al., 2000). Furthermore, core body temperature is sensed by the hypothalamic thermoregulatory system. However, to dissipate heat, the thermoregulatory system then sets off a number of circulatory regulations including sweat rate, body and skin blood flow shifts, cardiac output, respiratory rate, and a sensation of heat intensity (Romanovsky, 2007). Mainly this involves an increase in cardiac output function and redistribution of blood from the visceral organs to the skin and working muscles (Inbar et al., 2004). During exercise in heat, skin blood flow was reported higher than at resting level (Trinity et al., 2010). In addition, sweat glands increase evaporative heat loss via the four known mechanisms which is responsible for heat exchange at the skin surface (evaporation, condition, radiation and convection) (Yaggie et al., 2005).

Highly trained endurance athletes can tolerate a core temperature of 40ºC for prolonged periods of the time (Gonzalez-Alonso et al., 1999). The muscles' energy systems become more chemically effective with rise in muscle temperature during exercise (Gonzalez-Alonso et al., 2000).

Heat acclimation is documented to induce numerous physiological adaptations that theoretically could improve aerobic exercise performance including oxygen uptake, heart rate and core body temperature, blood lactate, plasma volume expansion (Lorenzo et al., 2010) and increase skeletal muscle force generation (Kodesh and Horowitz, 2010).

Heat acclimatisation is an adaptation that reduces physiological strain of heat stress, which may improve exercise capabilities and thermal comfort in athlete's bodies. Therefore, this heat adaptation is induced by repeated heat exposures exercise in the heat that are sufficiently stressful to elevate the temperature of the core body and the skin to elicit perfuse sweating. However, full acclimatisation can be only achieved only with repeated exposure and exercise in the heat (McClung et al., 2008). In addition, to avoid heat related disorders the acclimatisation process should start by training at a low intensity.

Lorenzo et al. (2010) examined the effect of heat acclimatisation on exercise performance in neutral (13°C, 30% relative humidity) and hot conditions (38°C, 30% relative humidity). Twelve trained cyclists performed tests of maximal aerobic power ($\dot{V}O_2max$), time-trial performance, and lactate threshold, in both neutral and hot conditions before and after a 10 day heat acclimatisation (cycling at 50% $\dot{V}O_2max$ in 40°C). However, heat acclimatisation improved time-trial performance by 6% in cool (879.8 ± 48.5 vs. 934.7 ± 50.9 kJ) and by 8% in hot (718.7 ± 42.3 vs. 776.2 ± 50.9 kJ) conditions. Additionally, heat acclimation increased resting plasma volume by about 6.5% (200 ml).

Challenging environmental conditions during races or training phases, such as heat, cold or altitude, pose particular risks to an athlete's health. Athletes' airway epithelium injury can be caused by exercising vigorously and by exposure to the cold resulting in airway surface dehydration because of inspired cold dry air (Bougault et al., 2009). Athletes should find a way of increasing the temperature and water content of the inhaled air during exercise to prevent this dehydration. Bolger et al. (2011) increased the temperature and water/humidity from 4°C, 37% relative humidity to 25°C, 94% relative humidity, and the warm condition limited the disruption of the airway epithelium induced by high level exercise; the study involved twenty one male athletes, where the participants performed on two separate days, 8 minutes aerobic capacity exercise tests near the maximal and CC16 was measured in urine (4.8 $ug.l^{-1}$ in warm and humid air compared to 16.8 19 $ug{\cdot}l^{-1}$ in cold and dry air). The reduction of airway dehydration in cold environments during exercising outdoors can be achieved by increasing the water content of the air inspired or

through nasal breathing. The use of heat and moisture exchange devices can be used in both situations, either directly in the mouth or as a mask (Beuther and Martin, 2006).

Theoretically, Stensrud et al. (2006) reported that even a small increase in absolute humidity will help to reduce the rate of water loss from the airways during exercise and consequently reduce the risk of airway injury. The only practical strategy for individual athletes exercising in cold environments to minimise the risk of airway injury is through the use of a moisture exchange device. Providing a protective effect against exercise induced upper respiratory tract infection (Beuther and Martin, 2006). Notwithstanding this, physiological demands of exercise are minimized in a cold environment compared to a hot environment (Gonzalez-Alonso et al., 1998). An example of this is in Crandall et al. (2008) where an increase of 13% in left ventricular ejection fraction in young participants exposed to a hot environment, where the heart rate was increased from an average 53 (beats min^{-1}) in the control group to 93 (beats min^{-1}) in the experimental group. In addition, it is widely recognised that the stress response increases during exercise as a result of exposure to a hot environment. In addition, it has been reported that exercise in a hot environment recruited higher numbers of circulating stress hormones and increased cardiac output (Satarifard et al., 2012). Several of these exacerbated physiological demands may possibly be responsible for eliciting a larger value of white blood cells when exposed to the heat, whereas both are potential mediating factors for neutrophilia (Mitchell et al., 2002; Niess et al., 2003).

2.6.7 Circadian rhythms and psychological response to exercise

In modern sport the use of sport psychology to enhance performance has increased significantly. Athletes, coaches and scientists have realised that, psychological preparation performs a key role in attaining optimum performance level (Gould and Maynard 2009). A better understanding of an athlete's behaviour has entirely changed the way in which athletes approach their events (Gould and Maynard 2009). Several studies have concluded that mood and alertness are very good predictor of performance (Terry, 1999; Lane, 2001; Totterdell and Leach et al., 2001).

Psychological state is known to improve following physical activity; there have been a number of physiological mechanisms proposed for this phenomenon including core body temperature, catecholamine and endorphin release (Weinberg and Gould 2007). There is a lack of understanding of the link between circadian rhythms at rest and during exercise, and psychological variables (such as mood states and anxiety). For athletic competition getting into the right psychological state is seen by many coaches, athletes and sport psychologists as an important part of the process of mental preparation, and the success or failure to do so is often presented as an attribution to explain performance outcome. Athletes may sometimes have to perform to the best of their ability at a specific time of day and under extreme environmental conditions (Lane et al., 2005). Holt and Dunn (2004**)** suggested that effects of environmental change tend to influence psychological functioning in the same pattern they affect physiological and sociological factors. Therefore, these provide a useful early indicator of the adverse effects of environmental stress that can lead to poor performance.

Psychological (mood) profiling can provide numerous benefits, such as improvements in physiological systems related to the cardio-respiratory, muscular, endocrine and nervous systems (Contrada and Baum, 2011). The clinical aspects of circadian rhythmicity in cognitive functioning, mood and feelings are potentially important for human mental function and behaviour (Fernald, 2008). In a study conducted by Florida-James et al. (1996) the profile of mood states (POMS, McNair et al., 1971) was used to measure mood fluctuation in twenty three student nurses undertaking shift work. The study showed a circadian variation in the nurses' mood profile both on day and night shifts with a significant effect of time on vigour, fatigue and confusion. Moreover, Lane and Lovejoy (2001) showed that exercise has an enhancing effect on mood. This enhancement can result from an increase in vigour with reduced anger, confusion, depression, fatigue, and tension post-exercise. Furthermore, in sport psychology human attention, including alertness and arousal, play an important role in performance. Arousal is a state of readiness for action that motivates a person to behave in a particular way (Weinberg and Gould, 2007).

The levels of arousal vary between sports and depend on the individual's characteristic and psychological preparations. Arousal levels vary between low, recorded at deep sleep to high excitement, were high arousal contributes to inhibited sport performance and low can lead to poor performance. Perkins et al. (2001) studied 28 elite athletes and showed that strength performance increased significantly when arousal was high. This increase in performance, as a result of high arousal, could be a motivational factor. Coaches, athletes and psychologists target optimal arousal; however, optimal arousal differs from high arousal. Both over-arousal and under-arousal can lead to poor performance. Athlete's execution of skills will be of a higher standard when arousal level is optimal for that particular task (the Drive theory, Clark Hull – 1943 to review this theories see Dewey, 2007).

There is a strong correlation between alertness and sleepiness, a better night time sleep leads to a better daytime alertness (Kayumov et al., 2000). Furthermore, a short nap prior to the commencement of shift work improved alertness (Purnell et al., 2002). On the other hand, jet lag sleep disorder decreases the level of the daytime alertness (Kayumov et al., 2000). Consequently sleep disorders or disruption have a negative impact on alertness and performance and the reduction of tiredness leads to an increase in mental alertness. Alertness increases during the day time with a peak around 10:00 hs and a decline during the night time. In Mah et al. (2011) peak performance in basketball players only occurs when sleep is optimal, with improved player reaction time, better mood, faster sprints and low fatigue with high vigour. The true mental performance can be masked by the endogenous regulating system and modulated by both wakefulness (light) and sleep (hormone regulation) (Mah et al., 2011). Physical and mental activity can be masking factors as well.

Changes in electroencephalogram signalling could be another factor of decreasing performance (central fatigue). This occurs with a reduction in the blood supply to critical parts of the brain indicating a reduction in arousal, alertness and motivation to exercise (Nybo and Nielsen, 2001c). The electroencephalogram signals power is an index of a metabolic process in the motor cortex that aims to maintain the core body temperature after muscle fatigue occurred. A link exists between ratings of perceived exertion during

exercise and electroencephalogram, cerebral blood flow and core body temperature. This may result from the influence of high core body temperature on nervous system function during prolonged exercise (Nybo and Nielsen, 2001c), and consequently reduce the motivation of the participant to continue the exercise.

Rate of Perceived Exertion (RPE) (Borg, 1982) is a recognised marker used during exercise tests to measure exercise intensity (Eston, 2012). RPE can be used as a predictor of exercise capacity and intensity, assessing changes in mood of training, and easily understanding pace and pacing strategy during exercise (Eston, 2012). RPE is the way of measuring the heaviness and strain of exercise (Borg, 1982). Several studies have shown that RPE increases linearly during exercise, such as in cycling (Nethery, 2002), running (Dantas et al., 2014), and during exercise in the heat or at neutral condition (Nybo and Nielsen, 2001a; Crewe et al., 2008). This RPE linear increase would indicate that the brain perceives the exercise as becoming progressively more demanding. Crewe et al. (2008) showed that RPE keeps increasing during exercise even though the work rate and the intensity remain constant. Additionally, there is a correlated increase of core body temperature and heart rate throughout exercise with RPE (Crewe et al., 2008). Crewe et al. (2008) showed that the rate of RPE increase was lower during exercise in a cold environment compared to a hot environment. Furthermore, and in accordance with an updated review by Drust et al. (2005) during intense exercise, or time to exhaustion, RPE peaks in the afternoon coinciding with the highest core body temperature mean.

2.7 Summary

Based upon reviewed studies, time of day effect on exercise is a factor that must be taken into consideration. This has a practical application in planning training sessions, racing, testing, and experimental investigations. Optimal performance is the paramount objective of athletic endeavour, and adjusting for time of day differences appears to be an important factor in attaining that optimal performance. Moreover, the higher core body temperature during afternoon exercise should probably be accounted for when scheduling intense exercise, training, competition and testing, especially during prolonged maximum exercise and in hot environments. Certain performance variables peak times are generally subjected to individual differences, and experimentation by the athlete is needed to find

the optimum performance time for these variables and how to train to maximise them at the sub optimum times.

Moderate exercise increases the number of circulating immune cells in the blood with heightened effectiveness leading to greater defence status. However, exercising at a high intensity for a prolonged time causes temporary stresses in the body. When the body is stressed in this manner the adrenal glands secrete cortisol and adrenaline (epinephrine). These hormones liberate energy stores, improve cellular metabolism and elevate the heart rate and blood pressure in athletes, and thereby affect sport performance. These hormones also have a significant impact on the effectiveness of the circulating immune cells. Many physiological, psychological and immune measures show a diurnal variation over the 24 hour day in the athletes' body.

It would appear that there is a lack of literature investigating the effect of circadian rhythmicity and environmental temperature on athletic performance. The main aim of this thesis is to address this area. It is hoped that this thesis will provide some practical insight and advice for coaches, athletes and scientists to better understand the circadian effect on physiological, immunological and psychological variables under different environmental conditions. Ultimately, an athlete's understanding of the risks to the immune system associated with high level activity at specific times of the day and the environmental conditions within which they train, can lead to improved performances.

CHAPTER 3:

GENERAL MATERIALS AND METHODS

All procedures used in the present study were approved by the Ethical Committee and Health and Safety department at Edinburgh Napier University. The timeline of the study trial is described below at specific sections (Figure 1).

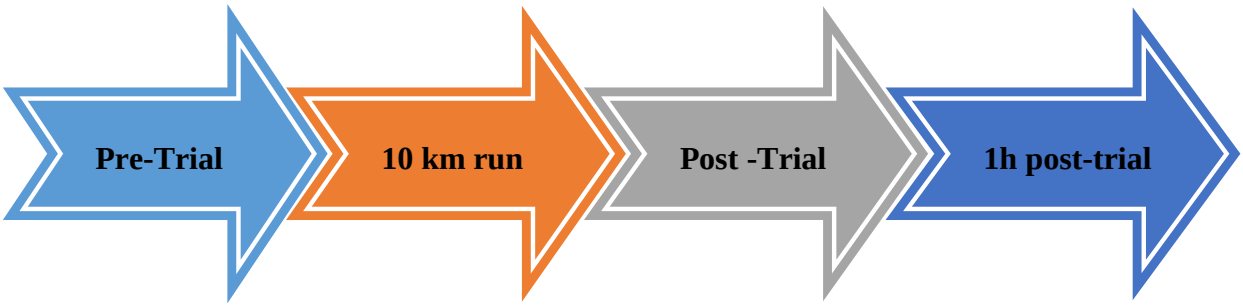

Figure 3.1: Timeline of the testing protocol. BP = blood pressure; HR = heart rate; CBT = core body temperature; Psychological Q = psychological questionnaire.

3.1 Human participants and experimental design

In no randomised order well trained runners; with a minimum $\dot{V}O_2max$ of 61 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$ were recruited for the study. Age range 18-43 years old, all participants were healthy, injury free and without respiratory tract infection within three weeks prior to the study. Participants not familiar to using a treadmill (Woodway, ergo ELG 55, Germany), were required to complete a familiarisation trial before starting the data collection. In addition, all participants were required to practice the nasal lavage procedure and lung function test in order to become familiar with these processes. All participants gave informed consent prior to the trial (Appendix 1). However, participants were free to withdraw at any time. In addition, the participants are experienced runners, there was no familiarisation or habitual trial to the condition that athletes will perform at.

3.2 V̇O2max trial

This test was carried out at least a week prior to the first trial, and aimed to measure participants' $\dot{V}O_2$max to ensure they fulfilled the minimum criteria for this study. Before the $\dot{V}O_2$max test participants completed a health questionnaire (Appendix 2 and 3). Blood pressure was taken to confirm the health condition of the subject prior to the test. The test used a standard methodology on a treadmill with online gas analysis (SBX/CPX, Master Screen. Germany). Starting at an initial speed of 10 $km \cdot h^{-1}$ and 0% gradient, speed was increased by 3 $km \cdot h^{-1}$ every 3 minutes until a maximum speed of 16 $km \cdot h^{-1}$ was reached. Gradient was increased every minute by 2.5% until maximum fatigue was achieved (based on Withers et al., 2000). The criteria for maximum fatigue and hence athletes' $\dot{V}O_2$max was attained when: $\dot{V}O_2$ reached a plateau, HR was 220 bpm minus the athletes' age, and the value for RER was greater than 1.10 (Withers et al., 2000).

3.3 Testing protocol – 10 km time trial run

After collecting all data required for the study at the rest state, and prior to the 10 km treadmill trial, participants went for a warm up easy run (between 5 to 20 minutes) on the treadmill at room temperature. Participants were required to warm up for the second trial in the same way (time and distance) as the first trial. After completing the warm up run participants stretched. In general all participants were asked to prepare as they would for a 10 km race.

The participants were asked not to engage in any intense training or race 24 h prior to the trial. Immediately preceding the trial each subject was required to complete a health questionnaire (Appendix 2 and 3) to assess their current health status. Participants presenting any cold or flu symptoms or chest problems were not allowed to participate in the trial and were recruited again only after making a full recovery. The trial consisted of a 10 km time trial run on a treadmill (Woodway, ergo ELG55, Germany) at two different times of the day (09:00 hs and 16:00 hs for Chapter 5 and 09:00 hs and 18:00 hs for Chapter 6), on two separate occasions. A minimum of 48 hours was required between each trial to allow participants to fully recover and avoid fatigue affecting the results. All participants were asked to prepare as they would for a 10 km race ensuring they were

sufficiently hydrated. Trials were performed under controlled environmental conditions; 6°C and relative humidity range (58 and 62%) for the first study (Chapter 5), and 28°C, 70% relative humidity for the second one (Chapter 6). Both studies were conducted in an environmental chamber (Weiss Technik, UK). Within the laboratory setting (the environmental chamber) the humidity is incontrollable when the temperature is below 10°C (Appendix 11). During the test, subjective Ratings of Perceived Exertion – RPE – on a scale of 6-20 (Borg, 1998), HR (Polar Electro, Finland) and running speed were recorded at the end of each kilometre completed. During the trial participants had free control of the speed they ran at, without knowing the actual value of the speed.

3.4 Experimental testing

3.4.1 Lung Function Tests

Lung function tests were carried out as described in Miller at al. (2005). A spirometer (Compact II: Type C, Vitalograph Ltd., UK) was used to carry out this test. Prior to the test calibration checks were taken, using a 3-L syringe. While the spirometer was calibrated anthropometric measurements were conducted (weight and height without shoes). These anthropometric measurements, age range and smoking history are required by the device (Spirometer) prior to the start of measurement.

Full demonstrations of all equipment used during the trial were given to the participants prior to the test taking place. The mouthpiece was placed in mouth making sure the lips were closed around it (no nose clip was used). In a standing position the participant inhaled air to capacity rapidly with a pause at the total lung capacity. The participant then exhaled maximally until no more air can be expelled. The test was performed 3 times with the best values recorded.

Lung function was measured pre-, post- and 1h post- the 10 km time trial. Values of forced vital capacity (FVC), which is the maximum amount of air a person can expel from the lungs after a maximum inhalation, forced expired volume in one second (FEV_1), which is the amount of air which can be forcibly exhaled from the lungs in the first second of a forced exhalation, the average expired flow over the middle half of the $FEF_{25-75,}$

which is the average expiratory flow over the middle half of the FVC and peak expiratory flow (PEF), which is the maximum speed of expiration, were measured.

3.4.2. Core body temperature

The validity and reliability of using sensor core body temperature pills, according to Byrne and Lim (2007) 10 of the 12 validation studies reported levels of agreement supporting the conclusion that using sensor core body temperature provided a valid index of core body temperature. An example in McKenzie and Osgood (2004) found that the core body temperature sensor pills were as accurate as rectal probe monitors with the study participants finding it to be much more acceptable.

To evaluate the accuracy and effectiveness of the Core Body Temperature pills Monitoring System, three temperature measurement devices were used: skin thermometer, tympanic thermometer and core temperature sensor. The aim is to assess the size and direction of the linear relationship between core temperature sensor device and both tympanic and skin device, for full details see Chapter 4. However, in field-based use McKenzie and Osgood (2004) reported the typical error was 6.5% during data collection with the principal causes seem to be electromagnetic interference or limitations in sensor transmitting range.

During the trial core temperature was measured throughout using Core Body Temperature pills Monitoring System (THermodot.USA). The core body temperature pills were stored according to the manufacture guidelines, such as avoiding storage close to electronic devices as this may affect the accuracy of the temperature pills. Prior to use the temperature pills were activated by removing the protective magnetic film and the recorder set to the appropriate reading program. In addition to this, the temperature pills, were checked for accuracy/reliability using a water bath to make sure that the temperature recorded by the Core Body Temperature Monitoring System matched with the temperature recorded in the water bath (see Chapter 4 for full details). All participants are required to sign a specific medical history related to temperature sensor pills according to HQ*int* wireless sensing system manufacturers' safety guidelines. Participants were

required to swallow the temperature pills one hour (Chapter 5) and two hours (Chapter 6) prior to the trial.

When the participants arrived to the trial venue they were asked to sit and refrain from any physical behaviour such as walking for at least for 10 minutes. After this when the participants are totally relaxed the first core body temperature measurement was taken (resting). In addition measurements are recorded at the end of each kilometre completed, post-trial and 1h post-trial. In accordance with the manufacture guidelines the temperature sensor pills will be discharged naturally from the body within 6 to 12 hs after the participant had swallowed it without any harm or other complications.

3.4.3. Haematology

Blood samples were collected both pre-, post- and 1h post- the 10 km time trial, in 5 ml tubes (BD Vacutainer Becton-Dickinson, Plymouth, UK). The samples were collected by venepuncture from the anticubital vein by a certified phlebotomist (Appendix 4). The samples were centrifuged for 10 min at 480 g at room temperature (Universal, 320R, Zentrifugen, Germany). The plasma was removed aliquoted into 5 ml eppendorfs and stored at -80°C until further analysis.

3.5 Biochemical Assays

3.5.1 Blood differential

Prior to the blood sampling procedure all participants have received a clear explanation of the procedure. All participants are required to be free from any infection or illness as these may affect the result generally and blood counting specifically (Appendix 3). All participants are asked to arrive at the laboratory in the same condition they would arrive for a race. An example would be having eaten their last meal at least 2 hours prior to the start time, to avoid any discomfort during the trial.

All participants were reported free from needle phobia. Participants were asked to lie down on a bed. A tourniquet was placed around the arm approximately 2 to 3 inches

above the antecubital fossa of the participant one minute prior to blood samples being taken (and no more than 2 minutes to avoid increasing risk for hemoconcentration). While the participants arm was extended with little or no flexion at the elbow the position of the puncture site was cleaned and with a sterile needle whole blood was collected from the antecubital vein by venepuncture. Afterward pressure applied to the puncture site with the participants instructed to keep the arm in a straight position, and maintain pressure on the puncture site for at least 3 minutes. After labelling the samples the puncture site was re-inspected to make sure bleeding has stopped, and to apply a bandage.

Blood differential counts were carried out using an automated cell counter (Sysmex, xs 1000i.USA). After calibrating the hymato-anylser with samples of the reagent (e-check-xs-level-2), the blood collected was placed in the rack for reading. The numbers and types of different cells within the blood were determined by the cell counting component and automatically saved ready for review, the samples were ran three times and the mean values obtained. The blood differential counts include, total RBC (total red blood cells), HGB (haemoglobin), HCT (haematocrit), PLT (platelet), total WBC (total white blood cells), neutrophil, lymphocyte, monocyte, basophil and eosinophil. In addition, and according to Ghys et al. (2009) the Sysmex (XS-1000*i*. USA) was able to generate a complete blood count and demonstrated a good analytical performance.

The blood samples were collected in a 6 ml tube **(**BD Vacutainer SST™) for the determination of Cortisol, CC16, HSP70 and IL-6. Whereas, for the blood differentiation counts, the blood samples were collected in a 2 ml tube. To avoid any inconvenience in blood collections, all the blood tubes used were labelled including participant name (code), time and which part of the trial.

The whole blood tubes were centrifuged for 15 min at 1000g at room temperature (Mistral 2000R, Sanyo, Leicester, UK). Afterward, the plasma was removed, aliquoted into eppendorfs (5 ml) and stored in a freezer at -80°C immediately until further analysis.

3.5.2 Biochemical analysis

Plasma CC16, IL-6 and cortisol were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits (R&D Systems Europe Ltd) and in accordance with the manufacturer's instructions. HSP70 was also measured using ELISA kits and in accordance with manufacture's instruction (Cambridge Bioscience Ltd). One hour prior to running the samples all reagents were brought to room temperature. Details of each method are described:

HSP70 (Heat shock protein 70) method

Seven tubes were labelled as follows: 17.5 $ng{\cdot}mL^{-1}$, 8.75 $ng{\cdot}mL^{-1}$, 4.38 $ng{\cdot}mL^{-1}$, 2.19 $ng{\cdot}mL^{-1}$, 1.09 $ng{\cdot}mL^{-1}$, 0.55 $ng{\cdot}mL^{-1}$, and 0 $ng{\cdot}mL^{-1}$. Then 250 µL of Standard and Sample Diluent was added to each one of the 7 tubes. The 35 $ng{\cdot}mL^{-1}$ standard was serial diluted 1:1 with Standard and Sample Diluent, the dilutions were performed by mixing 250 µL of the previous standard with 250 µL of Standard and Sample Diluent until the lowest concentration of 0.55 $ng{\cdot}mL^{-1}$ was achieved (Standard and Sample Diluent was used as the zero standard value). Wash buffer was diluted 1 in 10 with ultra-pure water. Plasma samples were diluted by mixing 60 µL of sample with 180 µL of Standard and Sample Diluent.

After plasma dilution, 100 µL of appropriately diluted standards or samples were added and covered with the adhesive strip. The plate was incubated for 2 hours at 37°C. Afterwards, plates were washed 3 times using washing solution (400 µL/well). After the final wash the plate was inverted onto water absorbent paper and tapped strongly. A second incubation followed (2 hours at 37°C). 100 µL of biotinylated antibody working solution was added to each well, followed by the washing process described previously.

After plate washing, 100 µL of Streptavidin-HRP Working Solution was added to each well and incubated for 30 minutes at 37°C followed by the same washing process described previously. 100 µL of TMB Substrate was added to each well and kept for 30 minutes in the dark at room temperature flowed by adding 100 µL of Stop Solution to stop the colour reaction. Then within a period no longer than 30 minutes the absorbance

was read using a microplate reader (MabTech, LT 5000ms, Elisa reader Essex.UK), with wavelength set on 450 nm. The concentrations were calculated using a standard curve and the average value obtained from the duplicate samples were automatically calculated.

IL-6 (Interleukin 6) method

Seven tubes were prepared for calibration to obtain the standard curve, 100 pg/ml, 50 pg/ml, 25 $pg{\cdot}ml^{-1}$, 12.5 $pg{\cdot}ml^{-1}$, 6.25 $pg{\cdot}ml^{-1}$, 3.12 $pg{\cdot}ml^{-1}$ and 0 $pg{\cdot}ml^{-1}$. To obtain a concentration of 100 $pg{\cdot}ml^{-1}$, 667 µL of Calibrator Diluent and 333 µL of IL-6 standard were added together. 500 µL of calibrator diluents were added to each remaining tube. The undiluted standard served as the high standard (300 $pg{\cdot}ml^{-1}$) and appropriate calibrator diluent served as the zero standard (0 $pg{\cdot}ml^{-1}$). Afterward 100 µL of Assay Diluents RD1W were added to each of the 96 wells and 100 µL of standard or sample were added to each well (performed in duplicate). Afterwards the plate was covered with the adhesive strip, and incubated for 2 hours at room temperature. The plates were washed 3 times using washing solution (400 µL/well). After the final wash the plate was inverted onto water absorbent paper or towel and tapped strongly. A second incubation (2 hours/room temperature), 200 µL of human IL-6 conjugate was added to each well, followed by the same washing process described previously. 15 minutes prior to the next incubation, substrate solution - colour reagents A and B are mixed together in equal volumes and were protected from light. 200 µL of substrate solution was added to each well and incubated for 20 minutes at room temperature and covered with aluminium foil to avoid direct exposure to sun light. To stop colour development 50 µL of stop solution was added to each well and within a period no longer than 30 minutes the absorbance was read using a microplate reader (MabTech, LT 5000ms, Elisa reader Essex.UK), with wavelength set on 450nm and reference of 550nm. IL-6 plasma concentrations were calculated using four parameter logistic curve fit (4-PL) software and the average value obtained from the duplicate samples were automatically calculated.

Cortisol method

Eight tubes prepared for the calibration standards were labelled: 10ng/ml, 5ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 $pg{\cdot}ml^{-1}$, 0.313 $pg{\cdot}ml^{-1}$, 0.156ng/ml and 0 $pg{\cdot}ml^{-1}$. To prepare a

concentration of 10 $pg{\cdot}ml^{-1}$, 900 µL of calibrator diluents RD5-43 was mixed with 100 µL of RD5-43 standard. 500 µL of calibrator diluents were added to each remaining tube. The appropriate calibrator diluent served as the zero standards (0 $pg{\cdot}ml^{-1}$). After 1 hour 150 µL of calibrator diluents RD-43 were added into non-specific binding wells. 100 µL calibrator diluents RD-43 added to zero standard.

Afterward 100 µL of standard and sample were added to each well (duplicate) and an extra 50 µL of cortisol conjugate was added to each well (generating a red colour) with an extra 50 µL of primary anti body solution added to each well excluding the non-specific binding wells, this created a violet colour, only non-specific binding wells remained red. After covering with the adhesive strip, the plates were incubated for 2 hours at room temperature on a microplate shaker at 500rpm. Afterwards, plates were washed 3 times using washing solution (400 µL/well). After the final wash the plate was inverted onto water absorbent paper and tapped strongly. 15 minutes prior to the next incubation, substrate solution, colour reagents A and B were mixed together in equal volumes and were protected from light. A second incubation (30 minutes/room temperature) 200 µL of substrate solution was added to each well, incubated at room temperature and covered with aluminium foil to avoid direct exposure to sunlight. To stop colour development 50 µL of stop solution was added to each well and within a period no longer than 30 minutes the absorbance was read using a microplate reader (MabTech, LT 5000ms, Elisa reader Essex.UK), with wavelength set on 450nm and reference of 550nm. IL-6 plasma concentrations were calculated using four parameter logistic curve fit (4-PL) software and the average value obtained from the duplicate samples were automatically calculated.

CC16 (Clara cells 16) method

Enzyme-linked immunosorbent assay (ELISA) kits (BioVendor, European Union and R&D System Europe, Ltd) were used to measure plasma CC16 following the manufacturer's instructions. First, plasma samples were thawed to room temperature and diluted by 1 in 24 using Sample Diluent. Standard solutions: 5 µl and 120 µl of Dilution Buffer for singlets and duplicated diluted samples (100 µl) were pipetted into the 96 microwell plate. Following microwell plate incubation at 25°C for 1 h, plates were

washed 3 times using washing solution (0.35ml/well). After the final wash the plate was inverted onto water absorbent paper or towel and tapped strongly. 100 µl of Biotin-antibody Conjection was then added to each well. A second incubation (1 h at 25°C) atop an orbital microplate shaker (300 rpm) followed by washing and drying of the microplates was completed before 100 µl of Streptavidin-HRP Conjugate was added. At this point the plate was covered with aluminium foil to avoid direct exposure to sun light and then the plate was incubated for a final time at room temperature for between 10 - 20 minutes (dependent on the ambient room temperature i.e. a longer incubation time of 20 minutes if the room temperature was less than 20°C). To stop colour development 100 µl of Stop Solution was added and within a period no longer than 5 minutes the absorbance was read using a microplate reader (MabTech, LT 5000ms, Elisa reader Essex.UK), with reference wavelength set on 450nm. CC16 plasma concentrations were calculated from the standard curve and the average value obtained from the duplicate samples.

3.5.3 Method of calculating diurnal variation in plasma volume changes (dehydration)

When the athletes are dehydrated the plasma volume decreases. The data obtained from this section were adopted from the result of cell counts differentiation using the automated cells counter (Sysmex, xs 1000i.USA). The method is fully described is section 3.5.1.

The method used to calculate the plasma volume changes (dehydration) is the same as described in Dill and Costill (1974).

Calculation of percentage changes in blood volumes:

Firstly: calculate BV_A (percentage change in blood volume at post- trial):

Where $BV_A = BV_B$ (HGB pre-trial/HGB post-trial). BV_B (blood volume at pre-trial) is given a value of 100

Secondly: calculate CV_A (blood volume at post-trial) from the formula:

$CV_A = BV_A$ (HCT post-trial).

Lastly: calculate PV_A (post-trial plasma volume) using the equation below.

$PV_A = BV_A - CV_A$. The concentrations of haemoglobin in red blood cells were obtained by dividing HGB by HCT.

3.6 Assessment of respiratory symptoms

Immediately post-trial athletes were asked to complete the "Assessment of Respiratory Symptoms"; a questionnaire designed by Schelegle and Adams, (1986) to assess respiratory symptom severity, (Appendix 5).

3.7 Statistical Analysis

Statistical tests were used to determine correlations, relationships, differences or similarities within and between datasets. All data collected from both studies were analysed using SPSS 20 software (IBM.UK). Specific statistical tests used to analyse the data within each chapter is provided in the respective methods section. A significance level of $P < 0.05$ was adopted throughout unless otherwise stated. Most of the results are represented as mean values ± one standard deviation, unless otherwise stated.

CHAPTER 4:

MEASUREMENT OF BODY TEMPERATURE IN ATHLETES USING DIFFERENT DEVICES

As we experienced a technical problem when collecting data for Chapter 5 and the impossibility of using the rectal device in runners. It is therefore unlikely, that a malfunction in 3 of the sensor pills developed after this time. A more likely explanation of the low temperature readings may be a result of the pills being ingested just one hour prior to the trial. In other hand using rectal device, is not applicable in many sports where athletes are moving fast, such as running. A common problem when using the rectal device is that it does not hold its position and falls off. The combined effects of the physical movement associated with running and sweating makes it nearly impossible for the rectal device to stay in the correct place. However, the main objective this chapter is to evaluate the accuracy and effectiveness of core temperature sensor device.

The use of a rectal temperature probe is not applicable in many sports where athletes are moving fast, such as running. A common problem when using the rectal device is that it does not hold its position and falls off. The combined effects of the physical movement associated with running and sweating makes it nearly impossible for the rectal device to stay in the correct position. An alternative method is therefore necessary, such as the ingestible core body temperature pill.

The main objective of this chapter is to evaluate the accuracy and effectiveness of the core temperature sensor device. With a comparison of temperature readings from the core temperature sensor device and both tympanic and skin devices.

4.1 Introduction

For assessing core body temperature the rectal device is often preferred and the recommended method by USA national athletic trainers' association (Binkley et al, 2002). Using this device, however, is not applicable in many sports where athletes are moving fast, such as running. A common problem when using the rectal device is that it does not hold its position and falls off. The combined effects of the physical movement associated with running and sweating makes it nearly impossible for the rectal device to stay in the correct place. In addition to this the discomfort and unpleasantness associated with using the rectal device suggests the need for an alternative method of measuring core body

temperature. For these reasons rectal devices were excluded from use in this study. Scientists, athletes and coaches use a variety of devices and sites on the human body to measure core body temperature.

Core body temperature rhythmicity has become the gold standard by which the function of the human circadian system is evaluated (Florida-James and Doggart, 2000). Core body temperature in humans varies during day time, but these variations are small, usually no more than 1.0ºC (37ºC +/- 1ºC). However, the thermoregulatory centre in the hypothalamus plays a very active role in keeping core body temperature within the normal range. Assessment of body temperature in humans is one of the oldest methods known of diagnostic disease or health (Sund-Levander and Groszinky, 2009). In addition, the accurate temperature measurement is important for appropriate intervention, when necessary, for patients and athletes. Clinically, rectal and oral temperatures are the most reliable indicators of core body temperature (Sund-Levander and Groszinky, 2009).

There are external (*climatic*) and internal (*metabolic*) heat source factors that may influence core body temperature such as: heavy exercise, illness, hot/cold environment. Ambient temperature, humidity, air movement, and radiant heat from the sun, as well as warm and cold surfaces, contribute to climatic heat stress.

During exercise core temperature measurement is an effective way to measure hypothermia or hyperthermia, to do this the thermometer must be practical, accurate, reliable and functional in all environmental conditions. In clinical studies rectal temperature is the golden standard of measurement because of the accuracy and reliability in all conditions, as illustrated in two classic studies by Casa (2007) and Ganio (2009). These studies had similar protocols and tested the same thermometer types: oral, axillary, aural, core temperature sensor, forehead, temporal, and rectal. The most notable difference between the studies was the setting, one was performed outdoors and the other indoors. Both studies showed that of all the thermometers tested rectal probe and ingestible telemetric pill (or core temperature sensor) were the most accurate and reliable compared to the rest of the devices used. Furthermore, several studies have reported that core temperature sensor is a valid estimate of core body temperature during exercise (Gant

et al., 2006; Casa et al., 2007) and that internal core body temperature versus other measures had the smallest bias.

The aim of the present study is to evaluate the accuracy and effectiveness of core temperature sensor device. Our aim is to assess the size and direction of the liner relationship between core temperature sensor device and both tympanic and skin device. Furthermore, to validate the accuracy and acceptability of Core Body Temperature Monitoring System (THermodot.USA).

4.2 Materials and methods

Nine male endurance runners (mean ± SD: 28±8 years, $\dot{V}O_2max$ range 64-74ml·kg^{-1}min^{-1}) took part in this study. The participants were asked to complete a general medical history questionnaire (Appendix 2) and a specific medical questionnaire (Appendix 6). All participants provided fully informed written consent before engaging with the experiment and were free to withdraw from the study at any stage.

The trial consisted of a 10 km time trial run on a treadmill (Woodway, ergo ELG55, Germany). All participants were asked to prepare as they would for a 10 km race ensuring they were sufficiently hydrated. Trials were performed under controlled environmental conditions 28°C, 70% relative humidity in an environmental chamber (Weiss Technik, UK). During the test, subjective Ratings of Perceived Exertion – RPE – on a scale of 6-20 (Borg, 1998), HR (Polar Electro, Finland) and running speed were recorded at the end of each kilometre completed.

Tympanic device

The tympanic digital device (Thermoscan ExacTemp IRT 4520; Braun, Boston) was placed in the external auditory canal with the probe aimed at the tympanic membrane. The digital reading occurred after 3 seconds and the temperature measurement was written down. The reading was collected at rest, every 1km ran, immediately post-trial and 1h post-trial.

Core temperature sensor device (internal)

The core temperature sensor device was measured at rest, throughout the trial and 1h – post-trial using Core Body Temperature Monitoring System (THermodot.USA). Participants swallowed the core temperature sensor pill 2 hours prior to the start of the trial. This method is fully described 3.4.2 section. The reading was collected at rest, every 1km ran, immediately post trial and 1h post-trial.

Skin Device

Was measured using a skin infrared device on the forehead. Using the method described in the instruction manual (Testo, 380-TI-UK) by placing the infrared on the forehead waiting 3 seconds for the digital reading to occur and the reading recorded. The reading was collected at rest, every 1km ran, immediately post trial and 1h post-trial.

4.2.1 Statistical Analysis

To assess the size and direction of the liner relationship between core temperature sensor device and both devices, tympanic and skin, a bivariate Pearson's correlation coefficient (r) was calculated using SPSS 20 software (IBM.UK). A significance level of $P < 0.05$ was adopted throughout unless otherwise stated. Most of the results are represented as mean values ± one standard deviation, unless otherwise stated.

4.3 Results

Table 4.1: The individuals (n=8) anthropometric measurement, HR and RPE.

Age (year)	29±10
Mass (kg)	65±7
Stature (cm)	180±8
Resting heart rate (beats·min^{-1})	58±13
Peak heart rate (beats·min^{-1})	181±11
Mean RPE	14±2
Peak RPE	18±2

All values are the mean ± SD.

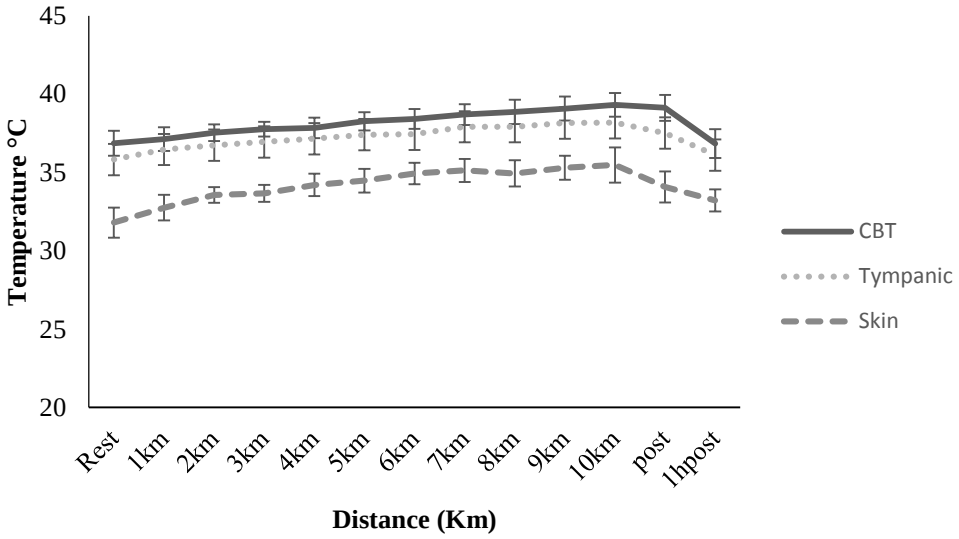

Figure 4.1: Comparison between three temperature measurement methods (CBT= core body temperature, tympanic and skin temperature).

Two-way repeated measure analysis of variance (ANOVA) was used to determine the significance difference between temperature devices. The result showed a significant difference between the 3 devices ($F_{2,24} = 671$, $p < 0.001$, partial $\eta^2 = .9$) with Bonferroni-

adjusted, post hoc test revealing that there is a significant difference between core temperature sensor device and tympanic device measurement and between core temperature sensor and the skin devices (P = 0.00). All three devices (and therefore the temperature measured locations) in this study showed the same trend and were affected by environmental condition and exercise. A significant positive correlation was observed between core temperature sensor and tympanic temperature and core temperature sensor and skin temperature (r (11) = 0.962, p = 0.00 and r (11) = 0.855, p = 0.00).

Table 4.2: Measures of validity of the three temperature measuring devices using core body temperature sensor as the reference.

	Mean	**Δ**	**Maximum**	**Δ**	**Minimum**	**Δ**
CBT	38.23±0.7	n/a	39.31±0.75	n/a	36.84±0.78	n/a
Tympanic	37.21±0.37	0.92	38.18±0.54	1.13	36.11±0.45	0.73
Skin	34.12±0.78	4.01	35.48±1.11	3.83	31.8±0.95	5.04

The 95% Confidence Intervals (lower; upper limits) for the three temperature measuring devices are as follows**:** CBT (37.6; 38.6°C), tympanic (37.0; 37.4°C) and skin (33.7; 34.0°C).

4.4 Discussion

The main purpose of this study was to evaluate the correlation between tympanic and skin device temperature compared to core temperature sensor device. There was a significant strong positive correlation between core temperature sensor with the two other devices (tympanic and skin). At rest, core body temperature was less than 1°C higher than tympanic temperature (0.92°C), higher by 1.13°C during the trial and after 1hours post-trial the difference was only 0.73°C. However, skin temperature was very different. In the present study the results suggest that the tympanic temperature device is more accurate

than skin thermometers in predicting internal temperature. Furthermore and in accordance to Kolka et al., (1993) tympanic temperature showed the same trend and responded rapidly, similar to the temperatures reported by the core temperature sensor. Several studies have reported that core temperature sensor is a valid estimate of core body temperature during exercise (Gant et al., 2006; Casa et al., 2007). Furthermore studies have found that the core temperature sensor versus other measures had the smallest bias.

The core body temperature monitoring system (THermodot.USA) represents a valid index of core body temperature that is convenient and shows excellent utility and strongly suggested for clinical and sport field use. It is clear that it is easy to use, more reliable, and may facilitate better overall management of studies that require accurate measurements of core body temperature. Therefore, it appears from the study data that the (THermodot.USA) monitoring system may be a viable device for further studies of core body temperature. However, one of the main concerns for using this core body temperature monitoring system (THermodot.USA) is the relatively high cost associated with using this device (cost £43 each, with a single use) that can make it less suitable for all users where it could be replaced by a tympanic device.

In addition, after reviewing the data collected for this chapter and the information in Chapter 6, it can be concluded that taking sensor pills one hour prior to the trial was insufficient for the pill to be transported to the bottom of the stomach to show the true core body temperature reading. It is therefore likely that the sensor pill position was much higher in the digestive system throughout the trial, such as in the oesophagus. It is therefore recommended that, in future research, sensor pills should be taken at least 2 hours prior to testing.

CHAPTER 5:

DIURNAL VARIATION IN PHYSIOLOGICAL AND IMMUNE RESPONSES TO INTENSE EXERCISE PERFORMED IN A COLD ENVIRONMENT

5.1 Introduction

Sport performance is complex and varies with respect to a number of factors, such as time of day and core body temperature. Highly trained athletes can be repeatedly exposed to cold air during winter training and racing, which can have an adverse effect on health and performance (Bougault et al., 2009). Wilber et al. (2000) evaluated athletes from seven different modalities in the 1998 U.S.A winter Olympic team and reported that 23% of the athletes showed airway inflammation. This exposure to cold air and other inhalants often causes upper and lower airway dysfunction and may result in airway epithelial injury (Gleeson et al., 2013). This dysfunction may be caused by dehydration and physical stress applied to the airways during maximum exercise in extreme environmental conditions.

Airway inflammation can be detected by an increase in inflammatory markers, such as neutrophils, macrophages, inflammatory cytokines and Clara cell protein (CC16). In Sue-Chu et al. (1999) during a comparison between inactive healthy control groups with cross-country skiers, an increase in white blood cell counts was observed in the control group, with a very high risk factor of asthma development. Moreover, 40 elite skiers compared with healthy controls showed an increase in T-lymphocytes, neutrophils and eosinophils (the author did not mentioned the season and the time the data were collected) (Karjalainen et al., 2000). In the skiers, neutrophil counts were more than 2-fold greater than in asthmatic participants. Similarly, runners showed an increase in neutrophils, in induced sputum, without an increase in eosinophils or lymphocytes (Bonsignore et al., 2001). The samples were collected in December during and after the Fourth Palermo International Marathon (Italy). Additionally, increased eosinophil and lymphocyte counts are likely related to environmental exposure factors, such as chlorine for swimmers or cold dry air for cross-country skiers and runners (Bonsignore et al., 2001; Helenius et al., 2005).

Physical exertion experienced from intense and prolonged exercise can cause significant stress on the respiratory system; possibly caused by hyperventilation and increased airway exposure to contaminants within inhaled air (Martin at al., 2009). Furthermore, the risk of URTI is elevated in athletes during periods of heavy training and in the period

of one to two weeks following participation in endurance races (Gleeson et al, 2013). The nasal cavity is the first line of defence against ambient cold and irritants. The upper respiratory tract, especially the nasal cavity is a good site to assess biomarkers, e.g. clara cell protein (CC16), for events related to airborne exposures (Gomes et al., 2011).

The research carried out and described within this chapter was designed to evaluate the effect of circadian rhythmicity on physiological and immunological parameters in a cold environment (UK winter average temperature of 6°C). It has been reported that circadian rhythmicity exists in the human immune system; many functions and parameters have been described as circadian rhythm dependent (Haus and Smolensky, 1999; and Vgontzas et al., 2003, more detailed in Chapter 2, section 2.4). In addition, CC16 concentration also presents a circadian variation, with higher values noted during the morning period and decreasing throughout the day (Helleday et al., 2006; Andersson et al., 2007, more detailed in Chapter 2, section 2.6.4).

Notably the underlying mechanisms of circulating blood cells and circadian changes in athletes in cold condition have not been fully understood. It is unknown how the circadian rhythm system and the immune system are communicating, but it has been suggested that this is achieved through circadian-controlled hormonal factors by the autonomic nervous system which regulate gene expression and protein activity (Mendez-Ferrer et al., 2008). Alternatively it could be possible that immune cells' local clocks are directly controlled by cellular immune functions (Keller et al., 2009). Circadian rhythm influence on immune function is an important factor in the regulation of cellular homeostasis where it is responsible for growth and aging (Reppert et al., 2002). Any change in clock genes can effect human sleep and lead to sleep disorders (Toh et al., 2001). In addition, core body temperature can also affect the individual's immune system in response to an exercise bout (Niess et al., 2006). To the best of our knowledge this the first study to investigate the zeitgeber effect on 10 km running performance.

In the research carried out and described within this chapter, the masking of circadian variables measured could be exogenously temporarily influenced by exercise and the cold ambient temperature tested at. In addition, frequently conditions such as the

environmental and experimental set up in which circadian rhythmicity measurements are taken are the main causes of masking and can obscure or modify the daily pattern rhythms and/or create circadian rhythm appearance (Kryger et al., 2005; Wirz-Justice, 2007).

Therefore, the aims of this study were:

To analyse the time of the day effect on running performance including immunological, physiological and psychological (fully discussed in Chapter 7) changes during exercise in a cold environment. The novelty of this research is that it investigates these parameters in a running experiment using highly-trained athletes to directly relate the findings to the competitive running population of the world. The hypotheses are:

Peak running performance will coincide with the peak of core body temperature in cold environmental conditions.

Physiological variables in well trained runners will present diurnal variation in a cold environmental condition and coincide with the peak of core body temperature.

Immunological variables in well trained runners will present diurnal variation in a cold environmental condition and coincide with the peak of core body temperature.

5.2 Materials and methods

Methods that were not described in Chapter 3 are fully explained in this section.

Eight male endurance runners (mean ± SD: age: 32 ± 5 years; $\dot{V}O_2$max: 71 ± 6 $mlO_2 \cdot kg^{-1}min^{-1}$; mass: 69 ± 4 kg; height: 178.0 ± 5.7 cm) took part in this study. The participants were asked to complete a general medical history questionnaire (Appendix 2) and a specific medical questionnaire (Appendix 6). All participants received detailed explanation about the study and signed an informed written consent form before engaging with the experiment. They were also fully aware that they could withdraw at any stage.

Core body temperature sensors were administered at least 1 hour prior to when the trial began as described in Domitrovich et al. (2010).

5.2.1 Experimental procedure

The exercise protocol consisted of a 10 km time trial run at 2 different times of the day: 09:00 hs and 16:00 hs (full details of the experimental condition can be found in Chapter 3, section 3). During the trials runners had access to control the speed, but they did not have access to the value of the speed. Blood sampling, lung function tests, were assessed at pre- immediately post- and 1h post-trial for both times of the day. Furthermore, heart rate and core body temperature were assessed through the trial and at pre-, immediately post-trial and 1h post-trial time points in both trials. Environmental conditions were controlled at 6°C the average UK winter temperature (metoffice.gov.uk, 2012). The relative humidity was not able to be controlled at this temperature or any temperature of less than 10°C in the climatic chamber (the humidity level ranged between 58 and 62%. See Appendix 11). Mood states were assessed pre-, post- and 1h post- the 10 km time trial using the Brunel Mood scale (BRUMS, Appendix 10). An assessment of anger, confusion, depression, fatigue, tension, and vigour, were rated on a 5-point scale anchored by 0 ("not at all") to 4 ("extremely").

5.2.2 Sample Collection

Lung function tests: full descriptions of the procedures used in this study are given in Chapter 3, section 3.4.1.

Blood samples: fully described in Chapter 3, section 3.5.1.

5.2.3 Biochemical analysis

CC16: method is fully described in Chapter 3, section 3.5.2.

Diurnal variation in plasma volume (dehydration): Full descriptions of the procedures used in this study are given in Chapter 3, section 3.5.3.

5.2.4 Statistical Analysis

Prior to statistical analysis all data were checked for normality. Data were analysed using two way repeated measure ANOVA with Bonferroni-adjusted, post hoc test to determine the difference at which time point the diurnal variation occurred and the difference between each trial time points (SPSS20 Statistical Software, IBM.UK). Statistical significance was accepted at $P < 0.05$. Results are represented as mean values ± standard deviation (SD).

5.3 Results

5.3.1 Performance variables

Seven participants out of eight completed both trials, 1 participant withdrew because of injury. There was no significant difference in athlete's performance for the two times tested, (Table 5.1). However, mean RPE was 15 ± 2 and 14 ± 2 in the morning and the evening, respectively.

Table 5.1**:** Mean RPE and mean running time diurnal variation.

	09:00 hs	**16:00 hs**
Mean RPE	15 ± 2	14 ± 2*
Mean time (mins:s)	33:40 ± 2:36	33:31± 2:40
Mean Speed(km. hr^{-1})	17.6 ± 0.75	17.8 ± 0.61

* Diurnal difference was observed with the variables. Values are mean ± SD

The individual running times for the 7 participants that took part in the study are presented in Figure 5.2. No significant difference between the two trials was observed (09:00 hs vs 16:00 hs). The 95% Confidence Intervals (lower; upper limits) for the time to complete the trials were at 09:00 hs 10 km running trial (1856; 2161 sec) and at 16:00 hs 10 km running trial (1863; 2159 sec).

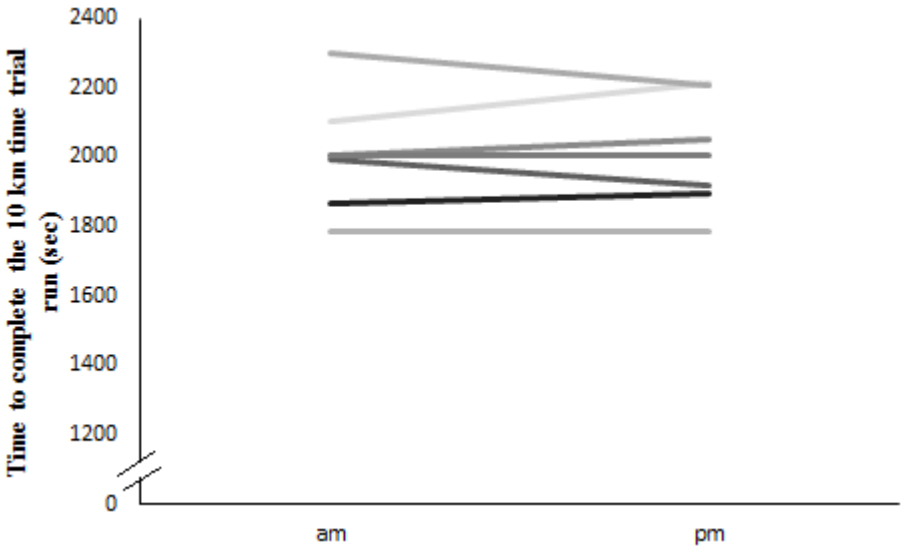

Figure 5.1: Diurnal variation in 7 individuals' running times (time in seconds).

5.3.2 Diurnal variation in core body temperature

A technical problem with the core body temperature sensor pills resulted in valid measurements for only 4 of the participants. Three participants recorded lower temperatures than the normal expected range (< 36°C) and were excluded. There are two potential explanations for these anomalous readings; either the long time the pills where stored (> 2 years) caused them to malfunction or the sensor pills has insufficient time within the gut to accurately record core body temperature.

Prior to the experimental stage of this research the temperature sensor pills where tested in a temperature controlled water bath for accuracy of measurement and were found to be functioning normally (see Chapter 3, section 3.4.2). It is therefore unlikely, that a malfunction in 3 of the sensor pills developed after this time. A more likely explanation of the low temperature readings may be a result of the pills being ingested just one hour prior to the trial. This was likely insufficient time for the pill to pass along the digestive tract, to deep within the internal digestive system to give the true core body temperature reading. It is most likely that the sensor pill position was much higher in the digestive

system throughout the trial, such as in the oesophagus. The remaining core body temperature sensors measured core body temperatures within the expected normal temperature range and therefore must be assumed to have travelled sufficiently along the digestive tract to measure core body temperature accurately. Two-way repeated measure analysis of variance (ANOVA) was used to determine diurnal variation in core body temperature and at the three time point intervals (pre, post and 1h post-trial). The result showed no statistical differences were found between trials (am and pm). However, a significant difference between time point trials was observed for core body temperature at both times of the day ($F_{2,4} = 5.16$, $p < 0.002$, partial $\eta^2 = .008$) with Bonferroni-adjusted, post hoc test revealing that the core body temperature has significantly changed pre to post ($p= 0.039$ and $p = 0.039$) at both times of the day (Figure 5.3b).

In addition, the mean core body temperature showed a significant diurnal difference throughout the trial ($P<0.00$), with mean higher values recorded during the morning trial compared with the evening trial (38.72±0.83°C at am and 38.32±0.78°C at pm) (Figure 5.3a). The range between the highest and lowest value in core body temperature was 2.31°C at am and 2.02°C at pm. The resting values, however, did not differ between trials.

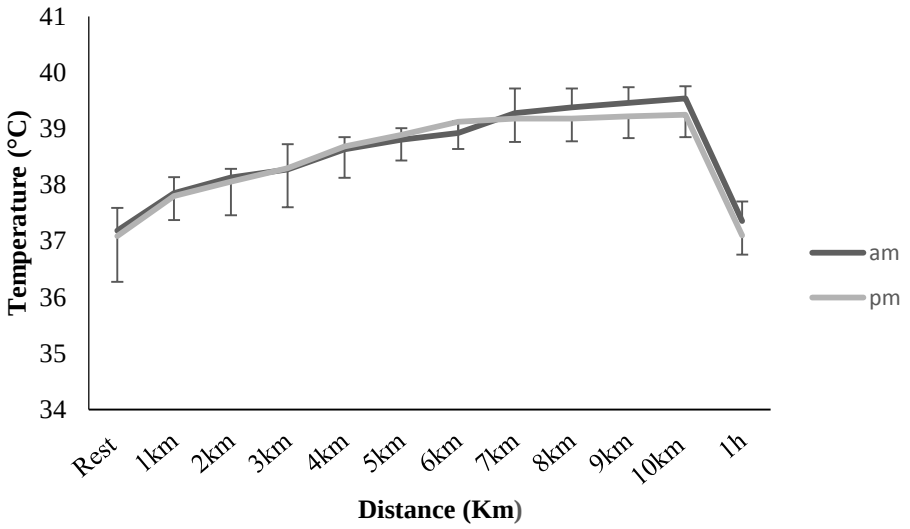

Figure 5.2a: Core body temperature (CBT) throughout the trials. Values are mean ± SD.

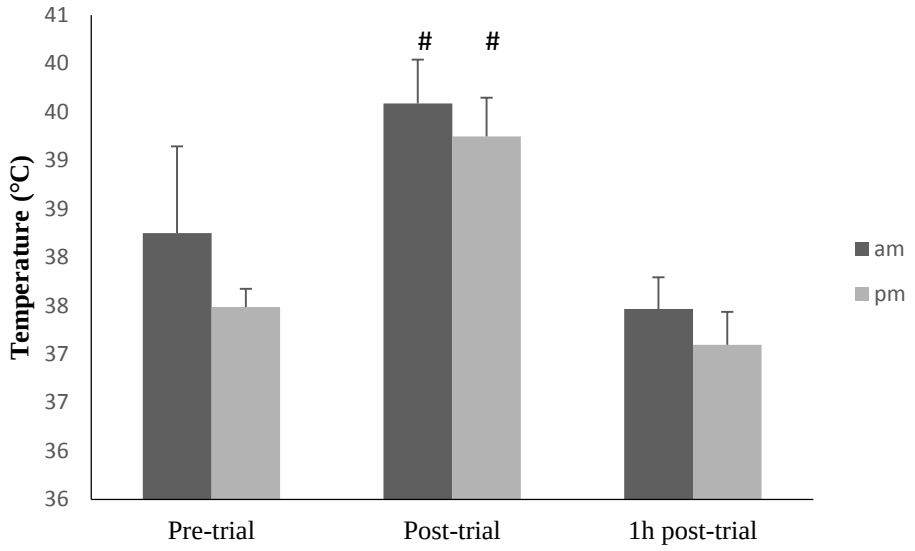

Figure 5.2b: Core body temperature at rest, post- and 1h post-trial at both times of the day. '#' denotes a significant different between trial time points ($P < 0.05$), values are mean ± SD.

5.3.3 Diurnal variation in heart rate

During the trial the mean heart rate demonstrated a diurnal variation ($F_{1, 6} = 23.5$, $p < 0.001$, partial $\eta^2 = .8$). The mean heart rate was significantly lower at 16:00 hs compared to 09:00 hs (174±8 at pm vs 176±7 beats min^{-1} at am). The mean maximum heart rate was recorded at the 10 km point for both trials (186±5 at am and 185±8 beats min^{-1} at pm). Figure 5.4 showed the mean and maximum heart rate response duirng the trial. In addition the results show that as the trial progresses the difficulty increases.

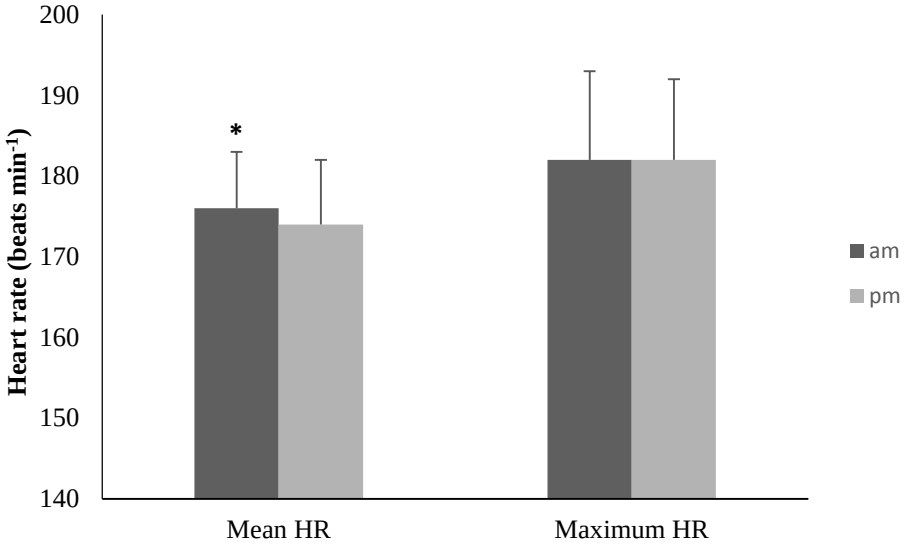

Figure 5.3: The diurnal variation in mean heart rate (mean ± SD). * Significant diurnal variation.

5.3.4 Diurnal variation in lung function variables

No differences were found between trials or time points (P>0.05) for the tested lung functions (Figure 5.5).

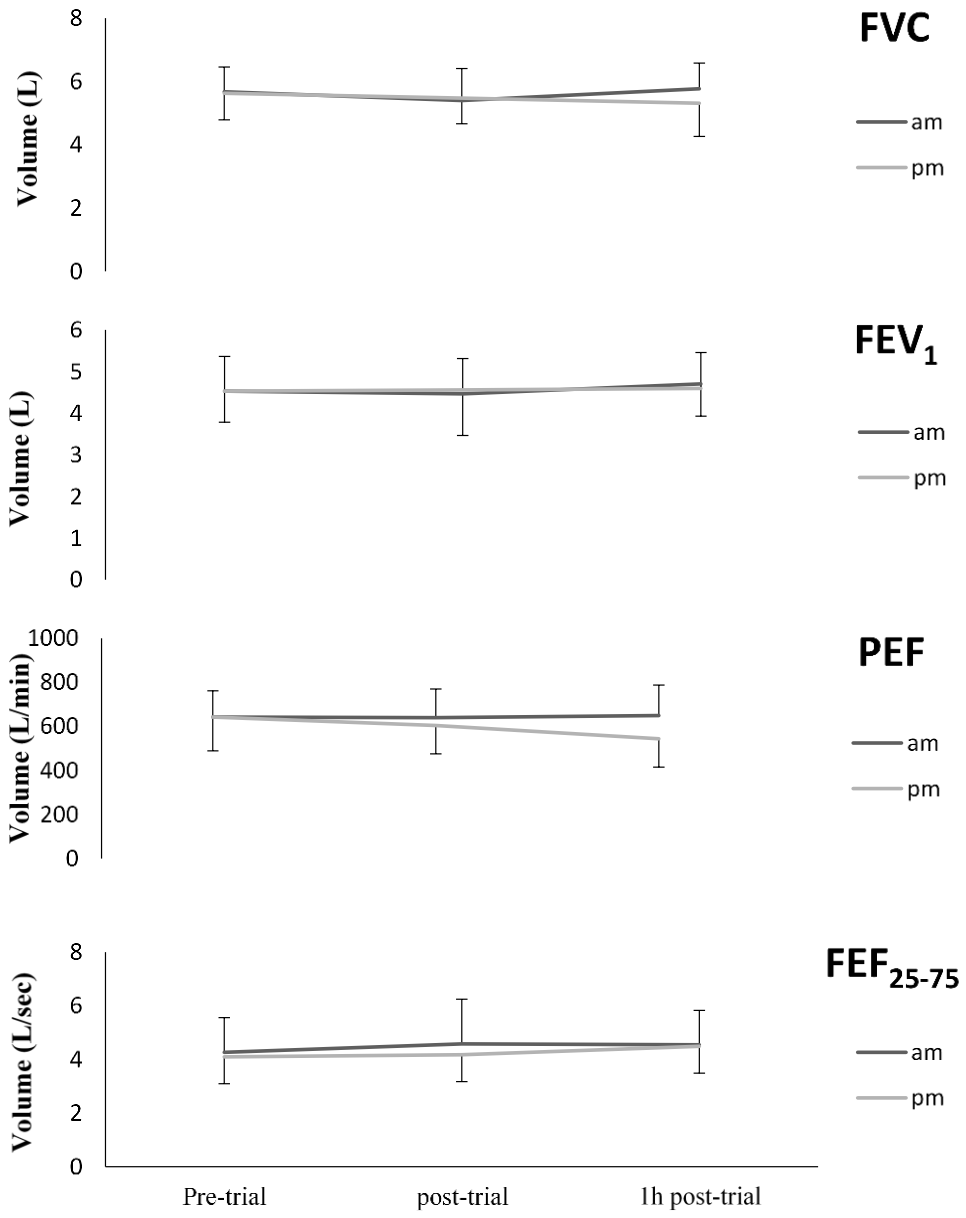

Figure 5.4: Lung function parameters in runners at pre-, immediately post- and 1h post-trial at both times of the day: 09:00 hs and 16:00 hs. Values are mean ± SD.

5.3.5 Diurnal variation in CC16

The results show that neither at rest or zeitgeber has an effect on CC16 in participant's diurnal variation (Figure 5.6). A significant difference between time point trials was observed for plasma CC16 at 09:00 hs ($F_{2,12}$ = 3.97, p < 0.001, partial η^2 = .4) with Bonferroni-adjusted, post hoc test revealing that the CC16 has significantly changed pre to post and post to 1h post-trial (p= 0.05 and p = 0.053) (Figure 4.6). In contrast to the morning trial, the evening trial showed no difference between time-points of the trial.

Higher values were measured immediately post-trial and 1 h-post- trial (29.19, 83.52 and 76.56 ug·L^{-1}) at 9:00 hs compared to 16:00 hs (48.60, 57.80 and 56.15 ug·L^{-1}). Evening resting plasma CC16 was higher than in the morning (48.60 and 29.19 ug·L^{-1}, 66%). In terms of phase response 09:00 hs, zeitgeber has a higher effect on CC16 compared to 16:00 hs (29.19 and 83.52 ug·L^{-1}). Additionally, CC16 at 09:00 hs had a slower return to the baseline level than at 16:00 hs (83.52 and 76.56 compared to 57.80 and 56.15).

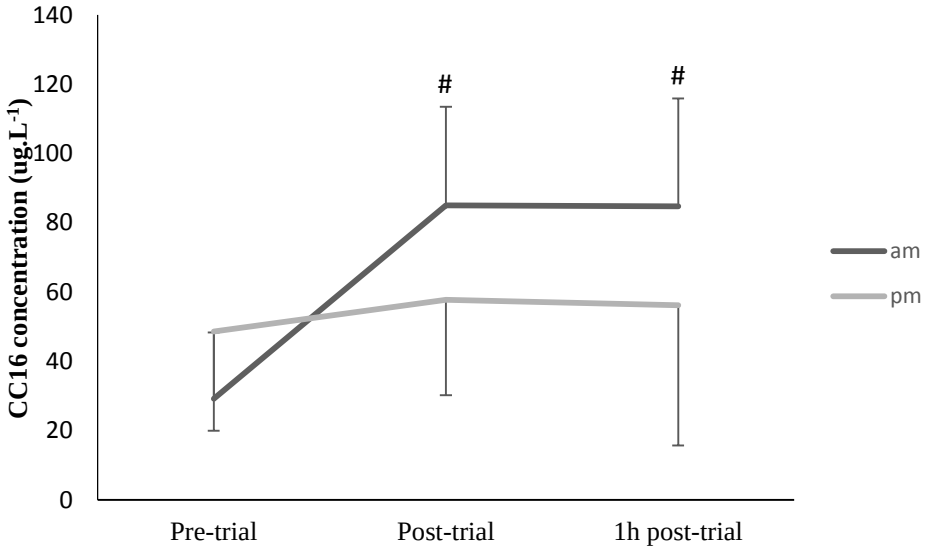

Figure 5.5: The diurnal variations in plasma mean CC16. # Significant increase in the morning trial (P<0.05).Values are mean ± SD.

5.3.6 Diurnal variation in WBC

A significant diurnal difference was found in WBC counts ($F_{1, 4} = 14.35$, $p < 0.002$, partial $\eta^2 = .8$) (Figure 5.7). Moreover, both trials showed a significant difference between time points ($F_{2, 8} = 13.3$, $p < 0.004$, partial $\eta^2 = .8$). With Bonferroni-adjusted, post hoc test revealing that WBC are more affected by zeitgeber in the evening trial ($p = 0.004$ and 0.003 at pm compared to $p = 0.004$ and 0.012 at am). WBC counts were higher at all-time points: pre-, post- and 1h post-trial for the evening trial compared to the morning trial. WBC increased at post-trial and remained higher at 1h post-trial compared to pre-trial. Phase responses showed that zeitgeber at 16:00 hs trial showed a greater effect on WBC compared to 09:00 hs trial (10.78, 6.38 x 10^9.l^{-1}, 70% and 7.81, 4.81 x 10^9.l^{-1}, 60%, respectively). At 1h post-trial WBC remained higher at 16:00 hs compared to 09:00 hs.

At 1h post-trial WBC remained higher at 16:00 hs compared to 09:00 hs (9 and 7.8 x $10^9.l^{-1}$, respectively).

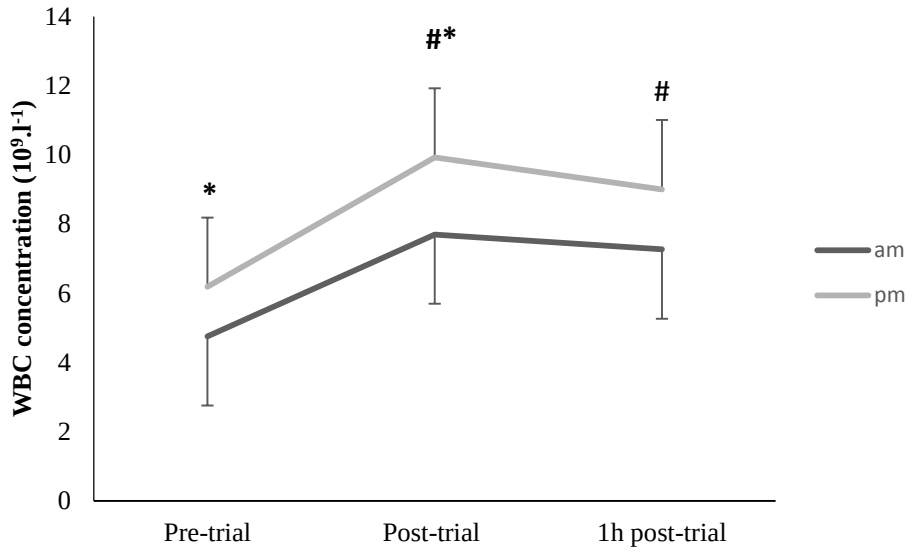

Figure 5.6: The diurnal variation in mean WBC concentration. * Significant diurnal variation at pre- and post-trial. # Significant increase between time points within both trials (P<0.05). Values are mean ± SD.

5.3.7 Diurnal variation in neutrophil

A significant diurnal difference was found in blood neutrophil counts ($F_{1, 6} = 6.26$, $p < 0.04$, partial $\eta^2 = .5$). With higher counts recorded at 16:00 hs compared to at 09:00 hs, at the three measured time intervals ($F_{1, 6} = 6.26$, $p < 0.04$, partial $\eta^2 = .5$) (Figure 5.8). With Bonferroni-adjusted, post hoc test revealing that neutrophils are affected more by zeitgeber in the evening trial ($p = 0.004$ and 0.003 compared to $p = 0.004$ and 0.012). However, neutrophil phase responses were higher at 09:00 hs (3.19 and 2.33 x $10^9.l^{-1}$, 37%) compared to 16:00 hs (4.53 and 3.37 x $10^9.l^{-1}$, 34 %). At 1h post-trial neutrophil counts remained higher at 09:00 (5.46 and 2.33 x $10^9.l^{-1}$, 134%) compared to at 16:00 hs (6.53 and 3.37 x $10^9.l^{-1}$, 94%).

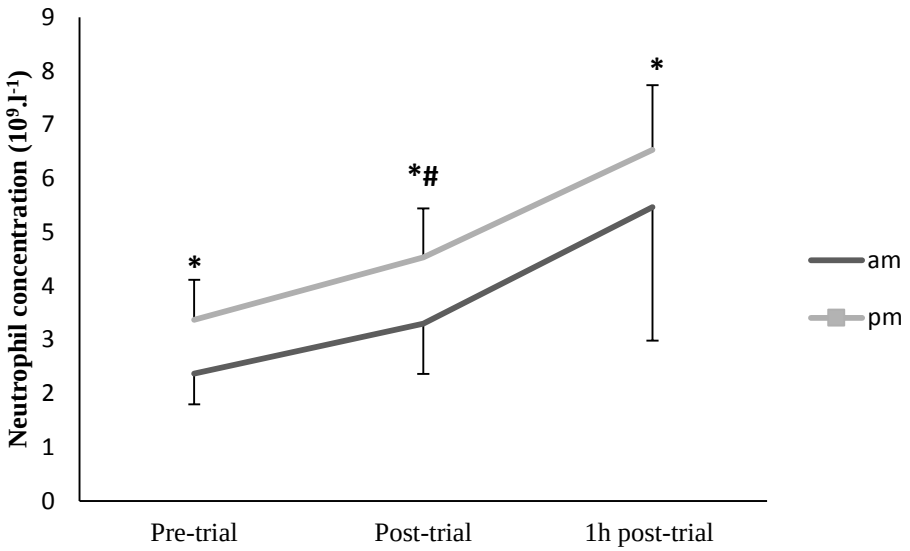

Figure 5.7: The diurnal variation in mean neutrophil concentration. * Significant diurnal variation at pre- and post-trial. # Significant increase between time points within both trials (P<0.05). Values are mean ± SD.

5.3.8 Diurnal variation in lymphocyte

A significant diurnal difference in blood lymphocyte counts was observed ($F_{1, 6} = 34.54$, $p < 0.001$, partial $\eta^2 = .9$). Both time point intervals of the day showed a significant difference ($F_{1, 12} = 15.72$, $p < 0.001$, partial $\eta^2 = .7$). With Bonferroni-adjusted, post hoc test revealing that lymphocytes are more affected by zeitgeber and showed greater counts occurred at 16:00 hs compared to 09:00 hs ($p = 0.018$ and 0.024 at pm compared to $p = 0.018$ and 0.112 at am) (Figure 5.9). Lymphocytes in response to the trial showed higher values at 16:00 hs compared to at 09:00 hs (3.65 and 1.67 and 4.38 x $10^9.l^{-1}$ and 2.08 x $10^9.l^{-1}$, 10% higher).

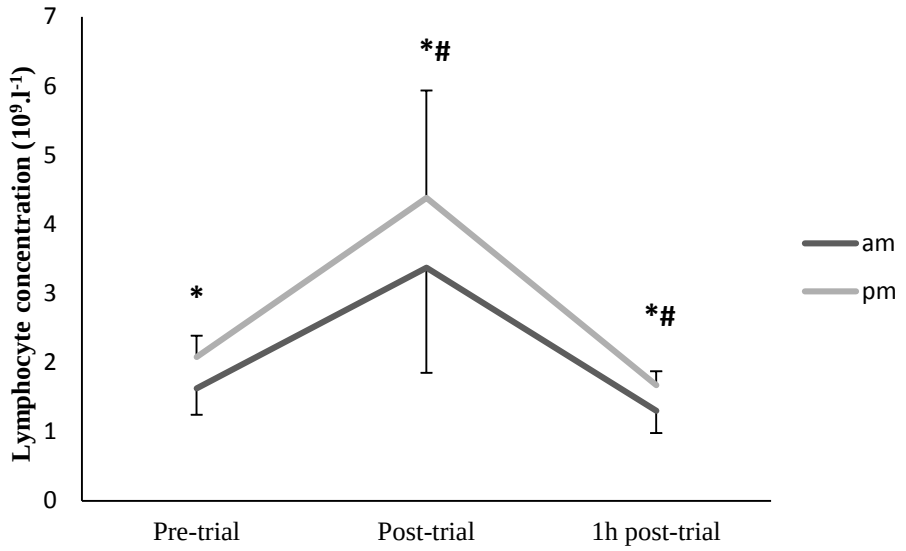

Figure 5.8: The diurnal variation in mean lymphocyte concentration. * Significant diurnal variation at pre-, post- and 1h post-trial. # Significant increase between time points within both trials ($P<0.05$). Values are mean ± SD.

5.3.9 Diurnal variation in white blood cell variables

The mean concentration of monocytes, eosinophils and basophils showed no significant difference between trials (Figure 5.10). The 95% Confidence Intervals (lower; upper limits) for the monocyte counts were: at 09:00 hs: pre-trial (0.40; 0.53), post-trial (0.42; 0.90) and 1h post-trial (0.41; 0.71) and at 16:00 hs: pre-trial (0.51; 0.58), post-trial (0.61; 0.92) and 1h post-trial (0.47; 0.75). The 95% Confidence Intervals (lower; upper limits) for the eosinophil counts were: at 09:00 hs: pre-trial (0.06; 0.48), post-trial (0.05; 0.90) and 1h post-trial (0.13; 0.31) and at 16:00 hs: pre-trial (0.09; 0.20), post-trial (0.08; 0.35) and 1h post-trial (0.10; 0.20). The 95% Confidence Intervals (lower; upper limits) for the basophil counts were: at 09:00 hs: pre-trial (0.02; 0.04), post-trial (0.04; 0.07) and 1h post-trial (0.03; 0.06) and at 16:00 hs: pre-trial (0.03; 0.04), post-trial (0.03; 0.06) and 1h post-trial (0.02; 0.05).

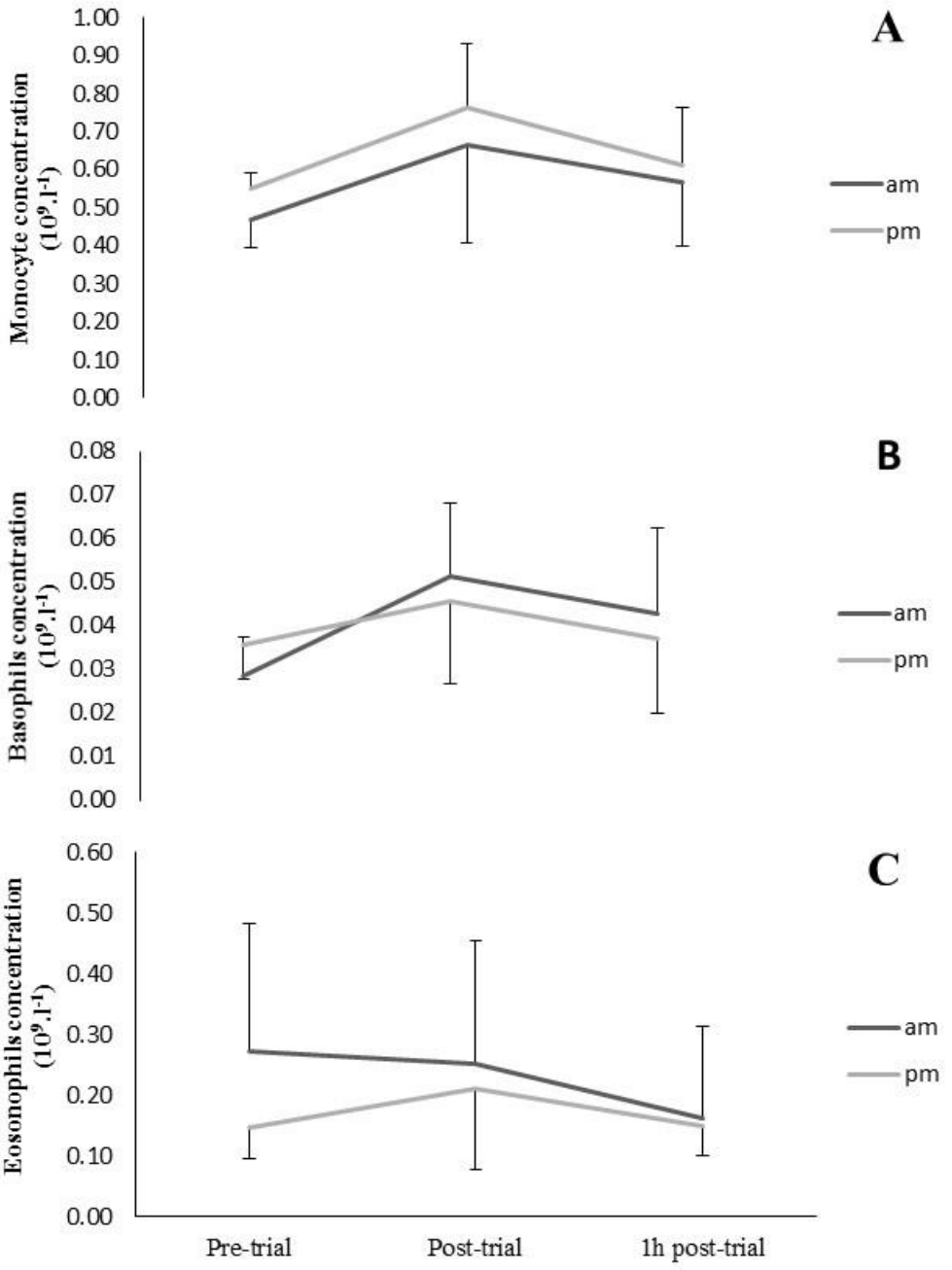

Figure 5.9: A, B and C: represent the response of monocytes, basophils and eosinophils, respectively, both trials. Values are mean ± SD.

5.3.10 Diurnal variation in red blood cell variables and platelet

The mean concentration of RBC, HGB, HCT and PLT showed both no diurnal variation and no significant difference between trials (Table 5.2). However, all recorded counts at pre- and post-trial where greater at 16:00 hs than at 09:00 hs, excluding PLT that were

higher at 16:00 hs. In contrast, at 1h post-trial all variables showed higher counts at 16:00 hs compared to at 09:00 hs.

Table 5.2: Diurnal variation in total red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT) and platelet (PLT), values are mean ± SD.

	Pre-trial			**Post-trial**			**1h post-trial**		
	09:00hs	**16:00hs**	**%Δ**	**09:00hs**	**16:00hs**	**%Δ**	**09:00hs**	**16:00hs**	**%Δ**
RBC	5.35±0.39	4.87±0.38	10%	5.23±0.49	5.04±0.17	4%	4.88±0.27	4.92±0.21	>1%
HGB	16.2±1.25	14.8±0.72	9%	15.9±1.64	15.4±0.59	3%	14.9±0.74	15.0±0.59	>1%
HCT	48.2±3.51	44.0±2.08	9%	47.4±4.91	45.7±1.81	4%	44.1±1.89	44.5±1.40	>1%
PLT	168±12	216±35	29%	288±44	275±49	20%	174±24	229±40	32%

5.3.11 Diurnal variation in plasma volume (dehydration)

Plasma volume was 53.83 ml and 52.27 ml at 09:00 hs and 16:00 hs, respectively. An increase of 0.26 g.100 ml^{-1} in HGB concentration was reported at 09:00 hs and an increase by 0.06 g.100 ml^{-1} at 16:00 hs. There was no diurnal variation observed in plasma volume dehydration (P = 0.803), the plasma volume was 3% lower in the evening compared to the morning.

5.4 Discussion

This study examined the effect of the time of day on 10 km running time trial treadmill run at 6°C. Physiological parameters of core body temperature and heart rate mean were both higher in the morning compared to the evening time-point. At 09:00 hs the mean core body temperature and heart rate were significantly higher by 0.4°C and 2 beats min^{-1}, respectively, compared to at 16:00 hs, therefore hypothesis 2 is rejected. No statistical differences (diurnal difference) were found for running performance between trials or for plasma CC16 concentration, on this basis hypothesis 1 and 3 are rejected. Interestingly however, a diurnal variation was observed for neutrophil and lymphocyte counts in response to the actual running trials immediately post-trial and 1h post-trial and therefore hypothesis 3 in this respect is accepted.

The performance result of this study does not corroborate previous published findings of a significant time-of-day variation in running performance (Martin et al., 2001). Atkinson et al. (2005) reported that better performance, in 16.1 km cycling trial, was found in the early evening. It is possible that the high level athletes involved in this study were used to running 10 km and therefore performed consistently regardless of the time of day. Additionally, most athletes participating in this study, trained twice a day, one session in the morning and the second session in the afternoon. Due to each participant's daily commitment (study or work) most morning sessions are easy runs, whereas intensity and high workload is reserved for the evening. Nevertheless, the 9 seconds mean difference in finishing time, is significant in terms of deciding the winner for a 10 km race. Furthermore, faster mean run in the present study performance in the evening rather than in the morning could be attributed to dietary status. However, in the present study masking factors such as dietary statuses was not controlled which may have an influence on circadian rhythmicity and sporting performance.

Core body temperature has been reported to influence exercise performance: enhanced when core body temperature is high (i.e. near its circadian peak), and worsened when it is low (i.e. near its circadian minimum) (Atkinson et al., 2005). Despite this study not finding a diurnal difference in performance, a diurnal difference in mean core body temperature was observed during the trial, with higher values in the morning compared with in the evening. However, at the pre-trial time-point no differences were found for these variables. These findings differ from those reported by Reilly et al. (2007) and all other research that found an opposite trend in core body temperature. It may be that these differences are due to the different techniques used to measure temperature: a wireless swallowed pill/device in this study compared to a rectal probe, tympanic or oral (Forsyth and Reilly, 2004; Reilly et al., 2007 and Hammouda et al., 2013). Therefore, a small study was carried out with this thesis to evaluate the accuracy and effectiveness of the core body temperature pills monitoring system. Three temperature measurement devices were used: skin thermometer, tympanic thermometer and core temperature sensor. The results showed that core body temperature pills monitoring system flow same trend as the both other measurement (Chapter 4). Furthermore, studies have found that the core temperature sensor versus other measures had the smallest bias (Gant et al., 2006).

However, Gant et al. (2006) reported that the wireless swallowed pill is appropriate or even more so, for use in exercise physiology research compared with a rectal device. Nevertheless, due to a technical problem and the participants excluded from the study presented an erroneous measurement below 36°C.

The ingestible thermal monitoring system was developed by a team at Johns Hopkins University in the late 1980s for NASA. Once the ingestible pill is swallowed by the participant (at least 1 hour prior to the trial following the method described in Domitrovich et al., 2010), the quartz sensor vibrates at a frequency relative to the body's temperature, transmitting a harmless, and low-frequency signal through the human body. A digital recorder monitored by the researcher displays the core body temperature reading. The pill is discharged safely from the digestive system approximately after 12 hours (HQinc manufacture guide line and THermodot). However, after reviewing the data collected for Chapter 6 and Chapter 4, it can be concluded that taking the sensor pills one hour prior the trial was insufficient time for the pill to be transported to the bottom of the stomach to show the true core body temperature reading. It is highly likely that the sensor pill position was much higher in the digestive system throughout the trial, such as in the oesophagus. It is therefore recommended that in future research the sensor pill is taken a minimum of 2 hours prior to testing.

During the morning trials the athletes' core body temperature increased more rapidly and achieved a higher temperature compared with the evening trials. These findings are supported by Aldemir et al. (2000) who showed that during sustained sub-maximal exercise, core body temperature rises more quickly in the early morning compared to late in the evening. This phenomenon could possibly be due to the body being in heat-gain mode in the morning; whereas in the evening, the body exhibits slow heat gain so that heat-loss is more pronounced (Aldemir et al., 2000). The athletes' bodies respond to these cold conditions by entering a preservation mode where the hypothalamus initiates a reduction in the volume of blood circulating near the surface to retain a greater volume of warm blood near the internal organs and maintain a higher internal temperature.

An elevated core body temperature could have a negative effect on the body's thermoregulation during maximal exercise. The fact that the mean core body temperature during the 09:00 hs run was higher compared to that of 16:00 hs suggests greater stress on the thermoregulatory system during the earlier morning testing. This is also reflected in the higher heart rate observed in the morning trial. This heart rate morning peak was 8% higher and could be in accordance with the body's physical demand during the trial. These results suggest that the increased morning heat production (heat storage) caused an extra strain on heart rate at this time of the day. These results should be considered when scheduling maximum exercise to avoid increased thermoregulatory demand and the feeling of fatigue (discussed in Chapter 7). These effects can have implications in the scheduling of training, testing and competition, especially for prolonged endurance events.

Athletes' heart rate in the present was significantly higher in the morning trial, which indicates that participants in the morning showed higher physical demand during this trial compared to the evening. In reality they are physically working harder to match the evening performance, as evidenced through higher heart rate and RPE in the morning trial compared to the evening. However, the present study result does not corroborate previous findings where heart was not affected by the time of the day (Scheer et al., 2010). In addition, from a clinical perspective the morning heart rate may be a cause for concern for cardiovascular patients that are attempting to practice exercise in the morning in cold condition.

RBC total counts, HCT, HGB, PLT and plasma volume (dehydration) did not show either a diurnal significant difference between morning and evening trials or at any time point during the trials. This result corroborates with Simpson et al. (2005) were RBC, HCT and HGB did not change in fourteen London marathon finishers. PLT showed higher counts at 16:00 hs at the three time points. The rate of PLT phase responses from pre- to post-trial was higher in the morning compared to the evening (36% vs 27%). This PLT result is in accordance with previous research that reported an increase in platelet counts ranging from 7% to 13%, immediately after 20 minutes of treadmill running (Davis et al., 1990). In addition, plasma volume was higher by 3% in the evening compared to the morning

(but did not reach the significance level). However, the highest decrease in plasma volume was recorded in the evening where better performances were also recorded. However, the decrease in plasma volume which accompanies dehydration (Jimenez et al., 1999) support the findings of Tam and Noakes (2013) and Wall et al. (2013) that have found dehydration has no effect on sport performance.

Likewise, the lung function parameters analysed in this study showed no significant diurnal variations or differences in the time-points. This result is contrary to the findings of Medarov et al. (2008) who reported that healthy individuals (n=4,756) have a significant circadian variation in FEV1 and FEV1/FEVC. The findings of the present study did not show diurnal variation in lung function which likewise may be due to the fitness of athletes used in this study. The morning data, in this study, were collected at 09:00 hs, potentially not early enough to notice diurnal differences in participants. A better assessment of diurnal difference would involve the first measurement to be taken straight upon waking, as it is known that bronchial calibre in all humans is narrower in the early hours of the morning (Kelly et al., 2004) and this may lead to a lung function nadir recorded in the early morning (straight upon waking).

To the best to our knowledge this is the first study investigating diurnal differences on plasma CC16 in highly trained runners (71 ± 6 $mlO_2 \cdot kg^{-1}min^{-1}$). The concentration of plasma CC16 did not show a significant difference between the two times of the day. This is in disagreement with the published findings of Helleday et al. (2006) who reported a decrease in serum CC16 concentration during daytime, with a significant drop between 11.30 hs and 22:00 hs within a healthy population group. This protein can be used as a specific biomarker of the airway epithelium integrity and an approach to estimate the degree of lung epithelial injury (Gomes et al., 2011). Therefore allowing the identification of URTI the most common medical condition affecting both highly trained and elite athletes, in particular those participating in endurance events (Bermon, 2007).

It is known that environmental conditions impact on the degree of airway epithelial disruption during high level exercise (Bolger et al., 2011**)**. Plasma CC16 concentration in this study at morning time shows a different trend than evening time; with evening resting

concentration higher by 66% than the morning resting concentration (did not reach the significance level). Whereas, morning post- and 1h post-trial CC16 concentrations where higher by 47% and 52%, respectively. Moreover, in the present study the degree of leakage of CC16 or lung function suppression was more noticeable in the morning compared to evening in cold conditions. Bolger et al. (2011) found after 8 minutes of exercise in dry air, epithelial injury was identified in participants. Furthermore, this may suggest a high effect of morning exercise on lung suppression (injury) compared to the evening during exercise in a cold condition and shows that a higher rate of ventilation occurred in the morning (this may be due to the high physiological strain reported in the morning).

This study supports the idea that workload training should be applied in the evening to minimize the risk of epithelial injury/URTI. In addition, participants in the morning may experience higher physical demand during the trial compared to the evening. A higher rate of cold air inhalation in the morning could be one of the main reasons that CC16 is higher at this time of the day compared to the evening. Additionally, a high concentration of this protein in the blood could be a sign of inflammation (Gomes et al., 2011). Hermans et al. (1999) found the concentration of CC16 in the blood increased as a result of pulmonary inflammation and increased the permeability of the lung epithelial barrier in animal model (the increase range was 46% to 246% in the treated group compared to the control group). In addition, Gomes et al. (2011) found a significant increase in CC16 concentration after an 8 km run in a hot and polluted environment (increased by 4% and 9%, in the control group and hot condition group, respectively). The authors suggested that exercise in extreme conditions will elicit early epithelial damage characterized by an increase in CC16 concentration in the airways.

In the present study, the reasons for the diurnal variation in the CC16 concentrations were not investigated, and nothing is known about the underlying mechanisms here. However, a few possible explanations for this result can be speculated on. According to McAuley and Matthay (2009), an increase in CC16 concentration could reflect the differentiation of distal lung epithelial cells into alveolar epithelial cells as part of the repair processes. Another explanation could be a difference in transepithelial leakage due to cyclic changes

in the tightness of the epithelial junctions. The CC16 difference could also be due to pulmonary hemodynamic: during the night, as a result of body sleep positions, CC16 may accumulate in the lung and/or a decrease in blood flow and reduced renal circulation may contribute to these observed differences in CC16 concentrations at the morning (Helleday et al., 2006). However, this increase in the epithelial damage and the decrease in lymphocytes (discussed in the next paragraph) may reduce the ability of the body to deal with the stresses of exercise in a cold environment. It may be that the open window theory could be more pronounced in the morning compared to the evening.

It is well known that a distinct circadian rhythm exists within the endocrine and immune systems (Dimitrov et al., 2009). For the present study a significant diurnal difference was observed in total white blood cells, neutrophil and lymphocyte counts. In contrast monocytes, basophils and eosinophils do not appear to be affected by time of the day. Monocytes showed a significant, pre- to post-trial difference at 16:00 hs. As expected, the exercise bout was sufficient to promote a change in lymphocyte counts increasing immediately post-trial compared to those pre-trial or at 1h post-trial at both times of the day (classic bi-phasic lymphocyte response) (Simpson el al., 2006). This classic bi-phasic lymphocyte response to exercise causes suppression in the immune system that can cause potential issues if the athlete does not allow sufficient recovery time before the next training session. Recurrent infections and immune-depression is common due to frequently repeated exercise without sufficient time for the athletes immune system to recover fully (Papacosta and Gleeson, 2013).

This result supports the findings of other published work: that intense exercise suppresses the immune system (Simpson et al., 2007b; Walsh et al., 2011). Neutrophil counts significantly increased 1h post-trial compared to pre- and post- trial at both times of the day. This post-trial increase in neutrophils and lymphocytes is considered to result in an "open window" (Kakanis et al., 2010) of decreased host protection, which can last between 3 and 72 hs and represents a vulnerable time period for the individual contracting and developing an infection (Nieman and Bishop, 2006). On the other hand, it could be that the highly trained participants of this study have an adapted immune system able to withstand infections even during severe physiological and psychological stress (S-curve

shape as described by Malm et al., 2006), while moderate and high exercise decreases the infection odds ratio. The higher neutrophil and lymphocyte counts at 16:00 hs compared to 09:00 hs is possibly due to the athletes' daily winter exposure and the cold environment (all participants were based in the UK). Another possibility may be the influence of the endocrine hormones as seen with regards to other immune cells (Dimitrov et al., 2009). For example T lymphocytes cells (naïve CD4+ and CD8+) showed nadir circadian rhythm with the day time driven by cortisol. Furthermore, it clearly indicates that exercising for a short duration (approximately 30 minutes) at high intensity has a greater effect on neutrophil and lymphocyte counts during exercise in a cold environment (37% and 34% neutrophil increase at 09:00 hs and 16:00 hs, respectively, and 112% and 111% lymphocyte increase at 09:00 hs and 16:00 hs, respectively). However, both chemical (supplements) and nutritional interventions have been recommended for athletes to minimise potential negative changes in immunity during periods of intensity training (for review see Nieman and Bishop, 2006). Another explanation for this is that athletes' in particular elite athletes often demonstrate airway inflammation in association with exercise (Walsh et al., 2011 and Figure 4.11). Exercise in a cold environment increases ventilation, increasing with the intensity and the duration of the exercise. This in turn increases the rate of water and heat loss through the respiration. At rest, ventilation decreases as does the cooling stimulus, resulting in a rebound vasodilatation. It is this process that increases the risk of airway inflammation in athletes (Anderson and Kippelen, 2008).

It can be suggested that participants taking part in the present study would be within the second suggestion (demonstrated by airway inflammation). Furthermore, the prevalence of airway inflammation is greater among elite athletes than in the general population (Bougault et al., 2009) and is higher among certain groups of athletes, such as distance runners and cyclists. The reason for this inflammation is unknown and this study has not investigated the mechanism behind it, but potentially it can be caused by exposure to cold air (Helenius et al., 2005). In addition, bronchial calibre in all humans is narrower in the early hours of the morning (Kelly et al., 2004), and this could be one of the high immunological morning recruitment factors. This study did not investigate directly URTI but is supported by previous studies of URTI (Gleeson et al., 2013). CC16 is linked to

epithelium injury that is higher in the morning. This study supports the finding that strenuous exercise suppresses the immune system in well trained athletes and that this dysfunction may lead to an URTI especially during inhalation of cold and dry air and is more pronounced at morning time (Whitham et al., 2006).

It is recommended that athletes perform their major race preparation in a warm humid condition rather than in cold dry conditions (see Figure 4.11). Also the implementation of new plans to reduce the risk of airway dehydration stress is therefore required to protect elite athletes' long-term respiratory health during cold winter months in the UK. According to these findings training or racing to minimise the risk of URTI in a cold environment is highly recommended to be carried out in the evening time where less physiological and immunological stress was found to occur (phase response).

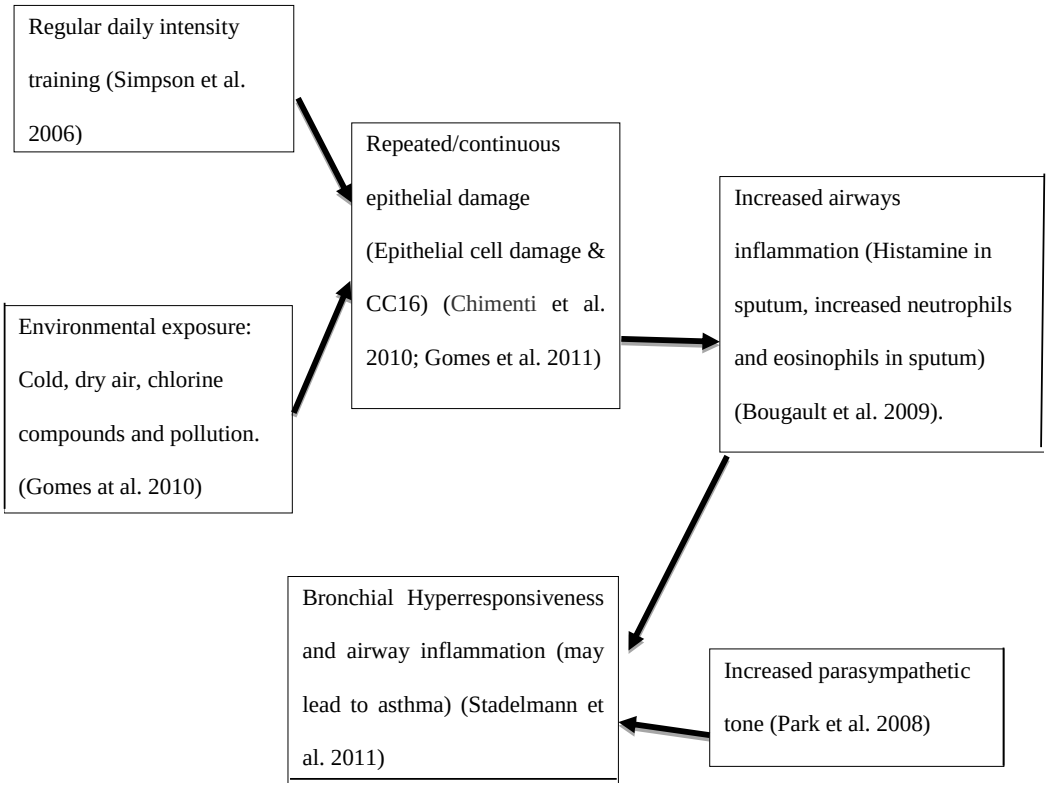

Figure 5.10: Factors involved in airway inflammation development in elite athletes.

5.5 Conclusion

In terms of running performance in a cold environment the findings of this study suggest that training at the same time of day has the potential to alter normal diurnal variation in 10 km running performance. Indeed, adaptation to training volume and intensity could be greater at the time of the day at which training is performed than at other times of the day (Chtourou et al., 2012). In addition, training performed in the morning can improve morning poor performances to the same level or even higher than the normal daily best, which is typically observed in the evening hours (Atkinson et al., 2005). Athletes are required to compete at a certain time of day due to the competition schedules, therefore, athletes and coaches may be advised to plan their training hours with the time of day at which one's critical performance is planned. In addition, the morning trial in cold condition caused more physiological and immunological strain compared to the same trial in the evening. However, this extra stress caused by zeitgebers could be a useful strategy for athletes and coaches to improve running performance in cold condition in the long term plan.

It can be concluded that a 10 km time trial run can result in different physiological and immunological responses depending on the time-of-day it is performed. Nevertheless, in highly trained runners these responses were not enough to impact performance. The participants of this study were highly trained athletes, training mostly two to three times a day during the pre-race season. Athletes, coaches and event organisers can program their race events or training to target the time when athletes perform best and should consider the immunological and physiological parameters that can enhance performance. There is a need to look at psychological factors that may affect running performance in elite athletes, and this is investigated in Chapter 7.

CHAPTER 6:

DIURNAL VARIATION IN PHYSIOLOGICAL AND IMMUNE RESPONSE TO INTENSE EXERCISE PERFORMED IN HOT AND HUMID ENVIRONMENT

6.1 Introduction

Mostly, fire fighters, athletes, army personnel and astronauts may be required to perform in extreme environment conditions (e.g. heat, cold, high altitude and microgravity). Sport endurance performance can be impaired in hot environmental temperatures compared with temperate conditions (Mohr et al., 2012). In fact, exercise to exhaustion is influenced by alterations in the initial core body temperature (Hsu et al., 2013). Exercise increases heat production with more than 70% of the generated heat transported to the skin for dissipation to the environment (Lim et al., 2009). The attainment of a critically high core body temperature has been proposed as the main factor limiting endurance performance in hot environments (Hunter et al., 2006**)**.

There are great physiological, immunological and psychological changes that occur in an athlete's body during exercise in the heat compared with the habitual environment (Casa et al., 2010). These changes also include thermoregulatory alterations in the circulatory and endocrine systems, cardiovascular strain, glycogen depletion as well as increased metabolite accumulation (Jentjens et al., 2002; Oeoepik et al., 2014; Racinais et al., 2014). Many interrelated physiological processes work together to maintain muscular function, sustain central blood pressure, regulate fluid volume, and maintain the body temperature within normal limits (Casa et al., 2010). Intense exercise in the heat can overload the body's ability to react properly to the stress imposed by athletes, and the result of this can be hyperthermia, dehydration, decrease in physical and mental performance, and even heat stroke (Casa et al., 2010).

The mechanisms dealing with prolonged exercise in heat involve several factors such as reductions in power output and muscle activation, and the rate of cerebral glucose uptake, the rate of fatigue which may primarily be associated with a change in the central fatigue system (central nervous system) (Boyas et al., 2013). This change in the central nervous system can lead to impairments in cardiovascular function that will diminish arterial oxygen supply to exercising muscles and increase peripheral fatigue (Gonzalez-Alonso and Calbet, 2003). Thus, body thermoregulation failure will lead to central fatigue, where the athlete's body is no longer able to recruit enough motor units to sustain the demand

of power output (inability to produce or maintain a desired power output) (Tucker et al., 2004). In addition, during exercise and due to the body's energy metabolism demand, heat accumulation in the athlete's body starts when the heat dissipating mechanisms are unable to cope with metabolic heat production, leading to an increase in body temperature (Lim et al., 2009).

In general, the rapid increase of core body temperature caused by the combination of heat and exercise lead to high internal temperature. This seems to be an independent cause of fatigue during exercise in hot environments (Mitchell et al., 2014). Therefore, body mechanism responses during exercise in heat, includes an increase in blood flow by vasodilation, vasoconstriction and maintenance of blood pressure (Gonzalez-Alonso and Calbet, 2003). In addition, muscles require more blood in order to meet energy requirements of the body, which will increase core body temperature as a result. This increase in core body temperature will have an effect on body functioning. The body's circulatory system regulates and transfers heat from active muscles to the skin. This will start a series of cooling down mechanisms, mainly by increasing sweating rate and vasodilation of the superficial blood vessels. In this case heat is lost via evaporation of sweat from the skin and transferred from the body to the environment (internal to external). However, an average individual may lose 3% of fluid without much decrease in exercise intensity (Wall et al, 2013). Therefore, a decrease in blood volume will lead to a slowdown in heat load dissipation as less blood will be supplied to the skin vessels for sweat production.

Athletes may experience hyperthermia due to the reduction of heat dissipation and from an impaired skin blood flow and sweating response when exercising in the heat at moderate intensities (Tucker et al., 2004). Three important components exist when exercising in heat: firstly, skin vasodilation, which is correlated to the external and internal heat production (Fujii et al., 2013). Secondly, muscle vasodilation that is dictated by the intensity of exercise and by the ambient temperature. Lastly, vasoconstriction of the internal organs that allows increased blood flow to the active tissues (Fujii et al., 2013). During exercise in heat an imbalance between sweat production and water intake often occurs, leading to dehydration (water deficit). This dehydration can have a negative

influence on thermoregulation and exercise performance in a hot environment (Marino et al., 2003).

Although a slight increase in skeletal muscle temperature will produce better performance (Florida-James and Doggart, 2000) this becomes markedly impaired when exercising at high intensity in a hot and humid environment. This reduced performance is directly associated with the failure of the cardiovascular system to maintain arterial oxygen delivery to the exercising muscles as well as the ability of the brain to sustain sufficient activation of the skeletal muscles. In addition, elevated brain temperature appears to be the main factor affecting motor activation of skeletal muscles and activity of the dopaminergic system, in addition, the relative influence from central fatigue is significantly enhanced during activities in the heat (Roelands and Meeusen, 2010).

To date heart rate is a known marker of exercise intensity in athletes (Lamberts et al., 2004), as heart rate can be used as reliable telemetric monitors during training sessions for immediate feedback to the athlete and coach, or for later analysis (Lamberts et al., 2004). In general, it is known that heart rate increases as a response to the heat, with core body temperature passing through phases during the day (diurnal variation): heat gain in the morning and heat loss in the afternoon. Heart rate increases both with exercise and during the phase of heat gain, additionally, dehydration causes heart rate to increase yet more. In contrast, heart rate decreases during the body's heat loss phase as an adjustment to improve exercise capacity during heat stress. This decrease in heart rate during this phase of heat loss is more effective in a hot environment compared to a neutral or cold environment (Sawka et al., 1999). Sawka et al. (1999) reported that on the first day of exercise in the heat, heart rate reached much higher levels than in neutral conditions, with heart rate beginning to decrease on the second day of heat exposure as a result of adaptation.

Crandall et al. (2008) showed an increase of 13% in left ventricular ejection fraction that occurs in concert with an increase in heart rate in young participants exposed to a hot environment where the heart rate increased from an average of 53 (beats·min^{-1}) in a control group to 93 (beats·min^{-1}) in an experimental group. Often major sporting events

are held during the summer such as the Olympic Games and the football World Cup. In general, endurance athletes perform at intensities of $\dot{V}O_2max$ (65–85 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$) in events lasting from 10 min to many hours (Gonzalez-Alonso et al., 2008). For instance, elite marathon specialists can run the 42.2 km marathon race under 2 hs 10 minutes at 80 to 90% of $\dot{V}O_2max$ speed. Moreover, elite cyclists can complete a 1 h trial at 85–95% of $\dot{V}O_2max$ (Gonzalez-Alonso et al., 2008). To sustain the body's physiological demand for more than 2 hs the heart is required to pump continuously at a rate of 22–27 L of blood min^{-1} (Gonzalez-Alonso et al., 2008). Furthermore, exposure to heat leads the human heart rate and cardiac output to increase, with blood vessels widening to divert warm blood to the body surface. Heat strain leads to 15 to 25% of the blood passing through the skin. Additionally, body physiological demand increases with more oxygen demand to sustain energy and heat metabolism (transporting blood from the deep tissue to the skin).

According to the IAAF medical handbook (2009) the risk of heat injury rises when competing above 21°C and 50% relative humidity. It is therefore important to understand the physiological and immunological changes that occur in response to exercising in the heat. This is particularly important considering the frequency of high level athletic competitions that occur in environmentally hot conditions such as the Athens Olympic Games 2004; Beijing Olympic Games 2008; FIFA World Cup South Africa 2010; as well as future fixtures to include Rio de Janiero Olympic Games 2016; and FIFA World Cup Qatar 2022.

The research idea behind this chapter was based on imitating the typical conditions of Rio de Janeiro where international athletes will gather for the 2016 Olympic Games, where atmospheric conditions of around 28°C and 70% relative humidity are expected (Metoffice.gov.uk 2012).

Several studies have investigated some aspects of this research, be it physiological, immunological or psychological diurnal variations or their response to different external environmental conditions. The novelty of this research is that it investigates these parameters in a running experiment using highly-trained athletes to directly relate the findings to the competitive running population of the world.

Aim: to analyse the time of the day effect on running performance including immunological, physiological and psychological (fully discussed in Chapter 7) changes during exercise in a hot and humid environment.

Hypotheses:

Better performance will occur in the afternoon coinciding with the peak of core body temperature in hot and humid environment.

Physiological responses to the running time trial will be higher in the afternoon coinciding with the peak of core body temperature in hot and humid environment.

White blood cells, IL-6, CC16 and HSP70 concentrations will be higher in the afternoon coinciding with the peak of core body temperature in hot and humid environment.

6.2 Materials and methods

Changing trial time from 16:00 hs to 18:00 hs:

The first study (Chapter 5) was completed at Edinburgh Napier University's Merchiston Campus. The University's regulation required that laboratory and testing facilities be vacated by 20:00 hs. Therefore to allow sufficient time to run the trial and the suite of complimentary measurements as well as essential laboratory analysis a start time of 16:00 hs was necessary. The University Life Science Department including all laboratory and testing facilities was transferred permanently from the Merchiston to the Sighthill Campuses. This change also coincided with an extension in the University's opening hours to 22:00 hs, this allowed for a later testing time of 18:00 hs for this trial (Chapter 6). Therefore, the second time point for the two studies are different, 16:00 and 18:00 hs. These two times were the latest possible sampling time period that would allow the completion of the trial and additional laboratory time to analyse samples immediately after completion of the trial. Furthermore, 18:00 hs reflects evening competition time and daily training time for our participants.

Further tests carried out in this study that were not described in Chapter 3 are fully explained in this section.

Thirteen male endurance runners (mean ± SD: 33±5 years, $\dot{V}O_2max$ range 61-79ml·kg^{-1}min^{-1}) took part in this study. The participants were asked to complete a general medical history questionnaire (Appendix 2) and a specific medical questionnaire (Appendix 6). All participants provided fully informed written consent before engaging with the experiment and were free to withdraw from the study at any stage.

6.2.1 Experimental procedure

In summary, the exercise protocol consisted of a 10 km time trial run at 2 different times of the day: 09:00 hs and 18:00 hs. During the trials runners could control the speed of their run, but they did not have access to the value of the speed. Blood samples, lung function test, heart rate, core body temperature, flexibility, strength, hand grip and muscle power were assessed pre-, post- and 1h post-trial for both times of the day. The specific environmental conditions were controlled by an environmental chamber at 28°C and 70% relative humidity; the same projected climate as Rio de Janeiro and the Olympic Games 2016 (Metoffice.gov.uk).

Blood samples were analysed for the following:

Inflammatory markers: plasma CC16, WBC, neutrophil, lymphocyte, monocyte, eosinophil, basophil, IL-6 and HSP70 (see Chapter 3 section 3.5.2, for more details).

Hormones: cortisol

Questionnaires were used to assess the following psychological variables*:* RPE, alertness and arousal (fully discussed in Chapter 7).

6.2.2 Sample Collection

Before the start of data collection, a demonstration of each of the techniques used were given by researcher.

Flexibility (Sit and reach box), power (Fusion sport & SMARTJUMP™/Australia), grip strength (Grip. A-T.K.K.5001. Japan), and back and leg strength (Back.A-T.K.K.5002. Japan) were measured pre-, post- and 1h post- trial.

Flexibility (Sit and reach box):

The participant is seated on the floor, with legs straight and shoes off. Feet were placed with the flat soles against the box. Then with straight hands (palms facing down), the participant reached forward along the measuring line as far as possible. The mean of three practices were recorded.

Power (Fusion sport & SMARTJUMP™/Australia)

The power was measured using vertical jump on a force platform mat. Participants stood with feet shoulder-width apart on the mat. Without flexing their knees (binding), jumped vertically as high as possible using both legs and arms to assist in projecting the body upwards. Body mass and height were entered into the device (Fusion Sport and SMARTJUMP™/Australia). The jump was repeated three times and the mean recorded. Before each jump, the force platform was zeroed.

Grip strength (Grip. A-T.K.K.5001. Japan)

In a standing position the participant holds the dynamometer in the hand to be tested (allowed to use only one hand), with the arm hanging vertically downwards and the elbow by the side of the body. The dynamometer handle is adjustable if required. The dynamometer handle should be held with the fingers and excluding the thumb with the base of the dynamometer resting on the heel of the palm. Participants were required to squeeze the dynamometer with maximum isometric effort without body movement. The mean of three practices were recorded. The test was repeated for the other hand.

Back and leg strength (Back.A-T.K.K.5002. Japan)

In an upright standing position with the feet shoulder width apart and the palms facing toward the body, arms hanging vertically down to hold the dynamometer bar with both hands. The dynamometer chain should be adjusted to allow the knees to be bent at approximately 110 degrees. At this position the participants back should be bent slightly forward at the hips, with head held upright. In this position participants are required to pull the chain as hard as they can and try to straighten their leg (back to 180 degrees).

Blood pressure measurement (Omron, 5-1, Japan)

At resting, post and 1h post-trial (BP) blood pressure was measured in the seated position by well-trained physicians using an electronic mercury sphygmomanometer (Wrist, Omron, 5-1, Japan). Participants were advised to wear a loose-fitting clothes like a t-shirt so that allow can to push the sleeve up comfortably if needed. The same arm was used for measurement at both times of the day and at all trial time points. With relaxed, straight arm and the hand palm facing up, a blood pressure cuff was placed approximately 5 cm above the elbow join. Afterward blood pressure was automatically measured and the result was displayed on the device screen. The measurement was performed at room temperature.

6.2.3 Nasal lavage method

At rest, prior to the testing, and directly after finishing the 10 km trial, nasal lavage was performed. Immediately prior to the nasal lavage procedure participants were required to perform a gentle massage to the nose (Naclerio et al., 1983). Participants then tilted their heads back to a 45° angle and elevated their palate, to close the nasopharynx, 5 ml of pre-warmed (40°C), sterile saline solution was introduced into the nostril and held for a duration of 10 seconds. The participant then brought their head forward and expired the lavage into a polyamide gauze-filtered funnel (size 100 mm) that separated the mucus from the solution and the samples were collected at the base in a 15 ml centrifuge tube. The nasal lavage procedure was performed again with the other nostril. The volume collected from both nostrils was stored in the same tube; the total value collected was recorded and immediately placed on ice until analysis (Gomes et al., 2010). The total

sample collected was centrifuged (Universal, 320R, Zentrifugen, Germany) at 480 g (*relative centrifugal force)* for 15 min at a temperature of 4°C. 200 µl was re-suspended with 50 µl of saline. From this suspension 25 µl was mixed with 10 µl of trypan blue. A 20 µl sub-sample from the mixture of nasal lavage suspension and trypan was used to conduct the cell count immediately under a microscope (Kyowa, Unilux-12. Japan) set at x10 magnification. A total of eight 1 mm^2 squares were counted: starting in the right top square of the haemocytometer. To determine the number of cells per ml the total cell count was multiplied by 1 x 10^4. The remaining 150 µl solution was re-suspended and used to count cell differentiation, using cytospin (Cytospin3, Shandon, UK). The cytospin machine was set at 120 rpm for 5 min. Due to the thickness of the filter it was better to run cytospin with 150 µl saline before running the samples to avoid lack of absorbance. Slides were left to dry before staining with Romanowsky, A, B and C stain (Raymond A. Lamb, UK). After this the slide was left to dry at room temperature. Lastly, according to the cells morphological appearance the cells were differentiated.

Calculating diurnal variation in plasma volume changes (dehydration), method of CC16, IL-6 and HSP70: as previously described in Chapter 3.

6.2.4 Statistical Analysis

Prior to statistical analysis all data were checked for normality. Data were analysed using two way repeated measure ANOVA with Bonferroni-adjusted, post hoc test to determine the difference at which time point the diurnal variation occurred and the difference between time points of the trial. Paired sample T tests used to determine the different diurnal variation in stature (SPSS20 Statistical Software, IBM.UK). Statistical significance was accepted at $P < 0.05$. Results are represented as mean values ± standard deviation (SD). Cohen's d was used to assess the size of the difference between two related sample means and can be calculated with the flowing formula: d= Mean 1 + Mean 2 / Sp. [Sp = Standard deviation 1+ standard deviation 2 / 2].

6.3 Results

6.3.1 Diurnal variation in anthropometric and physiological performance variables.

There was a significant diurnal variation difference in stature (P = 0.003, t = [12] = 3.87. Cohen's *d* for this test was 0.281 which can be described as small) (Table 6.1) where the participants were taller by a mean of 1.46 cm in the morning trial compared to the evening trial. Participant's fluid loss mean shows either no diurnal variation or trial time-point differences (1.02 kg in the morning trial and 0.79 kg in the evening trial). The 95% Confidence Intervals (lower; upper limits) for the participants mean fluid loss at 09:00 hs, pre-trial (67.6; 75.3kg), post-trial (66.4; 74.8 kg) and at 18:00 hs, pre-trial (67.3; 75.8 kg), post-trial (66.4; 74.7 kg). In addition, the level of dehydration was 3% higher at 09:00 hs compared to 18:00 hs (Table 6.1).

Table 6.1: Stature and body mass in both trials.

	09:00 hs		**18:00 hs**	
	Pre-trial	**Post-trial**	**Pre-trial**	**Post-trial**
Body mass (kg)	71.80±7.13	70.78±7.00	71.55±7.04	70.76±6.91
Body water lose (kg)	1.02±0.13		0.76±0.13	
Stature (cm)	180.96±5.38*		179.50±4.97*	

'*' symbol denotes a significant diurnal difference. Body water loss = pre- trial body mass – post-trial body mass.

6.3.2 Performance Variables

Each individuals (n = 13) time to complete both trials are shown in Figure 6.1. There was no significant difference between trials. The 95% Confidence Intervals (lower; upper limits) for the time to complete the trials were: 09:00 hs 10 km running trial (37:55; 43:16) and at 18:00 hs 10 km running trial (37:44; 43:54). Nevertheless, the mean running time to complete the 10 km trial at 09:00 hs was faster by 19 seconds than at 18:00 hs. The

mean times to complete the trial were 40 minutes and 22 second ± 4 minute and 20 seconds at 09:00 hs and 40 minutes and 41 second's ± 5 minutes and 03 second's. All values are the mean ± SD.

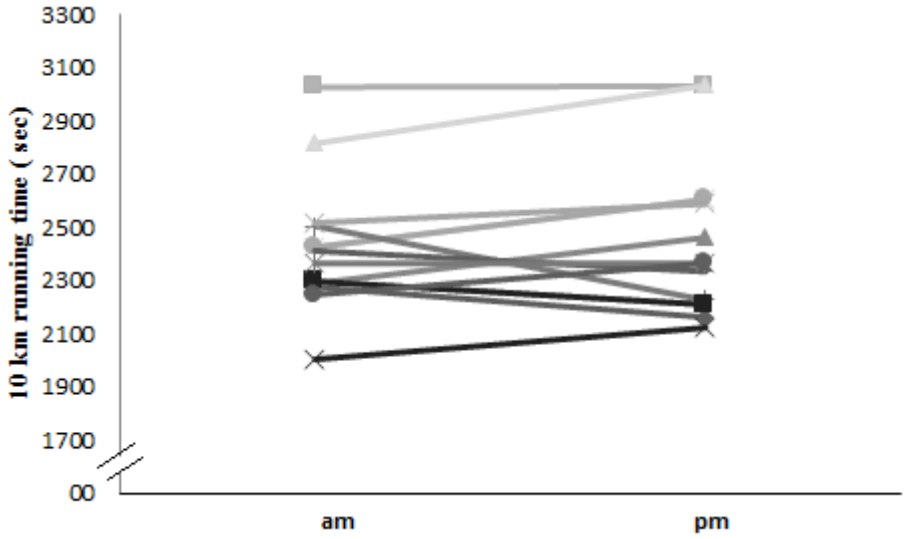

Figure 6.1: the 13 individual's time to complete the running trial at 09:00 hs and 18:00 hs. No diurnal variation was observed.

6.3.3 Diurnal variation in core body temperature

No significant diurnal variation was found between trials (P>0.05) for core body temperature in either at rest, at post-trial or at 1h post-trial (Figure 6.2b). The 95% Confidence Intervals (lower; upper limits) for the participants mean core body temperature at 09:00 hs, pre-trial (36.4; 37.1 °C), post-trial (39.1; 40.2 °C), 1h post-trial (36.1; 37.6 °C) and at 18:00 hs, pre-trial (37.0; 37.6 °C), post-trial (38.4; 40.3 °C), 1h post-trial (36.4; 37.3 °C). However, there is a significant difference between time trial point ($F_{2, 20}$ = 62.9, p < 0.001, partial η^2 = .9). with Bonferroni-adjusted, post hoc test revealing that the core body temperature has significantly changed pre-trial to post-trial (p= 0.00) at 09:00 hs and from pre-trial to post-trial and from pre-trial to 1h post-trial at 18:00 hs (p=0.00, p=0.00). Furthermore, resting mean core body temperature value was higher at pm than am by 0.57°C. In addition, the maximum value of core body temperatures was recorded in both trials at km 10; where the evening temperature was slightly higher than the morning value (39.72±0.57°C am vs 39.75±0.75°C pm). Whereas, morning immediately post-trial temperature value was higher than the evening value

(39.69±0.79°C am vs. 39.37±1.44°C pm), with a slower return to the baseline value in the morning compared to the evening. In terms of phase response which was significantly different ($F_{1, 10} = 55.1$, $p < 0.001$, partial $\eta^2 = .8$), core body temperature was higher in the morning (8%) compared to evening (6%). The ability of the athletes' body to remove a heat load is less in the morning compared to the evening and significantly different ($F_{1, 10} = 76.1$, $p < 0.001$, partial $\eta^2 = .8$), (+0.09 ºC at 09:00 hs and – 1.06 ºC at 18:00 hs), the removal of heat was calculated by the mean highest core body temperature recorded minus the lowest core body temperature recorded (Figure 6.2a and 6.2b).

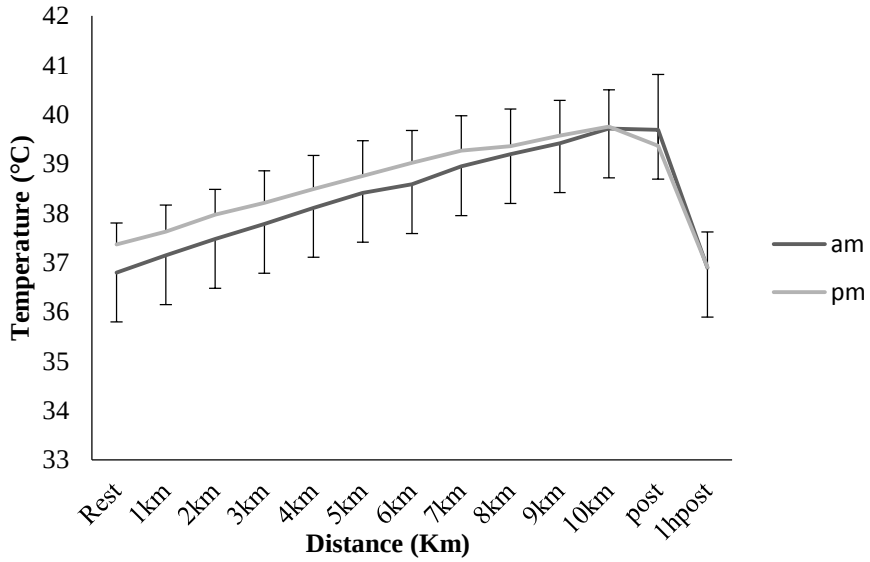

Figure 6.2a: Core body temperature measured throughout the trials. All values are the mean ± SD.

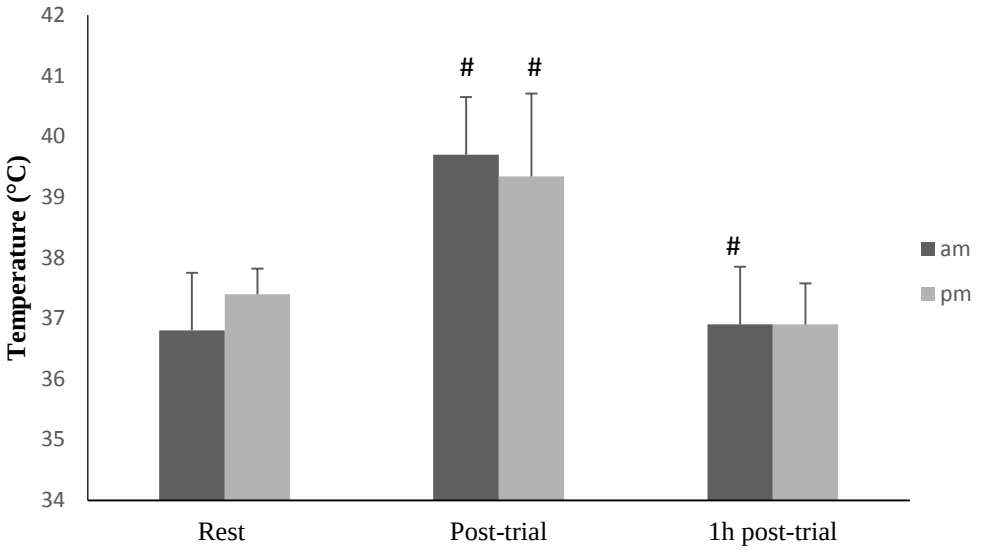

Figure 6.2b: Core body temperature at rest, post- and 1h post-trial at both times of the day. '#' denotes a significant difference between trial time points ($P < 0.05$), values are mean ± SD.

6.3.4 Diurnal variation in peak and resting heart rate and RPE

Two-way repeated measure analysis of variance (ANOVA) was used to determine diurnal variation in heart rate and at the three time point intervals (pre, post and 1h post-trial). The result showed no diurnal difference occurred between trials (am and pm). However, a significant difference between time point trials was observed for heart rate at both times of the day ($F_{2,24} = 875$, $p < 0.001$, partial $\eta^2 = .9$) with Bonferroni-adjusted, post hoc test revealing that the heart rate has significantly changed pre to post and 1h post-trial ($p = 0.00$) at both times of the day. Mean speed, peak heart rate (Figure 6.3), resting RPE and peak RPE did not show a significant difference between the two trials. Runner's heart rate throughout the trials is presented is Figure 6.3.

Table 6.2: The effect of the exercise trial in heat and circadian rhythmicity on heart rate, RPE and speed at 09:00 hs and 18:00 hs.

	09:00hs	**18:00hs**	**% difference**
Average Speed ($km \cdot h^{-1}$)	14.75±0.43	14.80±0.45	0.3%
Resting heart rate ($beats \cdot min^{-1}$)	50±7	53±7	6%
Peak heart rate ($beats \cdot min^{-1}$)	182±11	181±13	0.5%
Mean RPE	15±1	15±1	0%
Peak RPE	19±2	19±1	0%

Values are mean ± SD.

Phase response of heart rate was significantly different ($F_{1, 12} = 1356$, $p < 0.001$, partial $\eta^2 = .9$), zeitgebers effect on heart rate was more pronounced at the morning trial compared to the evening trial (p=0.00). A high heart rate amplitude was found at 09:00 hs trial compared to 16:00 hs trial (162±9 and 50±7 compared to 162±11 and 53±7) an increase by 264% at the 09:00 hs trial compared to an increase of 241% at 18:00 hs trial. At 09:00 hs the 1h post-trial was slower to return to baseline level compared to 18:00 hs (63±9 and 50±6 compared to 162 65±12 and 53±7).

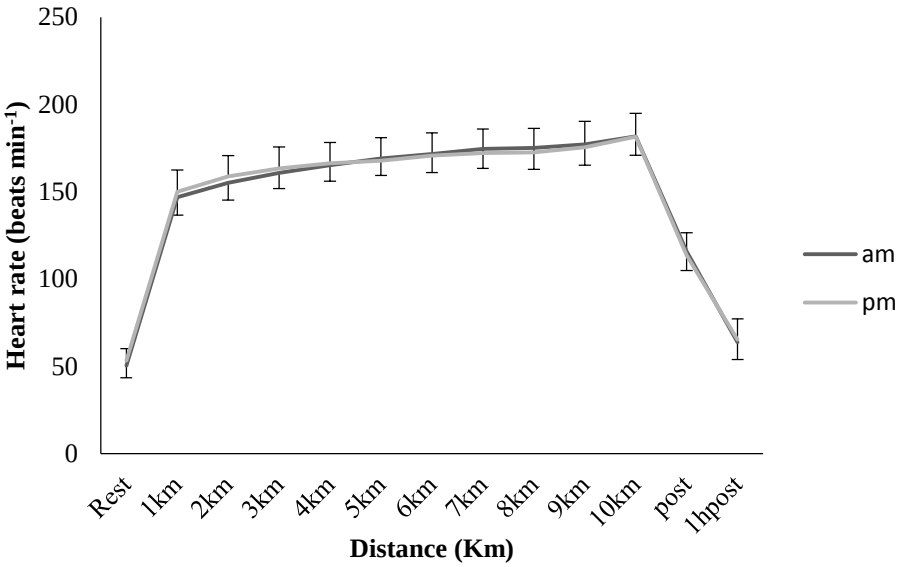

Figure 6.3: Runners heart rate throughout the trials at pre-, post- and 1h post-trial and on completion of each km ran at both times of the day. Values are mean ± SD.

6.3.5 Diurnal variation in hand grip

No differences were found between trials (P>0.05) for either dominant or non-dominant hand grip exercise (Figure 6.4). The 95% Confidence Intervals (lower; upper limits) for the participants mean non-dominant hand at 09:00 hs, pre-trial (40; 46 kg), post-trial (39; 45 kg), 1h post-trial (39; 45 kg) and at 18:00 hs, pre-trial (38; 46 kg), post-trial (36; 43 kg), 1h post-trial (37; 46). Mean dominant hand at 09:00 hs, pre-trial (41; 49 kg), post-trial (40; 46 kg), 1h post-trial (40; 48 kg) and at 18:00 hs, pre-trial (38; 48 kg), post-trial (38; 45 kg), 1h post-trial (39; 48 kg).

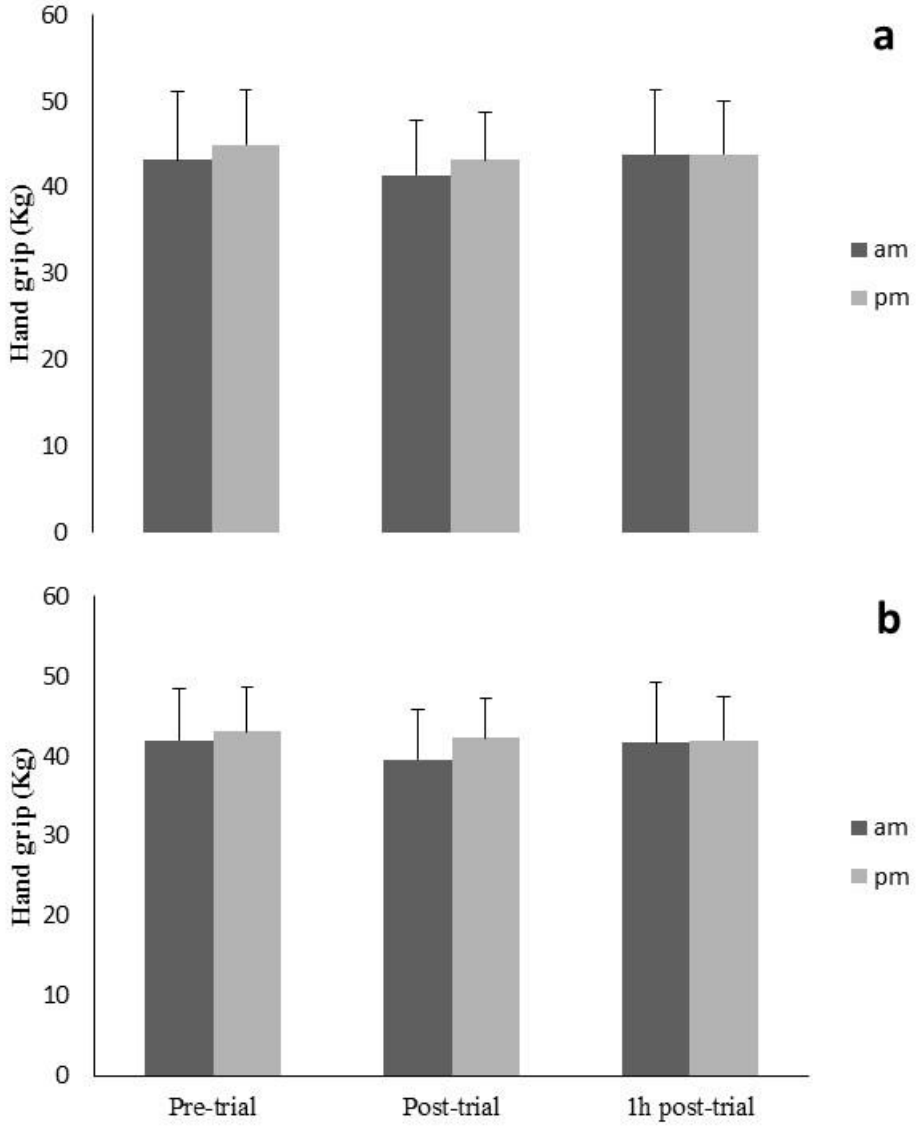

Figure 6.4: "a" dominant hand and "b" non-dominant hand grip in runners at pre-, post- and 1h post-trial at both times of the day: 09:00 hs and 18:00 hs. Values are mean ± SD.

6.3.6 Diurnal variation in back and leg strength

No differences were found between trials for strength (P>0.05) (Figure 6.5). However, a significant difference was observed between time point ($F_{2,24}$ = 6.84, p < 0.004, partial η^2 = .4) with Bonferroni-adjusted, post hoc test revealing that the strength has significantly changed post to 1h post-trial (p= 0.01) at 16:00 hs trial. However, the result showed an decrease on strength from pre-trial to post trial at both times of the day (140±48, 129±47 kg at 09:00 hs and 131±121kg at 18:00 hs) and at 1h post-trial participants showed an increase in strength (143±55 kg at 09:00 hs and 145±43 kg at 18:00 hs)

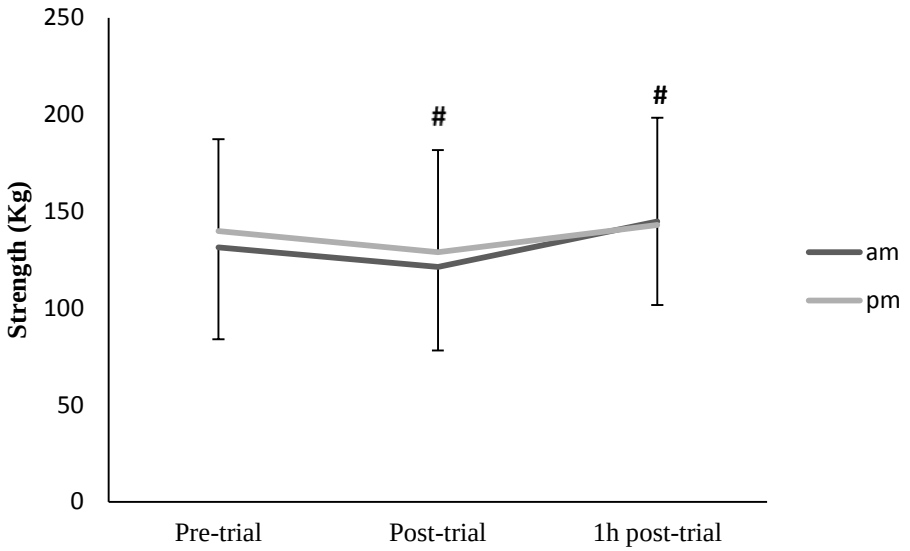

Figure 6.5: Strength in runners at pre-, post- and 1h post-trial at both times of the day. '#': denotes a significant difference between trial time points ($P < 0.05$). Values are mean ± SD.

6.3.7 Diurnal variation in vertical jump

No differences were found between trials ($P>0.05$) for the vertical jump, whereas, a significant difference was observed between time points ($F_{2,24} = 63.1$, $p < 0.001$, partial $\eta^2 = .8$) with Bonferroni-adjusted, post hoc test revealing that the vertical jump has significantly changed pre to post-trial at 09:00 hs ($p= 0.01$) and significantly changed from pre-trial to post trial ($p= 0.01$) and from post-trial to 1h post trial ($p= 0.01$) at 18:00 hs (Figure 6.6).

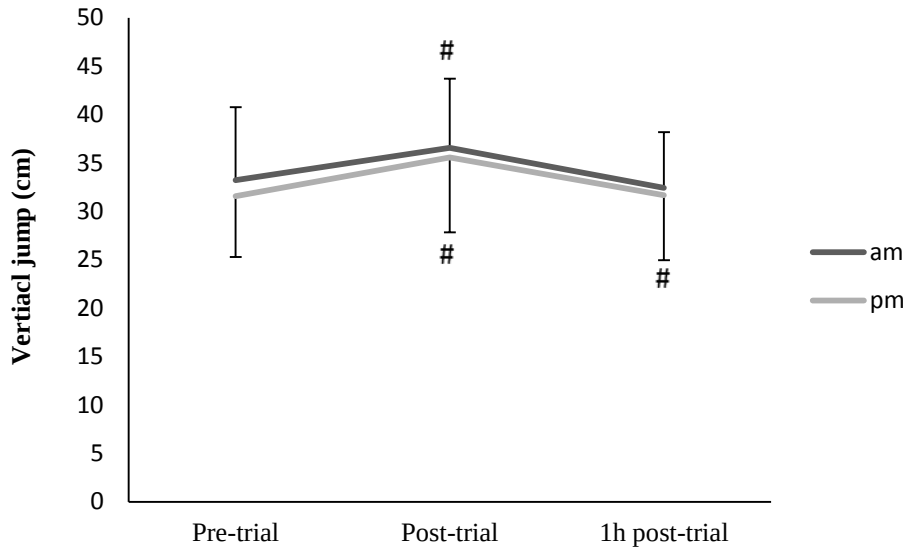

Figure 6.6: vertical jump in runners at pre-, post- and 1h post-trial at both times of the day. '#' denotes a significant different between trial time points ($P < 0.05$), values are mean ± SD.

6.3.8 Diurnal variation in flexibility

No differences were found between trials or for any measured time point (paired-samples T test, $P>0.05$) for flexibility (Figure 6.7). The 95% Confidence Intervals (lower; upper limits) for the participants mean flexibility at 09:00 hs, pre-trial (15; 25 cm), post-trial (18; 27 cm), 1h post-trial (18; 27 cm) and at 18:00 hs, pre-trial (17; 26 cm), post-trial (18; 27 cm), 1h post-trial (18; 26 cm).

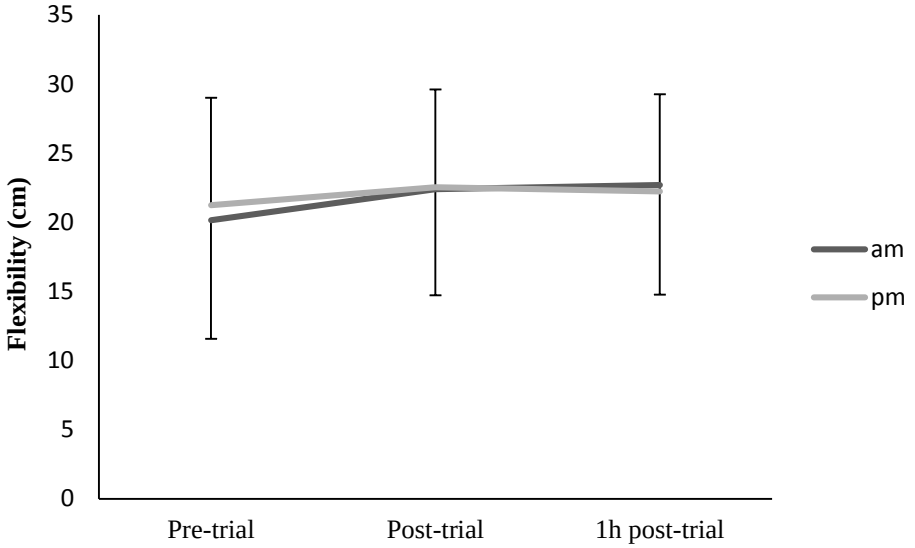

Figure 6.7: Flexibility in runners at pre-, post- and 1h post-trial at both times of the day. Values are mean ± SD.

6.3.9 Diurnal variation in respiratory symptoms

Respiratory symptoms reported by the participants after each of their trials are given in Table 6.3. Eleven from the thirteen participants that took part in this study reported a moderate shortness of breath during both trials. In contrast the other respiratory symptom parameters were either absent or minimal.

Table 6.3: diurnal variation in respiratory symptom questionnaire mean at post-trial for both measured times of the day.

	09:00 hs	18:00 hs
Shortness of breath	3	3
Cough	1	0
Excess sputum	1	0
Throat tickle	1	0
Raspy throat	0	0
Wheezing	1	1
Congestion	1	1
d/inspiration pain	0	0
Headache	1	0
Nausea	0	0
Eye irritation	0	0

Where 0 = Not present, 1 = Minimal, 2 = Mild, 3 = Moderate, 4 = Severe and 5 = Incapacitating. Values are mean.

6.3.10 Diurnal variation in lung function variables

No differences were found between trials (P>0.05) for the tested lung functions (Table 6.4). In the terms of phase response FEV_1, FEV1R, PEF showed a higher value in the evening trial compared to the morning trial (4%, 4% and 7%, respectively, at 18:00 hs compared to 3%, 0.95% and 1%, respectively), whereas, FVC and FEF_{25-75} showed the same response degree. At 1h post-trial, morning time showed quick return to baseline level compared to the evening.

Table 6.4: Lung function parameters in runners at pre-, post- and 1h post-trial at both times of the day: 09:00 hs and 18:00 hs.

	Pre-trial			**Post-trial**			**1h post-trial**		
	09:00 hs	**18:00 hs**	**%Δ**	**09:00 hs**	**18:00 hs**	**%Δ**	**09:00 hs**	**18:00 hs**	**%Δ**
FVC	5.44±0.94	5.38±1	1%	5.32±0.94	5.24±1.02	1%	5.36±1.17	5.31±0.99	0.1%
FEV_1	4.43±0.65	4.45±0.77	0.4%	4.56±0.68	4.57±0.92	0.0%	4.42±0.82	4.48±0.78	0.0%
FEV1R	0.82±0.07	0.83±0.07	0.2%	0.86±0.09	0.86±0.08	0%	0.83±0.08	0.85±0.08	0.0%
PEF	688±112	651±135	5%	689±123	670±125	2%	666±120	672±125	1%
FEF_{25-75}	4.37±1.05	4.52±1.11	3%	4.91±1.28	4.95±1.28	0.1%	4.44±0.96	4.78±1.27	8%

Forced vital capacity (FVC), Forced expiratory volume in 1 second (FEV_1), Forced expiratory volume in 1 second ratio (FEV1R), Peak expiratory flow (PEF) and Forced expiratory flow $_{25–75\%}$ ($FEF_{25–75\%}$). Where '#' indicates a significant difference between trial time points ($P < 0.05$). Values are mean ± SD.

6.3.11 Diurnal variation in blood pressure (systolic, diastolic and mean arterial pressure)

No differences were found between trials (P>0.05) for blood pressure in either systolic, diastolic or mean arterial pressure (MAP) measurements (Figure 6.8). In contrast, measured time points showed a significant effect of zeitgebers on systolic, diastolic blood pressure and MAP. Systolic ($F_{2, 22} = 11.1$, $p < 0.001$, partial $\eta^2 = .5$). with Bonferroni-adjusted, post hoc test revealing that the systolic blood pressure had significantly changed pre to 1h post-trial (p= 0.00) at both times of the day. Diastolic, ($F_{2, 22} = 4.48$, $p < 0.02$, partial $\eta^2 = .3$). with Bonferroni-adjusted, post hoc test revealing that the systolic blood pressure has significantly changed pre to 1h post-trial (p= 0.01) at 09:00 hs only. MAP ($F_{2, 22} = 9.5$, $p < 0.001$, partial $\eta^2 = .5$). with Bonferroni-adjusted, post hoc test revealing that the systolic blood pressure had significantly changed pre to 1h post-trial (p= 0.00) at both times of the day. Morning pre-trial systolic blood pressure compared to evening was higher by 3% and higher by 2% at 1h post- trial, whereas, systolic blood pressure remained unchanged at post-trial. Systolic blood pressure increased post-trial and decreased at 1h post-trial at 18:00 hs. In contrast to 18:00 hs the highest value at 09:00 hs was recorded at pre- trial. Both times of the day showed a decrease in diastolic blood pressure from pre- to 1h post-trial. Pre- and post-trial morning diastolic blood pressure

was higher by 5% and 1.5%, respectively, whereas, 1h post-trial evening diastolic blood pressure was higher by 2.5%. In terms of phase response to exercise in hot and humid conditions, MAP showed a significant decrease by 2% for the morning trial ($F_{1, 11} = 18.7$, $p < 0.01$, partial $\eta^2 = .6$) but was unchanged at the evening trial. Morning trial showed 14% MAP lower at 1h post-trial compared to the evening 1h post- trial (8%).

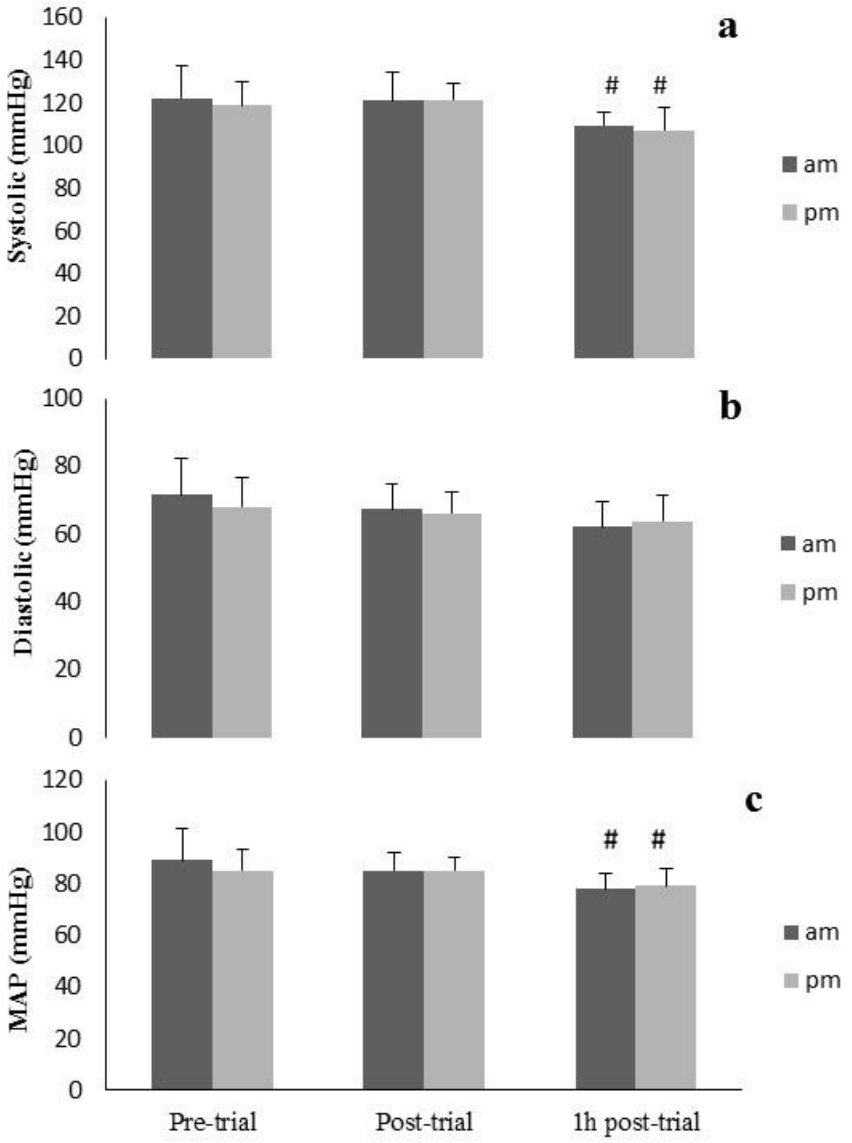

Figure 6.8: "a" Systolic blood pressure, "b" Diastolic blood pressure and mean arterial pressure (MAP) "c". Values are mean ± SD.

6.3.12 Diurnal variation in blood cells, markers of damage and inflammation

No diurnal variation was observed in any of the variables (Table 6.5). Platelet (PLT) time point showed a significant difference ($F_{2, 22}$ = 18.9, p < 0.001, partial η^2 = .6). With Bonferroni-adjusted, post hoc test revealing that the platelet significantly changed pre to post-trial (p= 0.00) at 09:00, and from pre to post-trial (p= 0.00) and from pre to 1h post-trial (p= 0.00) at 18:00 hs (Table 6.5). There was no diurnal variation observed in plasma volume dehydration (P = 0.809).

Table 6.5**:** Diurnal variation in total red blood cells (T/RBC), haemoglobin (HGB), haematocrit (HCT), platelet (PLT) and plasma volume (PV_A).

	Pre-trial			**Post-trial**			**1h post-trial**		
	09:00hs	**18:00hs**	**%Δ**	**09:00hs**	**18:00hs**	**%Δ**	**09:00hs**	**18:00hs**	**%Δ**
RBC	04.6±0.30	04.6±0.38	0.0%	04.8±0.36	04.7±0.53	3%	04.8±0.43	04.8±0.52	1%
HGB	14.3±0.67	14.2±1.00	0.4%	14.8±1.08	14.8±1.24	0.0%	14.7±1.36	14.4±1.48	2%
HCT	42.5±1.69	42.3±2.98	0.2%	43.7±2.79	43.7±3.05	0%	43.5±4.24	43.3±4.26	0.04%
PLT	200±35	204±44	2%	250±66 #	259±63 #	4%	198±39 #	211±57 #	6%
PV_A	-	-	-	55.61±3.37	54.21±4.77	3%	-	-	-

Red blood cells (RBC), haemoglobin (HGB), Haematocrit (HCT), platelet (PLT) and plasma volume changes PV_A. Significant differences between trial time points ($P < 0.05$) are represented by a '#'. Values are mean ± SD.

6.3.13 Diurnal variation in white blood cells

A significant diurnal difference was found in WBC counts ($F_{1, 11}$ = 24.8, p < 0.002, partial η^2 = .7). Moreover, both trials showed significant a difference between time points ($F_{2, 22}$ = 11.9, p < 0.001, partial η^2 = .5). With Bonferroni-adjusted, post hoc tests revealing that WBC are more affected by zeitgeber at evening trial (p = 0.00). WBC counts were higher at all-time points: pre-, post- and 1h post-trial for the evening trial compared to the morning trial (p=0.00) (Table 6.6). There was a diurnal variation also found in neutrophils, lymphocyte and monocytes: neutrophil, ($F_{1, 11}$ = 24.8, p < 0.002, partial η^2 = .6). Moreover, both trials showed significant difference between time points in neutrophil ($F_{2, 22}$ = 16.5, p < 0.002, partial η^2 = .6) with Bonferroni-adjusted, post hoc test revealing that neutrophil were significantly different from pre-trial, post-trial and pre-trial, 1h post-

trial at 09:00 hs (p=0.00 and p=0.00) whereas at 18:00 hs was significantly different only between pre-trail and post-trial (p=0.00). Lymphocyte, ($F_{1, 11} = 11.7$, $p < 0.001$, partial $\eta^2 = .5$), both trials showed significant difference between time points intervals ($F_{2, 22} = 27.5$, $p < 0.001$, partial $\eta^2 = .7$), with Bonferroni-adjusted, post hoc test revealing that lymphocyte were significantly different from pre-trial, post-trial and pre-trial, 1h post-trial at both time of the day (p=0.00, p= 0.02 and p= 0.00, p=0.00). Monocyte, $F_{1, 11} = 5.68$ $p < 0.03$, partial $\eta^2 = .3$), both trials showed significant difference between time point intervals ($F_{2, 22} = 4.92$, $p < 0.01$, partial $\eta^2 = .1$), with Bonferroni-adjusted, post hoc test revealing that monocyte were significantly different from pre-trial, post-trial and pre-trial, 1h post-trial at both times of the day (p=0.03, p= 0.02 and p= 0.03, p=0.02). However, basophil and eosinophil were not affected by either by time of the day (Table 6.6). Phase response in white blood cells showed a greater effect of exercise and the hot and humid condition in the evening on immune system variables compared to the morning. However, morning trial has greater effect on neutrophil count compared to the evening. White blood cells were slower to return to the baseline in the evening trial compared to the morning trial.

Table 6.6: Diurnal variation in white blood cells.

	Pre-trial			**Post-trial**			**1h post-trial**		
	09:00hs	**18:00hs**	**%Δ**	**09:00hs**	**18:00hs**	**%Δ**	**09:00hs**	**18:00hs**	**%Δ**
Total WBC	4.45±0.84	5.43±1.41*	22%	5.88±1.57#	7.44±1.66*#	26%	5.36±1.91#	7.31±2.5*#	36%
Neutrophil	2.14±0.57	03.01±1.02*****	40%	3.08±1.11#	4.26±1.18***#**	38%	3.56±1.76	5.08±2.09***#**	43%
Lymphocyte	1.63±0.46	1.78±0.46	9%	2.17±0.67#	2.6±0.63***#**	20%	1.33±0.32	1.6±0.46***#**	20%
Monocyte	0.44±0.13	0.46±0.19	4%	0.44±0.17#	0.55±0.25***#**	25%	0.35±0.12#	0.48±0.23***#**	37%
Eosinophil	0.18±0.14	0.16±0.12	12.5%	0.14±0.13	0.14±0.12	0%	0.1±0.09	0.12±0.1	20%
Basophil	0.03±0.01	0.03±0.02	0%	0.04±0.02	0.04±0.02	0%	0.03±01	0.03±0.01	0%

'*' indicates significantly different ($P < 0.05$), diurnal variation ($P < 0.05$) and '#' indicates a time point significant different ($P < 0.05$). Values are mean ± SD.

6.3.14 Diurnal variation in nasal lavage neutrophil counts

The mean recovery of the fluid at pre-trial was 6.1 ± 1.6 ml (am) vs. 6.0 ± 1.0 (pm), at post-trial 5.7 ± 1.9 (am) vs. 5.6 ± 1.1 (pm) and at 1h post-trial 6.4 ± 2.0 (am) vs. 5.6 ± 2.0 (pm). This value did not change significantly among the two trials. Neutrophils and

epithelial cells are the predominant cells in the nasal lavage. Lymphocyte, basophil, eosinophil and monocytes were also present in smaller numbers. The results for neutrophil counts in the nasal lavage at pre-, post- and 1h post-trial are presented in table 6.8. Neutrophils for all measured time points had higher counts in the evening compared to the morning trial but did not reach the significance level. Furthermore, total nasal cell counts showed no diurnal variation significance.

Table 6.7: Diurnal variations in nasal lavage neutrophil counts.

	Pre-trial			**Post-trial**			**1h post-trial**		
	09:00h	**18:00h**	**%Δ**	**09:00h**	**18:00h**	**%Δ**	**09:00h**	**18:00h**	%Δ
Total cell count (10^4)	23.1±20.9	22.1±18.0	4.5%	11.7±16.7	16.0±18.0	37%	5.7±4.22	11.2±10.28*****	96%
Neutrophil count (10^4)	0.9±0.9	1.5±1.6	67%	0.8±0.8	2.3±3.7	188%	0.8±0.7	1.1±1.1	38%
Neutrophil percentage	6.6±8.63	7.1±7.54		7.3±7.3	10.3±9.59		15±14.4	10.2±10.7	

Values are mean ± SD.

6.3.15 Diurnal variation in plasma CC16

Plasma CC16 showed no significant diurnal variation between trials (Figure 6.9). However, a significant difference between time points was evident ($F_{2, 20} = 11.9$, $p < 0.01$, partial $\eta^2 = .3$). With Bonferroni-adjusted post hoc test revealing that plasma CC16 is more affected by zeitgeber at the 3 time point intervals at 09:00 hs trial ($p = 0.00$; $p=0.00$) and only from pre to post at the 18:00 hs trial ($p=0.00$). There was a significant increase in plasma CC16 at post-trial for both times of the day (phase response) ($F_{1, 11} = 5.35$, $p < 0.01$, partial $\eta^2 = .3$) (35.2±7.33; 25.7±5.35 ug·L^{-1}) at am and at pm (34.5±13.1; 21.0±9.53 ug·L^{-1}). These elevated levels remained high at 1h post-trial for both trials compared to the baseline level (34.7±7.55; 25.7±5.35 ug·L-1) at am and pm (30.29±9.15; 21.0±9.53 ug·L^{-1}), respectively. The intra-assay coefficient of variation for the duplicate samples was 9.5% and 7% at morning and evening, respectively. One subject was eliminated from this intra-assay coefficient as it was erroneous. The exclusion of this sample was made on the basis that the value was an outlier (500% higher than the mean 113 vs 21 ng·ml^{-1}).

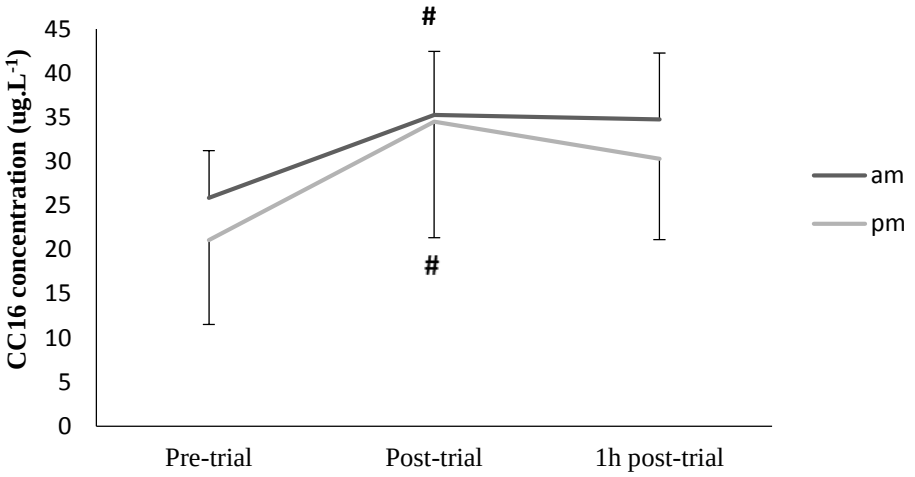

Figure 6.9: Plasma CC16 in both trials. Where '#' indicates significant difference between trial time points ($P < 0.05$). The coefficients of variation (CV) for these parameters were < 8 %. Values are mean ± SD.

6.3.16 Diurnal variation in plasma IL-6

Plasma IL-6 showed no significant diurnal variation between trials (Figure 6.10). However, a significant difference between time points were shown ($F_{2, 18} = 24.4$, $p < 0.01$, partial $\eta^2 = .7$). With Bonferroni-adjusted, post hoc test revealing that plasma IL-6 are more affected by zeitgeber at the 3 time point intervals at 09:00 hs trial ($p = 0.00$; $p=0.00$) and only from pre to post at 18:00 hs trial ($p=0.00$).

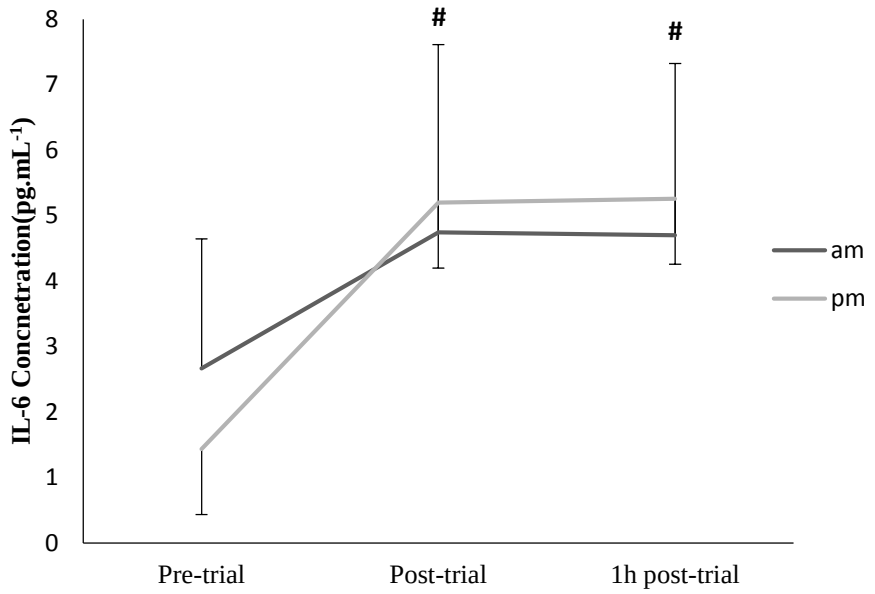

Figure 6.10: Plasma IL-6 diurnal variation. '#' indicates significant difference between trial time points ($P < 0.05$). The coefficients of variation (CV) for these parameters were < 10 %. Values are mean ± SD.

6.3.17 Diurnal variation in plasma HSP70

Plasma HSP70 did not differ between the two times of the day (Figure 6.11). Conversely, there was time point interval difference ($F_{2, 20} = 11.2$, $p < 0.001$, partial $\eta^2 = .5$). With Bonferroni-adjusted, post hoc test revealing that plasma HSP70 are more affected by zeitgeber from post to 1h post-trial at 18:00 hs trial ($p = 0.004$). However the zeitgebers in the present study caused a great masking effect on HSP70, especially in the evening trial were the value of HSP70 did not following the expected trend with exercise (HSP70 decreased rather than the expected increase post-exercise). The intra-assay coefficient of variation for the duplicate samples was 2.6% and 9.1% for morning and evening, respectively.

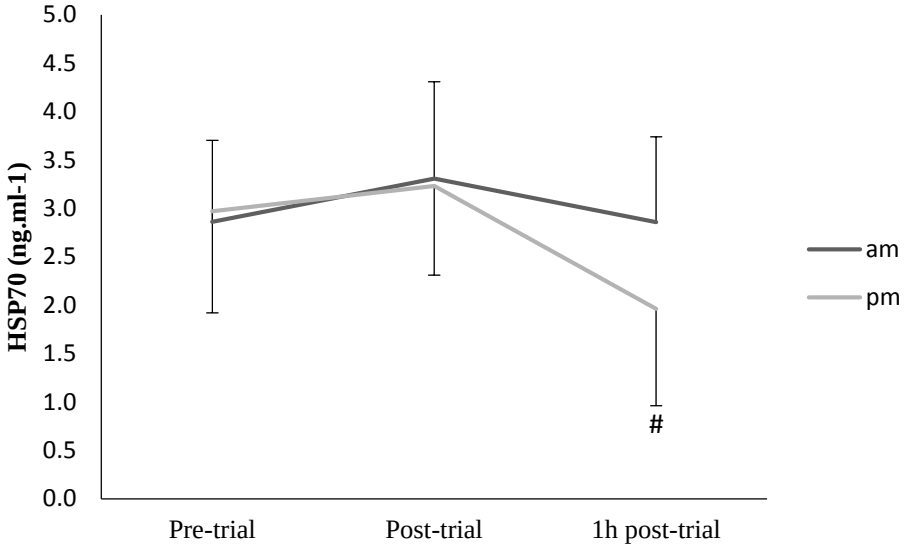

Figure 6.11: Plasma HSP70 diurnal variation. '*' indicates a significant diurnal difference between trials ($P < 0.05$). # denotes a significant difference between the time trials ($P < 0.05$). The coefficients of variation (CV) for these parameters were < 10 %. Values are mean ± SD.

Phase response in IL-6, HSP70 and CC16 presented in Table 6.9 showed a greater response of these variables to exercise in hot and humid condition in the evening compared to the morning. IL-6 was nearly 3 times higher in the evening compared to the morning. These variables showed a slow return to the baseline level in the evening compared to morning trial.

Table 6.8: Phase response in IL-6, HSP70 and CC16.

		IL-6 ($pg.mL^{-1}$)	%Δ	HSP70 ($ng.ml^{-1}$)	%Δ	CC16 ($ug.L^{-1}$)	%Δ
Pre to post-trial	**09:00hs**	2.67±1.98 4.75±2.87	77%	2.86±0.90 3.30±1.00	15%	25.9±5.35 35.2±7.23	35%
	18:00hs	1.43±2.56 5.20±2.94	263%	2.97±1.10 3.23±0.90	9%	21.0±9.53 34.5±13.1	64%
Pre to 1h post-trial	**09:00hs**	2.67±1.98 4.70±2.62	76%	2.86±0.90 2.85±0.90	1%	25.9±5.35 34.7±7.55	34%
	18:00hs	1.43±2.56 5.26±3.65	267%	2.97±1.10 1.96±1.00	-65 %	21.0±9.53 30.2±9.15	43%
Post-trial to 1h post-trial	**09:00hs**	4.75±2.87 4.70±2.62	-1%	3.30±1.00 2.85±0.90	86%	35.2±7.23 34.7±7.55	-1%
	18:00hs	5.20±2.94 5.26±3.65	1%	3.23±0.90 1.96±1.00	- 60%	34.5±13.1 30.2±9.15	-9%

Values are mean ± SD.

6.3.18 Diurnal variation in plasma cortisol

Plasma cortisol was not detected in samples due to a technical failure. Liaising with the manufacturer (R&D System) determined that the ELISA KIT (KGE008) purchased was not compatible with the shaker used in the laboratory (HEIDOLPH VIBRAMAX 100). The shaking motion produced by this machine causes vibrations that interfered with the process leading to no recovery of cortisol from the samples.

6.4 Discussion

This study examined the effect of the time of day on 10 km time trial treadmill run at 28°C and 70% relative humidity (conducted in an environmental chamber, Weiss Technik, UK). Participants mean running times for the two trials were not statistically different despite the morning trial being faster by 19 seconds. Surprisingly, core body temperature in this study showed no diurnal variation and therefore hypotheses 1, 2 and 3 can be rejected. White blood cells, neutrophil and lymphocyte were significantly higher in the evening trial and therefore for these measured variables hypotheses 2 and 3 can be rejected. All physiological parameters tested (heart rate, lung function, blood pressure, power, strength and flexibility), were not affected by the time of the day and therefore hypothesis 2 for these variables can be rejected. Likewise, IL-6 and CC16 as well as HSP70 were not significantly affected by the time of the day and therefore hypothesis 3 can be rejected for these variables. Nonetheless, running 10 km in hot and humid condition has greater effect on phase response in physiological (blood pressure and lung function) and immunological (white blood cell variable, IL-6 and CC16) variables measured at 18:00 hs compared to 09:00 hs. In contrast, heart rate, core body temperature and HSP70 rate of increase (phase response) were affected more at 09:00 hs confirming the morning high physiological strain due to exercise and the environmental conditions compared to the evening trial.

Surprisingly, the performance result of this study does not corroborate with previous published findings, where a significant difference in time-of-day variation in running performance has been reported (Martin et al., 2001; Athkinson et al., 2005 with both

studies conducted indoors). The mean morning running time was faster by 19 seconds than the evening, albeit not a significant difference.

Previous literature associates evening peak performance to core body temperature where the peak for both is reached. Core body temperature in the present study does not corroborate previous studies and do not show diurnal variation. Thus, the hypothesis for the association of better performance coinciding with the peak of core body temperature can be rejected when racing in a hot and humid condition (hypothesis 1). This poor evening performance could be attributed to the rate of muscle fatigue, muscle inflammation and muscle damage as these phenomena are reported to be higher in the evening (Hammouda et al., 2012a) and exacerbated by the unfamiliar hot and humid conditions. In addition, Hammouda et al. (2012b) reported higher creatine kinase (marker of muscle fatigue) in evening Wingate tests, and, Brancaccio et al. (2010) found the production of creatine kinase was in phase with the rhythm of oral temperature (higher in the evening).

The well trained athletes involved in this study regularly trained twice daily (morning and evening sessions). All participants in this study performed their intensity training in the evening due to daytime commitments to work or study. Participant familiarity of training at different times of the day has allowed them to become consistent performers regardless of the time of the day. This finding may indicate that daily morning training in our participants has the potential to entrain their circadian pacemaker. Another explanation of better morning performance could be attributed to the effect of exercise on advancing athletes circadian pacemaker. This is in accordance with Miyazaki et al. (2001) who found that, in participants who exercised, plasma melatonin was phase-advanced significantly during the waking period, whereas melatonin rhythmicity did not change in non-exercising participants. However, to date it is still not fully understood whether the phase shift is directly affected by exercise or a combination of other factors, such as the increase in body temperature as a response to exercise. Importantly, exercise can increase core body temperature rapidly, which seems to be an independent cause of fatigue during exercise in hot and humid environments. Furthermore, at these atmospheric conditions all participants failed to run fast, recording times of 3 to 10 minutes slower than their

personal best times. From this finding in hot and humid conditions it is suggested that a race distance of more than 5 km should be organised in the morning to allow athletes to perform their best.

The present study result is in agreement with the findings of Aldemir et al. (2000) and Waterhouse et al. (2007): that core body temperature increased significantly in the morning compared to the evening and the body's ability to remove a heat load is less in the early morning compared to the evening. This phenomenon could possibly be due to the body being in heat-gain mode in the morning compared to the evening when the body exhibits slow heat gain so that heat-loss is more pronounced. Moreover, heat storage is greater in the morning (heat gain mode), than in the late afternoon (heat loss mode). Fatigue during prolonged exercise in hot and humid conditions has been shown to occur at the same critical level of hyperthermia when the initial value and the rate of increase in body temperature are altered (Gonzales-Alonso et al. 1999). In the present study core body temperature at both times of the day could have a greater stress on the body's thermoregulation during 10 km run in 28°C and 70% relative humidity. To this, it can be concluded that athletic performance should not be linked to either circadian rhythm variation alone or core body temperature, but rather it should be related to environmental time of the day; i.e. sleep, diet, external temperature.

Core body temperature, heart rate, blood pressure, speed and RPE did not change between the two time trials. Blood pressure findings of the present study differ from Jones et al. (2006) where systolic blood pressure shows statistically significant 24-h variation. However, in the present study a reduction in diastolic and systolic blood pressure was found for the morning trial (pre- 122/71 mm Hg, post- 121/67 mm Hg and 1h post- 109/62 mm Hg). Mean arterial pressure (MAP) was not affected by the time of the day. However, post-exercise mean arterial pressure (MAP) was lower by 5 mm Hg in the morning compared to no changes in the evening. It can be concluded that the mean arterial pressure (MAP) shows highest reactivity when performed in the morning in hot and humid condition (Jones et al., 2008). Blood pressure in this study could be driven by elevated intravascular shear stress that occurred in the morning (Jones et al., 2009). In addition this reduction in blood pressure in the morning trial could be attributed to exercise alone as

literature has shown that blood pressure increases during morning time (Jones et al., 2009). Furthermore, from a clinical perspective the evening blood pressure may be a cause for concern for hypertensive individuals who are attempting to lower their blood pressure via exercise.

Heart rate in this present study did not show diurnal variation. This finding disagrees with Bessot et al. (2007) findings where diurnal variation in heart rate (aerobic power) in 15 endurance competitive cyclists (187.4 ± 9.8 beats min^{-1} at 06:00 hs and 190.3 ± 8.5 beats min^{-1} at 18:00 hs) were found. However are in agreement with Cruz et al. (2013) who found no difference in heart rate for each lap in both trials (3000 m and 5000 m) during three different times of day. It is well known that physiological demands during exercise increase and rise to the highest limit during exercise in hot and humid environments (Gonzalez-Alonso et al. 1999). In terms of phase response heart rate showed a significant response (increased by 23%) in the morning trial compared to the evening trial as a response to exercise in hot and humid condition. This showed that morning physiological strains were greater compared to the evening. This current study finding supports the idea that morning exercise showed higher physical demand compared to the evening (Cruz et al., 2013). In addition the recovery phases were slower in the morning compared to the evening (6%). Athletes and coaches should take into consideration this phenomena (high morning heart rate with slow recovery) when planning intensity training for the athletes in the morning time. From a clinical perspective the morning heart rate may be a cause for concern for the risk of cardiovascular events, were it is reported to be more pronounced in the morning hours compared to the rest of the day (Scheer et al., 2008).

In the present study lower resting heart rate was present in all participants in the morning (lower by 6% compared to the evening). In addition maximum heart rate in the morning was higher only by 1 beat min^{-1}. It can therefore be speculated that the cardiovascular system was exposed to same morning and evening exercise stressor in the present study. It must be noted that all participants reached maximum heart rate during both trials at km 10. Therefore, the hypothesis of heart rate increase at the phase of heat gain and a reduction at heat loss can be rejected in a hot and humid condition for high level athletes (Aldemir et al., 2000). Likewise, it has been reported that the dehydration level increases

heart rate during exercise in a hot and humid environment (Armstrong et al., 2011). However, in the present study there was no difference between morning and evening rate either of body fluid loss or plasma volume hydration.

Running a 10 km trial in hot and humid conditions at intensity close to exhaustion may result in sweat output exceeding water intake thereby causing a body water deficit (hypohydration). The IAAF medical handbook (2009) states that the risk of heat injury rises when competing above 21°C and 50% relative humidity. Body mass loss among participants was higher in the morning trial compared to the evening but was not significantly different (1.02 kg vs 0.79 kg). Additionally, prehydration (water drank) prior to the trial was higher by 79% in the evening compared to the morning. This higher water intake in evening compared to the morning may be due to the physical and mental demands that occur through daytime activities since waking up time. In the present study at both times of the day participants did not exceed 1.5% fluid loss as a result of the trial. However, the highest rate of dehydration was recorded in the morning where better performances were also recorded. Nevertheless, some authors have found that dehydration has no effect on sport performance such as reported by Tam and Noakes (2013) and Wall et al. (2013); where in Wall et al. (2013) 3% dehydration had no effect on 25 km cycling performance in hot and humid conditions (33°C and 40% relative humidity). The study result presented here in this research using runners as participants and of Wall et al. (2013) with cyclists suggests that the previous position on exercise and fluid replacement has to be revisited.

Flexibility, strength, hand grip and power were not affected by the time of the day in this study. However, in terms of values some differences exist within the variables. The idea that poor morning performances are associated with stiffness of joints following sleep is rejected (Chtourou et al., 2011) and support the finding of Jones et al. (2000) were the least flexible runners are also the most economical. Strength results did not present significant diurnal variation in runners and this contradicts previous studies where the strength nadir occurred in the morning and the peak occurred in the evening (Chtourou et al., 2012). However, in terms of values, evening pre and post-trial demonstrated a 6% and 6.3% higher value compared with the morning. This could be attributed to core body

temperature that was somewhat higher in the evening, additionally, it can be suggested that less stiffness and better muscle elasticity could occur in the evening. Similarly, hand grip of both the dominant and non-dominant hand did not show a significant diurnal difference. Amplitude of 4% and 3% for dominant and non-dominant hand, respectively, were recorded at rest in the evening. The lack of diurnal variation in the present study in strength and hand grip could be attributed to the type of participants in this study being endurance runners that only engage in basic strength resistance training.

Flexibility was not affected by the time of the day in this study. This finding is in accordance with Edwards and Atkinson's (1998) study that showed no diurnal change in variable movement measured (lateral movement of the spine and ankle plantar, spinal hyperextension and dorsi-flexion). As expected resting flexibility in the evening was better than in the morning and this reflects the daily pattern of body temperature with the lowest levels in the morning and highest in the evening. Whereas, post-trial flexibility was very similar for both time trials 1h post-trial morning flexibility was superior by 1cm than the evening. These results may elicit the idea of the benefit of warm up to enhance morning performance. Florida-James and Doggart (2000) and Atkinson et al. (2005) showed that in morning test sessions warm-up had a positive effect on performance where participants are able to increase core body temperature to a level comparable to that of an afternoon session. Pre-race warm-up may result in increases in muscle and core temperatures, blood flow, arousal and alertness. In addition, it also reduces myocardial ischemia (poor oxygen supply to the heart muscle), delays fatigue and the premature onset of blood lactic acid accumulation during high intensity exercise, and reduces the risk of injury (Wittekind et al., 2012). In addition in the present study lower morning flexibility in athletes were associated with better performance and this supports the findings of Jones et al. (2000) who reported the least flexible runners tend to be more economical when running at submaximal speeds. The author linked this finding to the greater stability of the pelvis with reduction for further muscular activity as the foot made contact with the ground.

An unexpected finding for vertical jump was greater morning values compared to the evening for the three measured time points (however, it did not reach the significance

level). This finding contradicts previous findings where the nadir was reported in the morning and the maximum in the evening (Chtourou et al., 2011). This higher morning result may, in part, be due to participant adaptation to morning exercise. Alternatively, it could be due to peripheral mechanisms (muscle power and fatigue) being more pronounced in the evening because of the increases in physical and mental fatigue that occur through daytime activities.

All physiological variables measured is this study did not show diurnal variation. The training level of participants used in this study (train twice daily, first session before 09:00 hs and second session after 18:00 hs) combined with exercise time of the day adaptation may have caused a prolonged masking effect on the physiological variables measured (Edwards et al., 2005). The masking of circadian variables could be attributed to the external influences (exogenous), heat and humidity, core body temperature and lastly athlete adaptation to the time of the day exercise, or more likely a combination of some or all of these factors. It is difficult to define (detect) circadian rhythms in physiological variables relevant to sport performance and underlay their mechanisms at the same time (exercise, exercise adaptation will mask any underlying mechanism). Furthermore, Lung function variables in the present study did not show diurnal variation and contrasts previous findings (Spengler et al., 2000; Medarov et al., 2008). Lung function was not affected by the type of exercise, nor by the hot and humid environment. In terms of value difference, most of the lung function variables only showed a slight increase at post- and 1 h-post- trial compared to pre-trial (< 3%). Furthermore, 11 from 13 of the participants in this study reported shortness of breath at the end of both time trials. We can speculate two explanations for this. Firstly, it can be associated with the discomfort of the exercise workload and the hot and humid condition. The second hypothesis is more likely and states that this discomfort of breathing reported at post- exercise by participants is caused by airway inflammation (narrowing of the airway). However, this inflammation was not caused by this trial, but rather this inflammation is a pre-existing (chronic) condition from daily training and exposure to cold air in the UK were the athletes that took part in the study are based. It has been reviewed in Chapter 2 that airway inflammation is associated with exercise, predominantly in elite athletes (Bougault et al., 2009).

Likewise variable RBC total counts, HGB, HCT and PLT do not exhibit diurnal variation. With RBC total counts, HCT and HGB not showing a significant difference between the morning and evening trials at any time point during the trials. This result agrees with Simpson et al. (2005) were RBC, HCT and HGB did not change in fourteen London marathon finishers (RBC 4.9, 5 and 5 $x10^{12}1^{-1}$) at pre-, post- and 24h post-marathon, respectively. PLT levels, however, showed a significant difference between time points being higher post-trial at the morning than the evening by 25% and 27%, respectively. PLT levels then returned to the approximate baseline level after 1h post- trial (below baseline level at 09:00 hs by 0.1% and remained higher at 18:00 hs by 3%). Therefore, this increase in circulating PLT at post-exercise may be caused by the combination of the intensity of the exercise and the hot and humid conditions in which the trials were performed. Additionally, this increase in platelet counts in whole blood for athletes after intensity exercise could indicate a prothrombotic situation (blood clots in blood vessels) in athletes (Hilberg et al., 2003) and therefore this prothrombotic situation were more pronounced in the morning trial. This present study's findings are in accordance with published research that reported an increase in platelet counts ranging from 7% to 13%, occurring immediately after 20 minutes of treadmill running (Davis et al., 1990). Furthermore, in Singh et al. (2006) platelets counts were higher in trained athletes compared to sedentary population after 60 minutes of cycling at 70% of $\dot{V}O_2max$ (235 x $10^9.l^{-1}$ vs. 208 x $10^9.l^{-1}$). In addition, it could be due to the increased O_2 uptake during exercise which is associated with oxidative stress that plays a role in increasing platelet counts in participants after exercise (Singh et al., 2006).

In contrast to physiological measurements, immunological variables showed a level of circadian rhythmicity, and the mechanism behind many of these rhythms can be viewed as exogenous, endogenous or a combination of both. A significant diurnal difference was observed in total WBC counts, neutrophils, lymphocyte and monocyte with an increase in WBC and neutrophils occurring from baseline level to 1h post-trial. Furthermore, total WBC, neutrophil, lymphocyte and monocyte counts were higher in the evening compared to the morning at all-time points (pre-, post- and 1h post-trial). This is in agreement with Cooper et al. (2010), which found after water immersion (39.5°C) core temperature rose

by at least 1°C; this increase of core body temperature resulted in a greater WBC circulation including neutrophils and monocytes.

Niess et al. (2003) linked elevated white blood cells in a hot environment to the elevation in circulating stress hormones. It is therefore, to some extent, this immune system suppression that can provide a weakened immune system, more likely to follow the evening trial, with this period of weakened immune defence lasting up to 72 hs (Kakanis et al., 2010). The evening high counts in WBC variables may be due to a combination of endogenous (core body temperature) and exogenous (exposure to heat and humidity) putting extra stress on the immune system.

The present study is, to date, the first to analyse the neutrophil diurnal variation and response to hot and humid conditions in well trained athletes undertaking a 10 km running trial using the nasal lavage method. The nasal lavage method is used as a less costly tool for the assessment of upper-airway inflammation in adults and children and is easily tolerated by the participants (Nikasinovic-Fournier et al. 2002). Furthermore, numerous studies have used this method to assess airway inflammation in healthy or unhealthy populations (Ciprandi et al., 2004; Gomes et al., 2011). Nasal lavage total cell concentration in this study did not show a diurnal difference between the two trials. Evening nasal lavage neutrophil counts were elevated compared to the morning at the three measured time points (pre- 67%, post- 188% and 1h post-trial 38%). This result agrees with the findings that blood neutrophil numbers are higher in the evening. This evening elevation in neutrophil counts could be attributed to the change in higher circulating hormones such as cortisol and epinephrine released during exercise. The mechanisms by which hormone release elicit increases in the circulating neutrophil count vary. Mechanisms responsible for heat-induced neutrophilia include increased secretion of hormones and an increased cardiac output (Niess et al., 2003). Increased neutrophil content of the nasal lavage fluid indicating that exercise also induces nasal inflammation. This increase in the airway neutrophil count following exercise was more pronounced in the evening and could be due to an oxidative stress process that occurred in the airway tissues in the athletes and this is due to the high increase in the production of the reactive oxygen species through the respiratory process (Corradi et al., 2002).

Plasma level of IL-6, CC16 and HSP70 were not affected by the time of day. However, CC16, IL-6 and HSP70 in the present study showed an increasing pattern immediately post- and 1h post-exercise, which indicate zeitgeber's positive effect on these measured variables. This present study somewhat agreed with the published findings of Helleday et al. (2006); Andersson et al. (2007) and Tufvesson et al. (2013) who reported a decrease in CC16 concentration during the daytime, with plasma CC16 increasing as a response to exercise volume and intensity. To the best to our knowledge this is the first study investigating the diurnal variation on plasma CC16 in highly trained runners in a hot and humid condition. It is known that environmental conditions impact on the degree of airway epithelial disruption during high-level exercise (Bolger et al., 2011). Moreover, in the present study the degree of leakage of CC16 can be affected by the hot and humid environment. This corresponds with Bolger et al. (2011) where urinary excretion of CC16 in a hot and humid environment remained constant (did not change dramatically) compared to a cold environment (250% higher at 4°C and 37% relative humidity compared to 25°C and 94% relative humidity). Furthermore, the results of Bolger et al. (2011) indicated that exercise disrupts the airway epithelium of all athletes not only the athletes with exercise-induced bronchoconstriction (EIB). However, warm humid air inhalation limits the airway injury and epithelial cell perturbation compared to cold dry air (a comparison between CC16 in cold air "Chapter 5" and warm humid air "Chapter 6" is discussed in Chapter 8).

Inhalation of warm humid air limits airway epithelial cell perturbation and according to CC16 manufacture suppliers (R&D Systems, UK) our result lies within the healthy population range (4.91-38.1 ng/ml). In contrast the results from the cold study (Chapter 5) fall outside of this range suggesting greater epithelial damage had occurred. Thus, CC16 remained stable in this study and could reflect epithelial injury low risk, which occurs less under hot and humid conditions compared to cold conditions (Chapter 5). Additionally, it may lead to better performance in this condition, after acclimatisation, as heat acclimatisation reduces physiological strain, improves the ability to exercise in a hot environment, and reduces the incidence of heat illness and stroke (Lorenzo et al., 2010). To avoid exercise confounding effects, it can be suggested that plasma CC16 samples wherever possible should be collected in at least a 24 h cycle to observe the diurnal

variability. The aim of the present study was not to assess the reasons behind the diurnal variation in the CC16 concentrations, and nothing is known about the underlying mechanisms here. This is a key area for future research.

Plasma IL-6 concentration in this study did not show significant diurnal variation. This finding contradicts previous work (Vgontzas et al., 2003) that found, for a healthy population, an IL-6 nadir in the morning. The findings of this research support those of DeRijk et al. (1997) who found IL-6 levels were almost identical between morning and evening after exercise. There was no diurnal significance difference recorded after the phase response period. However, evening IL-6 was higher than the morning both immediately post- and at 1h post-trial by 10% and 12%, respectively. Furthermore, a phase response of 60% in the morning and 264% in the evening from baseline level to post-trial was recorded. The elevated evening IL-6 concentration could be due to an increase in total white blood cell counts (including neutrophil and lymphocyte) in the evening as these cells are known to express IL-6 (Febbrai and Pedersen, 2002). This cytokine plays an important role as a mediator in the development of an inflammatory process. Indeed, Vincent et al. (2014) showed a correlation between muscle damage and plasma IL-6 levels when comparing eccentric and concentric cycle exercise. It is known that exercise duration, intensity and the muscle mass involved in the exercise are factors influencing plasma IL-6 concentration (Wallberg el al., 2011).

As previously mentioned, in the evening, the athlete's body is in heat loss mode rather than heat gain mode. Exercise and heat together delay this process by adding extra stress on the athletes' body leading to an imbalance in heat storage. We can suggest that the combination of exercise intensity, core body temperature as well as the hot humid environmental condition resulted in higher circulating white blood cells (neutrophil and lymphocyte) and IL-6 after exercise in the evening compared to the morning (< 200% higher). It has been suggested that the release of IL-6 in exercise is related to the occurrence of muscle damage and this increase in IL-6 levels during exposure to a hot and humid environment responds as an anti-inflammatory cytokine in athletes (Lim et al., 2009). Vincent et al. (2014) showed that muscle damage exists straight at post- eccentric contractions. Collectively, this supports the idea that the induction of IL-6 is linked with

the initial disruption of muscle fibres. However, our finding did not support previous study trends; where IL-6 after 1h post-trial remained somewhat stable compared to immediately post-trial. However, the amount that IL-6 increases is correlated to exercise intensity, duration, muscle mass and power involved in the mechanical work. In the present study participants adaption to intense exercise minimised the occurrence of muscle damage and the recovery period was quicker (stop releasing IL-6). The higher resting IL-6 found in the morning trial could have been driven by cortisol (peak in the morning). It is highly recommended to measure IL-6 in a 24 h cycle post-exercise for better understanding of the changes that may occur. The present study may bring a new finding that IL-6 responds differently in this climatic condition in well trained long distance athletes.

No diurnal variation was found for HSP70. However, HSP70 in the current study responded differently to what was anticipated by remaining at the same level post-trial and decreasing by 66% 1h post-trial compared to pre-trial levels at 18:00 hs. At the morning trial HSP70 increased by 15% as response to the trial and returned to the baseline level after 1h post-trial. On one hand, HSP70 concentration at rest is in agreement with Sandstrom et al. (2009) were HSP70 reported higher in the evening (21:00 hs) compared to the early morning (05:00 hs). On the other hand, Sandstroem et al. (2009) study showed another peak of HSP70 at 09:00 hs and was statistically significant from all other time points except 21:00 hs (HSP70 measured at 09:00 hs, 13:00 hs, 17:00 hs, 21:00 hs, 01:00 hs and 05:00 hs).

These findings contrast with Fehrenbach et al. (2000) who found an increase in HSP70 post-half marathon at 3 hs and remained elevated 24 hs after. The low level recorded in the present study post-trial can be due to the duration, the temperature degree and humidity level of the trial: a 10km time trial run in hot and humid conditions that were not sufficient to alter HSP70 in highly trained athletes. Thus we can assume that the production of HSP70 in highly trained athletes is more linked to the duration, the intensity and the adaptation to exercise. This is in agreement with Fehrenbach et al. (2000) where HSP70 in trained participants was significantly lower compared to the untrained

individuals (approximately 20% difference). Our study is the first study to report a decrease in HSP70 1h post-exercise in a hot humid condition.

It can also be speculated that the higher HSP70 in the morning post- and 1h post-trial coincided with physiological strain during exercise that occurred more in the morning (stress caused by exercise). The result from this study showed an inverse relationship between HSP70 and neutrophil at 1h post-trial and was more pronounced for the evening trial. We suggest that the peak of circuiting neutrophils 1h post-trial leads to a decrease in circulating HSP70. This inverse relationship between HSP70 and neutrophils occurred at post-trial too, particularly for the evening trial. This may mean that neutrophils express persistently active elastase (enzyme from the class of proteases) on their surface, which is remarkably resistant to inhibition by naturally occurring proteinase inhibitors, especially at inflammation sites (Owen et al., 1995). The increase of neutrophils occurring at post- and 1 h post-trial may lead to the degradation of HSP70 (loss potential beneficial effects), this process which could make the cell surfaces more fragile and more prone to rupture (Martin-Ventura et al., 2007).

In addition, the combination of exercise intensity, metabolic stress, physiological demand and increase in core body temperature is known to lead to an increase in HSP70 production or activation (Fehrenbach et al., 2000). Likewise the decrease in core body temperature, cardiovascular strain, and the exercise intensity may be paralleled with the decrease in circulating plasma HSP70 at 1h post-trial in the present study. In addition, this decrease in HSP70 during 1h post-trial could be considered as direct evidence of decreased physiological demand in these well trained athletes. The quick return of HSP70 after 1h post-trial to the baseline level concentration in response to exercise seems to be a result of adaptation mechanisms to regular endurance training in the participants that took part in this study (physiological stress controlled) (Grebenyuk et al., 2010). This response of HSP70 is unlikely as Fehrenbach et al. (2000) found that exercise of a similar nature (half marathon) produced greater HSP70 1h post-trial. It is therefore more likely that subject adaptation to high intensity and ‘stressful’ exercise has enabled their bodies to recover more quickly and to react less severely to the perceived stressful situation of the intensity of the exercise (S-curve).

6.4.1 Summary

It can be concluded that a 10 km trial ran in a hot and humid environment can result in different physiological and immunological responses depending on the time-of-day when it is performed. Higher physical strains are reported in the morning trial (heart rate and core body temperature). Nevertheless, immunological variables are affected more at evening trials. Heart rate and core body temperature showed better recovery at the evening trial, whereas, lung function variables, blood pressure and immunological variables were better at the morning trial in terms of recovery.

CHAPTER 7:

PSYCHOLOGICAL CIRCADIAN RHYTHM IN HIGHLY TRAINED ATHLETES IN TWO DIFFERENT ENVIRONMENTAL CONDITIONS

Different questionnaires were used in the two main studies undertaken in this thesis; therefore no direct comparison of the data in the two studies can be made.

7.1 Introduction

All humans have the same basic brain, senses and emotional structure, although every human behaves and responds differently to a raised situation. The reason behind this differentiation of acting differently to similar situations is due to the compilation of different characteristics of each individual that makes each personality unique. In short, performance personality plays a key role in sporting events that present a particular type of stressful yet exhilarating situation. Hence, there is an important role of sport psychology and why mental training and self-regulation programmes based on research evidence should be applied in this situation to enhance performance (Gould and Maynard, 2009). East African domination of distance running is well known and is due to a higher quality of physiological factors in the regional population (Hamilton, 2000). Psychological and mental strength also has a big impact in their performance. Furthermore, it may create a stimulus that can have significant positive consequences on performance (Hamilton, 2000).

Generally speaking all participants whether at a major or minor competition will experience stress pre-event and this stress severity differs from one person to another. Indeed a little stress improves performance, whereas uncontrollable or severe stress decreases performance and may eventually cause athlete break down (Gould et al., 2002). A combination of better self-control of psychological and physiological parameters can lead to better performance. Psychological indicators include arousal, alertness and mood, whereas; physiological indicators include levels of stress hormones in the body (Perkins et al., 2001; Totterdell and Leach et al., 2001; Chiodo et al., 2011).

According to Watson (1989, page 3) "Mood is a relatively short term feeling state or emotional tone which can involve various specific types of positive and negative emotions such as anger, tension, vigour, confusion, depression and fatigue''. Several studies have concluded that mood is a good predictor of performance (Terry et al., 1999;

Chiodo et al., 2011). For athletic competition getting into the right mood is seen by many coaches, athletes and sport psychologists as an important part of the mental preparation process (Terry, 2000). The lack of mood preparation or preparedness is often presented by athletes as an attribution to explain the performance outcome (Terry, 2000). Furthermore, athletes may have to perform at different times of day and under extreme environmental conditions. Holt and Dunn (2004) have suggested that effects of environmental change tend to influence psychological functioning sometimes even before they affect physiological factors. Therefore, these provide a useful early indicator of the adverse effects of environmental stress that can lead to poor performance. Psychological (mood) profiling can assist many physiological aspects of performance and provide numerous benefits, such as improvements in physiological systems related to the cardio respiratory, muscular, endocrine and nervous systems (Gonzalez-Bono et al., 1999).

Berger and Motl (2000) showed that exercise has a mood-enhancing effect. This is typified by increased vigour with reduced anger, confusion, depression, fatigue, and tension following exercise. Furthermore, in a study conducted by Florida-James et al. (1996) POMS was used to test twenty three student nurses undertaking night shift work. The study showed a circadian variation in the nurses' mood profile both on day and night shifts with a significant effect of time on vigour, fatigue and confusion.

The second study discussed in Chapter 6 used the Stanford Sleepiness Scale (SSS) to assess arousal and alertness circadian rhythm (Hoddes et al., 1973). It is known that subjective measuring of arousal, fatigue and alertness are vulnerable to masking influences and thereby obscure the circadian rhythmicity. However, it is important to understand the concept of the masking effect when considering the assessment of circadian rhythms and exercise (Kryger et al., 2005). Alertness in athletes is the degree at which they can respond quickly (quick action), as well as, how they can reliably use selective and sustained attention. Krauchi et al. (1997) demonstrated a relationship between core body temperature, alertness, and melatonin.

In Dijk et al. (2012) increasing core body temperature led to a greater state of alertness. Although, there is a strong correlation between alertness and sleepiness, a better night

time sleep may lead to a better daytime alertness (Kayumov et al., 2000). Furthermore, a short nap prior to the start of shift work can improve alertness (Purnell et al., 2002). On the other hand, jet lag sleep disorder will decrease the level of the daytime alertness (Kayumov et al., 2000). Collectively it is clear that sleep disruption or disorders have negative impacts on alertness and performance, however, with the reduction of tiredness mental alertness will increase. Alertness increases during the day to a peak around 10:00 hs and declines during the night time. In Mah et al. (2011) peak performance in basketball players only occurred when sleep was optimal, with improved player reaction time, better mood, faster sprint with high vigour and low fatigue.

Arousal is a state of readiness for action that motivates a person to behave in a particular way (Vandercammen et al., 2014). Arousal levels vary between low, recorded at deep sleep to high excitement. It also plays an important role in sports performance, where high arousal contributes to better performance. Perkins et al. (2001) analysing 28 elite athletes observed that strength performance increased significantly when arousal was high. This increase in performance, as a result of high arousal, could be a motivational factor. Coaches, athletes and psychologists are targeting optimal arousal; however, optimal arousal differs from high arousal, both over-arousal and under-arousal lead to poor performance (Perkins et al., 2001). Athletes' performance reaches a peak when arousal level is optimal for that particular task (Chang et al., 2012b).

There are several theories that attempt to explain the relationship between arousal, stress and their effects on sport performance such as: inverted U theory (Yerkes and Dodson - 1908), the Drive theory (Clark Hull, 1943), the Flow theory (Mihaly Csikszentmihalyi, 1975), the Zone of Optimal Functioning theory (Yari Hanin - 1980), the Reversal theory (Michael Apter - 1982) and the Catastrophe theory (John Fazey and Lou Hardy - 1988) (to review these theories see Dewey, 2007). However, drive theory (optimal arousal and anxiety) is the best theory that is most applicable to modern sport performance.

Few studies have looked at the circadian rhythm in arousal and sport performance (Schmidt and Collette, 2007). However, it is clear that a relationship exists with performance peaking at a certain level of circadian arousal (Yoon et al., 1999). In general,

peak sport performance is reported to occur in the evening time (Atkinson et al., 2005), albeit with the caveat of the evening scheduling of athletic competitions. There is a significant correlation between the peak of memory task and the circadian rhythm of alertness and arousal (Schmidt and Collette, 2007); and additionally Atkinson and Spiers, (1998) reported that participants showed lowest performances in the early evening in both accuracy and speed tests.

The Rating of Perceived Exertion (RPE) is a standard marker used by coaches and scientists during exercise tests and training to measure exercise intensity (Eston, 2012). As RPE indicates the degree of exhaustion and strain elicited by the physical activity (Borg, 1982) it can be used as a predictor of exercise capacity, assess changes in training workload, intensity, and allow for a better understanding of workloads, pace and pacing strategy for future training or racing (Eston, 2012). Several studies have shown that RPE increases linearly during exercise such as in cycling (Nethery, 2002), during walking and running exercise (Bath et al., 2011), and during exercise at different environment conditions such as in the heat (Nybo and Nielsen, 2001c). The RPE linear increase during exercise indicates that the brain perceives that the exercise is becoming progressively more demanding. Crewe et al. (2008) demonstrated that even if work rate remained constant RPE increased linearly during the period of exercise. RPE correlated strongly with core body temperature and heart rate throughout the exercise, and it was additionally shown that the rate of RPE increase was less during exercise when exposed to a cold environment compared to a hot environment (Crewe et al., 2008). RPE shows no circadian rhythm at day light during moderate intensity exercise, with the highest RPE mean value occurring at around 05:00 hs (nocturnal). This peak coincided with the lowest core body temperature mean where during intensity exercise or time to exhaustion RPE peaked in the afternoon; this also coincided with the highest mean body temperature (Morris et al., 2009).

The novelty of this research is that psychological states are investigated using highly-trained athletes to directly relate the findings to the competitive running population of the world.

Hypotheses:

Runner's at rest will score high in the evening and will coincide with the peak of core body temperature in cold condition.

Alertness should be high in the morning at resting state and core body temperature and exercise will be drivers of alertness.

Optimal arousal and high alertness will coincide with the peak of core body temperature.

7.2 Methods

Seven male endurance runners (mean ± SD: 32 ± 5 years, 71 ± 6 $mlO_2 \cdot kg^{-1}min^{-1}$, 69 ± 4 kg, and, 178 ± 5.7 cm) took part in the cold study (Chapter 5) and thirteen male endurance runners (mean ± SD: 33 ± 5 years, $\dot{V}O_2max$ range 61-79$mlO_2 \cdot kg^{-1}min^{-1}$) took part in the hot and humid study (Chapter 6). The participants were asked to complete a general medical history questionnaire and a specific medical questionnaire. In addition, they signed an informed consent form, and ethical approval was attained from the local ethics committee. The study was conducted in accordance with the Declaration of Helsinki.

Preliminary measurements: Fully described in Chapter 3.

10 km Trial: Fully described in Chapter 3.

BRUMS: Mood was assessed using the 24-item BRUMS scale (Terry and Lane, 2003) that assesses Anger, Confusion, Depression, Fatigue, Tension, and Vigour. The participants reported their mood state before, immediately after and 1 h after the 10km time trial runs at both times of the day.

The BRUMS is comprised of 24 items, anger, confusion, depression, fatigue, tension and vigour. Each subscale contains four items. After the answer a score range 0-16 will be obtained. The items in each subscale are:

Anger: annoyed, bitter, angry and bad tempered.

Confusion: confused, mixed up, muddled and uncertain.

Depression: depressed, downhearted, unhappy and miserable.

Fatigue: worn out, exhausted, sleepy and tired.

Tension: panicky, anxious, worried, and nervous.

Vigour: lively, energetic, active and alert.

After obtaining the raw score it was converted to a standard score as described in Terry and Lane (2003) (see Appendix 10)

Overall Mood Variation

A general mood score was calculated from the 6 mood scores (anger, confusion, depression, fatigue, tension, and vigour), where a score of 200 represents an average score for an athlete population; higher scores meaning a worse overall mood. To frame the scores into context, the worst mood score possible would be 500 and the best mood score possible would be minus 100. The overall mood is calculated as follows: (confusion + depression + fatigue + tension) – Vigour.

Arousal and alertness questionnaire

Both arousal and alertness questionnaires were conducted for both trials and for each of the measured time points: pre-, post- and 1h post-trial. The Stanford Sleepiness Scale records an alertness score from 1 to 10; 1 indicating “as tired as I’ve ever felt” to a score of 10 indicating “as alert as I’ve ever felt” (Hoddes at el., 1973). The arousal questionnaire (Stanford Sleepiness Scale and Appendix 7) is based on a scale of 7 relating to how the individual is feeling; for example, the first question states “Feeling active and vital, alert

and awake" compared to the seventh question stating "Almost in reverie, sleep onset soon, lost struggle to remain awake".

Statistical Analysis

Prior to statistical analysis all data were checked for normality. Data were analysed using two- way repeated measure ANOVA with Bonferroni-adjusted, post hoc test to determine the difference at which time point the diurnal variation occurred and the difference between trial time points. Statistical analyses were conducted using SPSS 20 software (IBM.UK), and a significance level of $P < 0.05$ was adopted throughout, unless otherwise stated.

7.3 Results

7.3.1 Mood state

There was no difference in the athlete's performance (Chapter 4), although there was a higher RPE reported during the morning trial as shown in Table 7.1.

Table 7.1: Mean RPE and mean running time diurnal variation.

	09:00 hs	**16:00 hs**
Mean RPE	14.9±2.11	14.5±2.13*

* Diurnal difference was observed with the variables. Values are mean ± SD.

7.3.2 Diurnal Variation of Psychological Measures

Table 7.2 illustrates that there were no statistically significant differences in overall mood scores.

Table 7.2: Mean Mood state at 09:00 hs and 16:00 hs.

	Pre-test		Post-test		1h post-test	
	09:00 hs	**16:00 hs**	**09:00 hs**	**16:00 hs**	**09:00 hs**	**16:00 hs**
Overall mood	192.6±2.06	182.4±2.8	194.6±4.94	186.5±5.04	185.5±3.57	190.1±4.49
Anger	48.5±3.74	49.7±12.4	51.5±13.9	45.0±0.00	52.3±16.2	46.1±2.86
Confusion	51.5±10.2	44.8±7.57	44.0±3.7	43.1±3.02	46.5±8.40	45.3±8.16
Depression	47.5±4.90	45.0±0.00	49.0±7.58	47.7±7.18	47.3±6.72	47.1±5.31
Fatigue	48.3±10.5	47.1±4.53	53.6±10.4	52.2±12.3	47.6±7.03	49.5±6.28
Tension	44.5±5.55	40.4±4.04	39.6±4.07	37.4±1.13	40.3±4.93	39.0±3.63
Vigour	47.7±11.7	4.7±10.2	43.1±8.56	39.0±9.11	48.7±8.19	37.0±7.48

Values are mean ± SD.

Diurnal Mood Variation for Pre-, Post- and 1 h post- 10km Time Trial

An average pre- run mood score of 192.6 was recorded at 09:00 hs and 185.1 at 16:00 hs, meaning the mood score was better in the afternoon than the morning at rest. However, there was no significant difference between these scores (p = 0.623) (Figure 7.1).

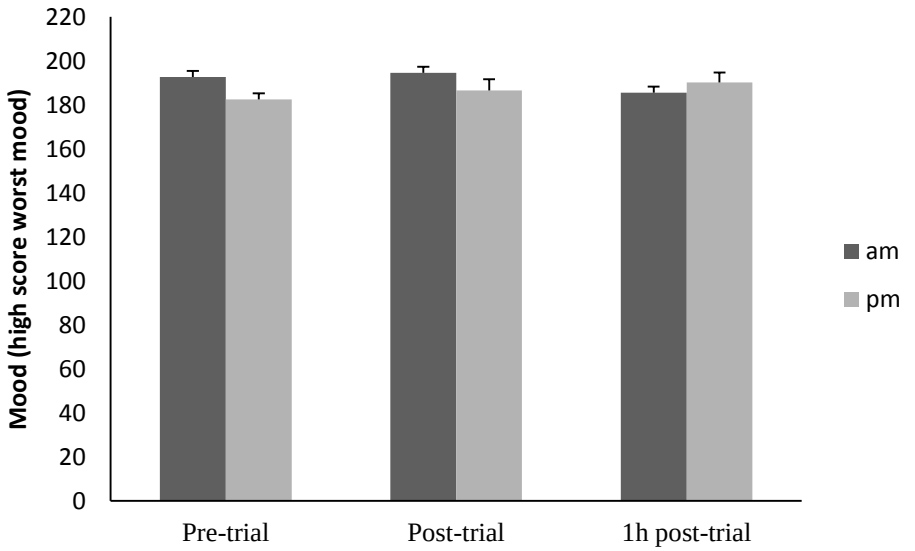

Figure 7.1: Diurnal variation in overall mood. Values are mean ± SD.

Pattern of General Mood Variation between 09:00 hs and 16:00 hs

The results indicated that there was no significant difference or effect size for combined (09:00 hs and 16:00 hs) mood scores between pre-, post- and 1 h post-trial. However, while there was no significant interaction effect. This most likely represents the different pattern of mood change 1 h after the 10km run, experienced in the morning compared to the afternoon (see Figure 7.1). In other words, there appeared to be a worsening of mood 1h post-trial in the afternoon, but an improved mood 1 h-post- morning time trial. However, note that pre-, post- and 1- h post- for both 09:00 hs and 16:00 hs all presented better than average mood for an athlete population (Terry, 2000).

Diurnal Mood Variation for Pre-, Post- and 1 h Post- 10km Time Trial

There were no statistically significant differences for pre- 10 km time trial mood scores (Figure 7.2). There was tendency towards a worsening of mood for all these features in the morning.

For mood scores, post- 10 km time trial, again there were no statistically significant differences between morning and afternoon for any of the mood scores (Figure 7.2) with a trend of higher tension and vigour in the morning compared to the afternoon for post-trial mood.

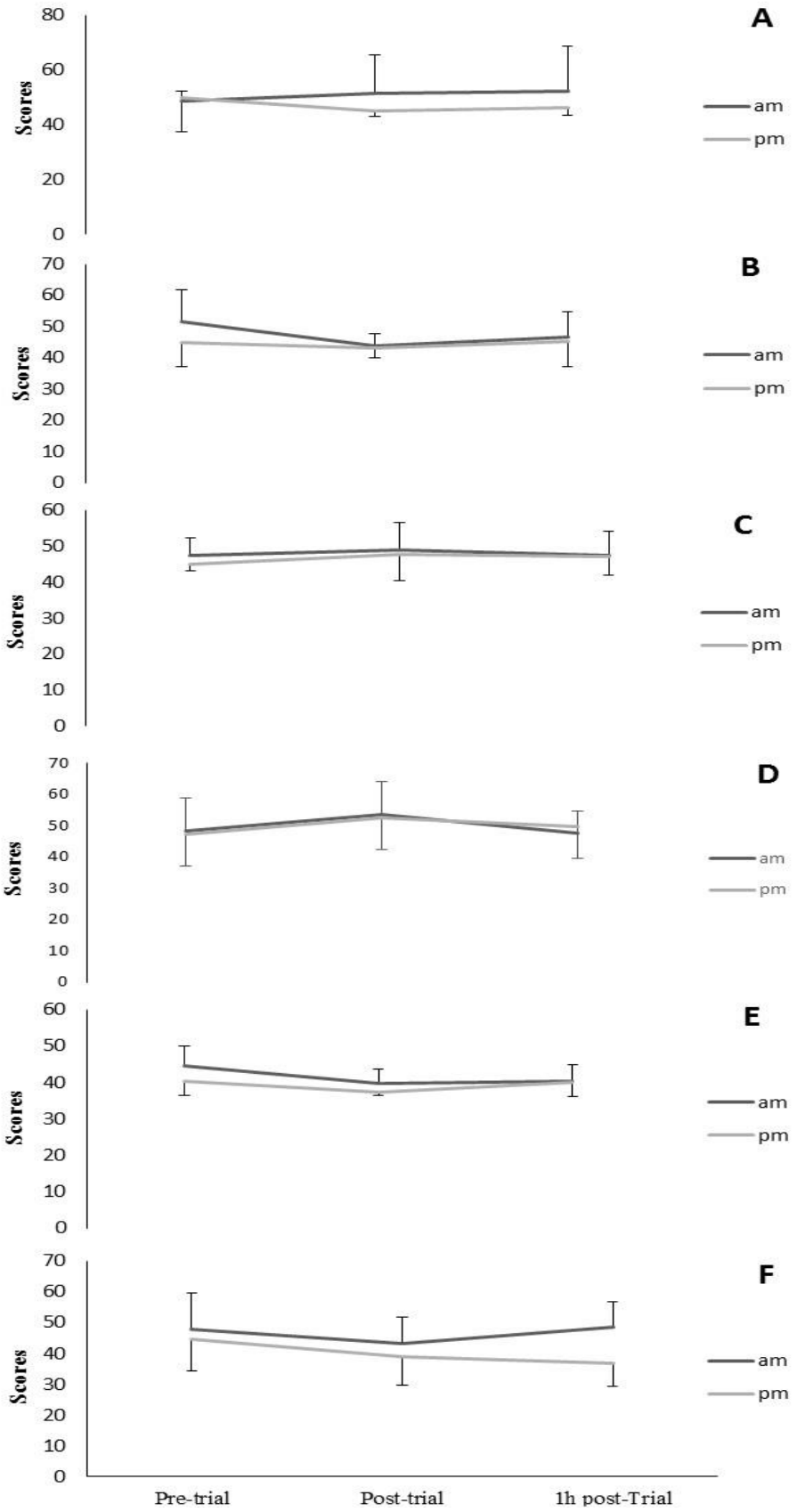

Figure 7.2: BRUMS variables were A, anger, B, confusion, C, depression, D, fatigue, E, tension and F, vigour. Values are mean ± SD.

7.3.3 Arousal and alertness

Alertness did not differ between the two times of the day (Figure 6.11). Conversely, there was time point interval difference ($F_{2, 22}$ = 11.1, p < 0.001, partial η^2 = .5). With Bonferroni-adjusted, post hoc test revealing that alertness is effected by zeitgeber at both times of the day (p=0.00 and p= 0.00). In terms of phase responses, the evening showed a lowered alertness compared to the morning (60% and 12%, respectively) with better morning 1h post-trial alertness.

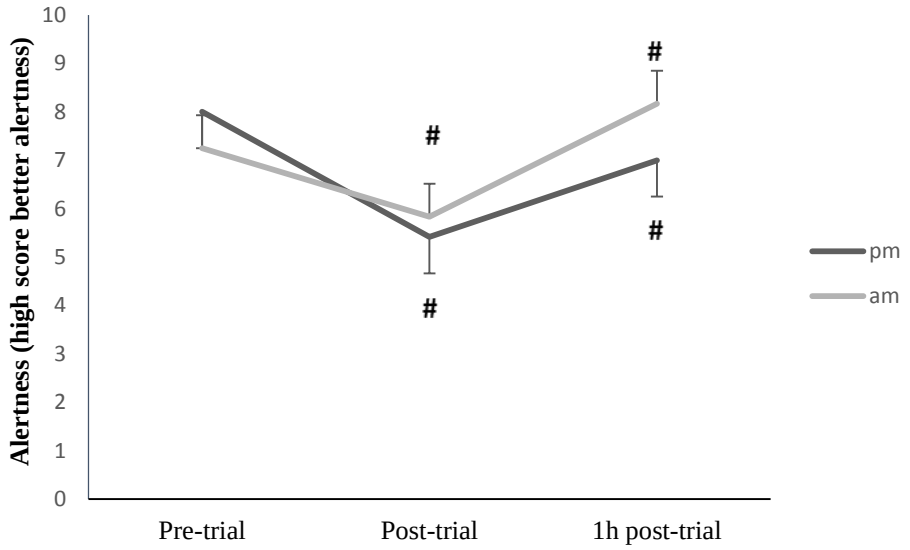

Figure 7.3: Alertness diurnal variation between both trials. Significant differences between trial time points ($P < 0.05$) are represented by a '#'. Values are mean ± SD.

No diurnal variation was evident for arousal. Nevertheless, a significant difference was observed for the measured time points for both trials ($F_{2, 20}$ = 8.24, p < 0.001, partial η^2 = .5). With Bonferroni-adjusted, post hoc test revealing that arousal is effected by zeitgeber at both times of the day from pre-trial to post trial (p=0.01 and p= 0.01). In addition, the same arousal pattern occurred within the trials.

The mean answer score for both times of the day at rest was the second answer which was: "Functioning at a high level but not at peak. Able to concentrate". At post-trial the mean score answer for both times of the day was the same: "Relaxed, awake but not at full alert. Responsive''. Whereas, at the 1h post-trial 09:00 hs the mean score answer

remained the same as at post- trial, in contrast at 18:00 hs the mean answer was the same as at pre-trial.

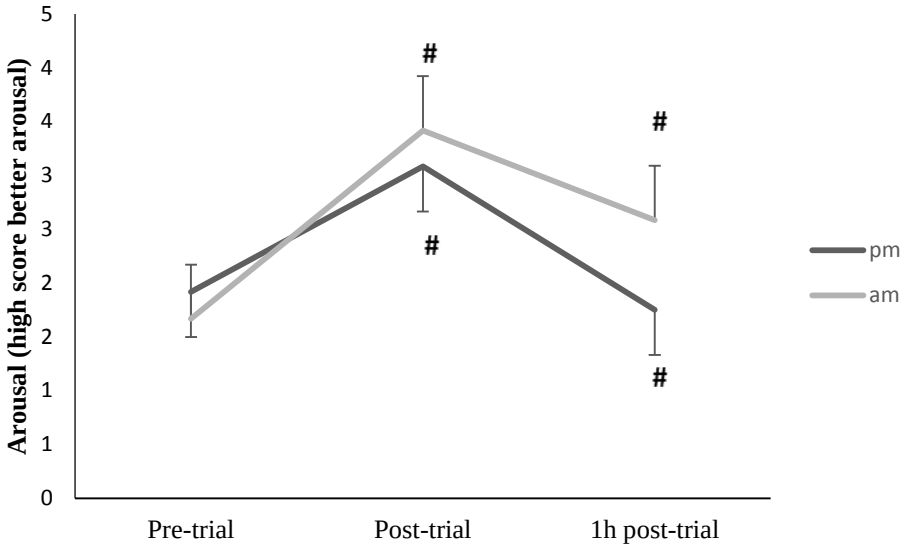

Figure 7.4: Arousal diurnal variation at both trials and at time point during the trial. Significant differences between trial time points ($P < 0.05$) are represented by a '#'Values are mean ± SD.

7.4 Discussion

The purpose of both these studies was to investigate the diurnal variation in mood, alertness, arousal state and perceived exertion (RPE) in highly trained distance runners. This study demonstrated that despite no statistical difference in the time taken to complete the 10km run at the different times of day in both studies, the athletes' RPE was significantly higher in the morning trial in the cold study (Chapter 5), whereas, RPE in the hot and humid study was not significant (Chapter 6). Mood score was better in the afternoon than in the morning at rest (Chapter 5). However, there was no significant difference between these scores. Therefore, hypothesis 1 of a greater resting mood in the evening to coincide with peak core body temperature is rejected. Alertness was not affected by the time of the day. On the basis of these findings the second hypotheses of morning high resting alertness is rejected, however, hypothesis 2 also states core body temperature and exercise as drivers of alertness which is accepted. Mean performance was better in the morning and corroborates with low arousal and alertness and therefore hypothesis 3 is rejected. The result from Chapter 6's study showed no significant difference between mood scores. However, the mood score was better in the afternoon

than in the morning at rest and at post-trial by 4% and 4.3%, respectively. Whereas, at 1 h post-trial mood score was better in the morning than the afternoon by 3%.

This study's participants are competitive athletes and as such it was anticipated mood state to be characterised by high levels of vigour and tension, with low levels of anger and arousal (Terry, 2000). Vigour was low in the morning trial compared to the evening. The patterns of change between pre-, post- and 1 h post-trial indicates that for tension, confusion and vigour there was a reduction of mood pre-trial to post-trial and then a slight increase 1h post-trial. Although it is worth noting that there was an interaction effect for morning to afternoon. For depression and fatigue there was broadly an increase from pre- to post- then a 'recovery' between post- and 1-h post-trial, more so for fatigue than depression. Overall mood scores for anger stayed relatively stable; however differences occurred when diurnal affects were considered. Mood was higher at the post-trial time-point in both trials, this finding supports past research that suggested exercising at a greater exertion level leads to greater mood change (Rocheleau et al., 2004).

Moods are transitory emotional states that can be influenced by a range of personality and environmental factors. As observed with general mood state, there was an interaction effect in the pattern between morning and afternoon; where at the 1-h post- time point mood was observed to worsen in the afternoon whereas it improved in the morning. It may be that because this study was carried out in Scotland the low daily daylight/sunlight hours may have played a role in this interaction effect between morning and evening (data for this study was collected during the autumn and winter season). Early morning light sets the body's clock to gear up for the day's activities; in contrast the lack of light causes a delay in the normal cycle. On the other hand the recovery time for the afternoon was greater than in the morning.

Confusion, depression and tension were worse in the morning pre-trial, when the test was carried out close to waking time. These findings may reflect the personal nature of the athletes where they could be categorised as "evening" people (i.e. people with a natural tendency to prefer evening activities as opposed to morning activities. Generally, most of the participants in this study were intermediate types). The increased confusion,

depression and tension could therefore reflect a lack of sleep. Mah et al. (2011) reported that extended sleep in basketball players indicate that optimal sleep is likely beneficial in reaching peak athletic performance as a result of mood improving. At the post-exercise time point both higher tension and higher vigour was recorded in the morning. The results of this study suggest that in general mood in the morning time is worse for pre-trial but more positive during the recovery period.

The results of the present study (Chapter 5) are not consistent with Barkhoff et al. (2007) who reported ideal performance states (called iceberg profile) characterized partly by high levels of vigour versus low levels of anger. In the present study vigour at each time point was lower than the anger level. Potentially these findings can be explained due to the nature of the participants exercise levels of high intensity exercise, training load, reduced rest phases and general increased aerobic capacity that may result in a greater capacity to work within periods of psychological strain or stress. Two top level artistic roller skaters participated in Barkhoff et al. (2007). The training champion repeatedly exhibited more activation (vigour) and less anger before and after competition compared to the competitor type.

Moreover, experienced athletes learn over time to take hardship and turn it into motivation: this can count as one of the keys to success (Koumpoula et al., 2011). On the other hand, Mehta and Josephs (2006) suggested that increased testosterone post-competition may be responsible for positive mood. Other findings suggested that confidence, competitiveness, and a motivation to win are associated with increases in both cortisol and testosterone (Suay et al., 1999). Moreover, there is evidence that active people experience more mood benefit at higher levels of exertion than inactive people (Blanchard et al., 2001; Tieman et al., 2002).

Core body temperature has been reported to influence exercise performance: enhanced when core body temperature is high (i.e. near its circadian peak) and worsened when it is low (i.e. near its circadian minimum). In addition there is strong evidence for a relationship between mood and sports performance (Terry et al., 1999). The correlation in these data can perhaps be seen when confusion, depression, and tension worsen in the

morning at pre-trial and this may be affected by core body temperature diurnal variation. Despite this there was no difference in performance in the present study (9 seconds faster in the evening trial). Lane (2001) reported that vigour was associated with facilitating performance regardless of depression. In the current study, running performance in the afternoon was slightly faster than in the morning (9 seconds), in contrast vigour shows high values in the morning at all testing stages, pre- post- and 1-h post-trial compared to the same trial at 16:00 hs. Vigour, in contrast, is often associated with facilitating performance.

According to Lane (2001), depressed mood represents unpleasant deactivation and should be inversely related to vigour, which is typified by pleasant activation. In this study depressed mood showed an opposite trend to vigour, at the recovery period (1h post-trial) at 09:00 hs: a decrease in depressed mood led to an increase in vigour. In contrast 16:00 hs a depressed mood recovery was not obvious, this affected negatively vigour's recovery scores. Moreover, Lane (2001) reported that depressed mood and fatigue should be positively correlated, as both are associated with low arousal and unpleasantness. Often depressed mood is associated with reducing vigour and increasing confusion and fatigue, but in this study vigour and confusion responded positively, where fatigue showed the same trend as depressed mood. Depression was higher at post-test for both times of the day and is correlated with RPE, in contrast vigour scores showed a decrease at post-test for both times of day. As a general overview, exercise in the morning has a positive effect on vigour. In this current study athletes may tend to use vigour as the basis for setting a challenging goal with low vigour associated with depressed mood.

The higher RPE during the morning exercise found in the present study is in accordance with Martin et al. (2001). RPE is often used to monitor exercise intensity; therefore any diurnal variation in RPE implies an altered perception of exertion of the actual exercise intensity. Deschenes et al. (1998) on the other hand found RPE to be unaffected by the time of day during maximal exercise. The results from this study showed an association between fatigue, depression and RPE; which are higher in the morning at pre- and post-test. This morning worsening in variables can be associated with the cortisol level at this time of the day that reaches a peak at approximately 08:00 hs. Cortisol in this study was

not measured, however it has been found to play a leading role in the behavioural consequences of psychological stressors, including negative affective states, and altered memory and behaviour when the individual faces a threat (Buchanan et al., 1999; Erickson et al., 2003). It is well known that high-intensity physical exercise evokes significant mental responses that involve both the sympathetic nervous and adrenal gland (hormonal systems). Cortisol is positively correlated with both the duration and the intensity of physical workload (VanBruggen et al., 2011). However, the participants in this study are daily training athletes who are accustomed to higher training stress levels (especially when doing interval and workloads).

Generally RPE is an indicator of mental and physiological recovery, and the results showed that recovery is faster in the morning than in the afternoon: this may be associated with the body's heat loss mechanism in the afternoon rather than the heat gain in the morning. Moreover, a link could be made between lower hydration in the morning compared to the afternoon, where RPE results indicate that the morning trial was harder than in the afternoon. It can be suggested that training in the morning rather than the afternoon has a more positive effect in terms of general mood state for northern latitude populations.

Both arousal and alertness were not diurnally significantly different between the trials (Chapter 6). However, the levels of alertness are higher in the morning compared to the evening trial at post and 1h post-trial. Mean performance was better in the morning and corroborates with low arousal and alertness. In terms of phase response alertness was affected negatively by exercise and climatic condition at both times of the day, with worst scores recorded at the evening trial (10%). Whereas, arousal was positively affected by exercise and climatic condition, with higher scores recorded in the morning (20%). Furthermore, both alertness and arousal recorded higher (less worse) scores at post- and 1 h post-trial in the morning. This may indicate that the morning trial and trial conditions have a positive effect on the arousal and alertness (less negative effect) during the recovery period. Thus, athletes and coaches should take this into consideration when planning daily training, especially with elite athletes that train more than one time a day. The patterns of change between pre-, post- and 1 h post-trial indicates that alertness was

higher for the evening trial at rest but for the morning trial higher at both post- and 1 h post-trial. This may be due to core body temperature, where the body is in a heat gain mode during the morning and a heat loss mode in the afternoon.

Sleep is known to have large effects on alertness and performance, thus it was expected that resting morning alertness scores would be worse. This could be due to the degree of light and the production of melatonin, as exposure to light has been shown to increase alertness and core body temperature (Chang et al., 2012a). In addition, exercise and core body temperature was reported to modulate neurobehavioral function and increase alertness and performance in humans (Peluso et al., 2005). However, the findings of the present study disagree with the previous statement as alertness declined below resting level as a response to the trial and increased back to resting level after 1h post-trial. This decline in alertness could be attributed to the rate of physical exhaustion that participants reached at the end of the trial, and this phenomenon was more pronounced for the evening trial. Furthermore, recovery time (1 h post-trial) alertness coincided with the increase of core body temperature during the day. This showed that the morning recovery in terms of mental health is better than in the evening when the light concentration decreases.

Only two participants from 13 stated they were classified as a ‘morning person’ from this it was anticipated that the mean resting level of alertness would be low in the morning and this was verified by our result showing the morning resting level lower by 14% compared to the evening. However, greater alertness is linked to better sport performance (Mah et al., 2011). In the current study, mean running time was faster in the morning where alertness is lower. Arousal as well as alertness did not show diurnal variation between trials (09:00 hs and 18:00 hs), although, for some of the measured time points a decrease at post-trial with a further decline after 1h post-trial was recorded. Arousal in this study showed an opposite trend than alertness. In addition, exercise caused an increase in arousal and a decrease in alertness, with a decrease in alertness and an increase of arousal at 1h post-trial at both times of the day. Indeed, recovery time (1 h post-trial) shows a significant diurnal variation between both trials, with morning arousal at this time point higher than the evening.

The results presented in the present study are not consistent with the literature that states high alertness and optimal arousal are associated with better performance (Colquhoun, 1972). However, in this study morning low resting arousal and alertness (did not reach the significance level) were corroborated with better performance. Worse performance in the afternoon may be due to the participants exceeding the optimal arousal level and as such may enter the anxiety level at this point. An explanation for the differences recorded in his study may be due to the participant's fitness levels. That participants accustomed to frequent, high intensity exercise, may have a greater capacity to control psychological strain or stress at any time of the day that they trained at. This would be learned over time and create the ability to turn seemingly negative psychological factors into positive, even motivational factors. This ability of sport professionals to perform under physiological stress can count as one of the key to success. An alternative explanation to these observed differences could be due to hormonal changes; where findings from other research have suggested that confidence, competitiveness, and motivation to win are associated with increases in both cortisol and testosterone (Suay et al., 1999). Moreover, there is evidence that active people experience more psychological benefit at higher levels of exertion than inactive people (Blanchard et al., 2001; Tieman et al., 2002). Mostly likely combinations of these factors contribute to the unique psychology and adapted ability of high level athletes.

In conclusion (Chapter 5) highly trained runners showed that mood state has no effect on running performance at an environmental condition of 6°C. Mood state in athletes showed different trends for different times of the day, nevertheless the running performance was not significantly different diurnally. Denissen et al. (2008) found significant negative relationships between climatic condition and mood scores. However, in our study participants are based in the UK and are acclimatised to run under the conditions set in the trial. It could be that in the morning participants were able to increase core body temperature to a level that was comparable to that of an afternoon session by doing warm up (Florida-James and Doggart, 2000). Furthermore, the participants in this study were highly trained athletes, training mostly two to three times a day during the race season were they develop their body's adaptation to exercise at its best at any time of the day they will race at it.

The athletes RPE were higher in the morning trial but their mood did not show a difference between the two trials. In addition this finding may conclude that athletes in this study are able to control their mood in terms of performance. In addition, no diurnal variation in RPE, arousal or alertness in the hot and humid study (Chapter 6) was found. None of these factors affected running performance of highly trained athletes in hot and humid conditions.

CHAPTER 8:

GENERAL DISCUSSION

8.1 General Discussion

This chapter summarises the results of this thesis with a general discussion of the findings and their relevance. This is done by examining the practical implications of the time-of-day effect on physiological, immunological and psychological responses to a time trial run in hot and humid, and cold environments. Finally, thesis limitations and the importance and direction of future studies are outlined.

The primary aims of this thesis were:

To investigate the diurnal immune response and physiological differences in a cold environment in well trained runners.

To investigate the diurnal immune response and physiological differences in a hot and humid environment in well trained runners.

To investigate the psychological circadian rhythm in highly trained athletes in two different environmental conditions.

Main finding of the thesis: Table 8.1 highlight the main finding of the thesis comparing the effect of the time of the day, exercise and environmental condition on the running performance, physiological and immunological measure.

Table 8.1: The main findings of this thesis.

Measurements	**Cold condition**			**Hot and humid condition**		
	09:00 hs	**16:00 hs**	**Phase response**	**09:00 hs**	**18:00 hs**	**Phase response**
Running time	-	-		-	-	
Core body temperature	x		am	-	-	am
Heart rate	x	-	am	-	-	am
Lung function	-	-	-	-	-	-
Systolic blood pressure	N/M	N/M	N/M	-	-	-
Diastolic blood pressure	N/M	N/M	N/M	-	-	-
Mean arterial pressure (MAP)	N/M	N/M	N/M	-	-	am
WBC		x	pm		x	pm
Neutrophil		x	am		x	am
Lymphocyte		x	pm		x	pm
CC16	-	-	am	-	-	pm
IL-6	N/M	N/M	N/M	-	-	pm
HSP70	N/M	N/M	N/M	-	-	am

‘x’ symbol denotes a significant diurnal difference. ‘-’ symbol denotes no significant diurnal difference. ‘N/M’ not measured.

Take home message

The data presented in this study reinforces the reasoning that core body temperature remains unchanged with exercise and diurnal conditions in aerobically trained athletes. As such, the rejection of the hypothesis of association between optimum core body temperature and improved performance is recommended. Core body temperature as a marker of optimum physiological readiness is therefore an inadequate tool. However, changes to hormonal or physiological parameters would appear to be a more accurate and reliable means to measure circadian phase in response to high intensity exercise at diurnal divergent time-points.

Racing and training in the evening in cold condition should have a positive effect on performance with less complication on athlete's health, since phase response in immunological variables are higher in the morning (such as CC16). This can cause airway injury and epithelial cell perturbation in athlete's airway. Contrary to cold condition, in hot condition athletes are advised to race and train in the morning where better performance occurred with the least immune suppression. Furthermore, mean performance time/physiological variables were better in the morning and correlated with low arousal and alertness. It was affected negatively by exercise and hot condition at both times of the day, with worst scores recorded at the evening trial.

The findings of this research combined with published literature recommend that athletes seeking to perform at a certain time of the day should apply maximal overload training at that specific time. This will phase shift the body to adapt and respond better at this time culminating in better performance.

In summary, in choosing between the two different climatic conditions for racing, best performance (time) may be achievable in cold condition, although hot and humid conditions present the optimum environment to preserve health.

Elite athletes not only compete against one another but also against tough environmental conditions. Challenging environmental conditions such as heat or cold during racing or training phases pose particular risks to an athlete's health. In modern sport history the Olympic Games, World Championships and major races have drawn attention to a number of climatic effects on running performance (Goubault et al., 2001; Fitch et al., 2008). The summer competition calendar events such as the Olympics, Outdoor World Championships and some major road races are usually held in hot and humid conditions (Goubault et al., 2001). Equally the winter competition calendar including the World Cross Country Championship and the Winter Olympics that invariably calls for protection against the cold.

A 10 km trial run either in a hot and humid condition or in a cold condition (28°C and 70% relative humidity, or cold 6°C and 60% relative humidity, respectively), can result in different physiological, immunological and psychological responses depending on the time-of-day when it is performed. Nevertheless, in both environmental conditions these responses were not enough to impact on performance for highly trained runners (did not reach the significance level). Despite no significant diurnal differences in performance the mean running time was faster in the morning, in hot and humid conditions, compared to the evening: this contradicts with previous literature (Atkinson et al., 2005). This new finding in running performance can be linked to the high standard of athlete used in this research that are used to training and competing at all times of the day. This finding may indicate that daily morning training in our participants has the potential to entrain their circadian pacemaker. Another explanation of better morning performance could be attributed to the effect of exercise on advancing athletes circadian pacemaker. This is in accordance with Miyazaki et al. (2001) who found that, in participants who exercised, plasma melatonin was phase-advanced significantly during the waking period, whereas melatonin rhythmicity did not change in non-exercising participants. However, under cold conditions the mean time to complete the trial was slightly higher in the evening (9 seconds faster) and this is in accordance with previous findings (Atkinson et al., 2005, Martin et al., 2007; for a review see Reilly and Waterhouse, 2009).

In hot and humid conditions, a combination of low alertness, arousal and core body temperature at resting state resulted in a mean faster running time in the morning compared to the evening. This result contrasts the previous literature where evening performance dominates. However, this study found that core body temperature was not significantly different between morning (09:00 hs) and evening (18:00 hs) which contradicts previous research where core body temperature peaked in the evening (Atkinson et al., 2005). In addition, the slower evening performance in hot and humid conditions could be attributed to increases in physical and mental fatigue that occur through daytime activities. Gonzalez-Alonso et al. (1998) demonstrated that the increase in core body temperature causes fatigue in trained participants during prolonged exercise in hot and humid environments. Furthermore, time to exhaustion in hot environments is inversely related to the initial temperature and directly related to the rate of heat storage. In addition, core body temperature increased significantly in the morning compared to the evening in both studies (Chapter 5 and 6). This may reduce the body's ability to remove a heat load in the early morning compared to the evening, suggesting a greater stress on the thermoregulatory system during morning testing (Aldemir et al., 2000).

Zeitgeber has less effect on physiological variables during exercise in cold conditions compared to the same exercise in hot and humid condition, confirming previous research findings (Abbiss et al., 2010). However, it must be noted that different participants took part in each of the studies carried out in this thesis (cold or hot and humid) and therefore it is difficult to make definitive conclusions between the environmental conditions. Performance for most of the participants under the hot and humid conditions was at least a 5% slower than their personal best. It can be concluded that running in a cold environment, in which the participants are familiar with training in (6°C is the UK average winter temperature), resulted in better performance compared to participants who ran in the unfamiliar (non-acclimatised) hot and humid conditions (was not significant). However, the participants in this study were based in Scotland and are not acclimatised to hot and humid conditions, and are used to cold conditions due to Scotland’s weather.

High phase response were observed in mean core body temperature and mean heart rate during exercise in the morning trial compared to the evening trial in both studies. This

higher core body temperature in the morning suggests greater stress on the thermoregulatory system during the earlier morning testing. These findings differ from those reported by previous research such as in Atkinson et al. (2005) who found an evening high core body temperature.

Mean heart rate in the cold study showed significant diurnal variation with a morning peak of 7% that could be due to greater physical demand during the trial when compared to the evening. Conversely, in a hot and humid environment the heart rate did not present diurnal variation. A lower resting heart rate was reported at morning trial in hot and humid condition, and with a slow return to the baseline level after 1h post-trial, which suggests a slow morning recovery. These findings could be a good practical implication for athletes and coaches in terms of recovery when planning their daily training program. In addition, zeitgeber lowered MAP by 5 mmHg in the morning trial compared to no changes in the evening. It can be concluded that running 10 km in a hot and humid condition results in the MAP having the highest reactivity when performed in the morning and these changes in MAP could be driven by elevated intravascular shear stress that occurred in the morning (Jones et al., 2008; Jones et al., 2009a). Other physiological measured variables (strength, flexibility and vertical jump) were not affected by the time of day either at rest or after the appliance of zeitgebers.

Resting plasma CC16 showed higher value in the morning compared to the evening in warm and humid condition (did not reach the significance level). In contrast, in the cold study resting CC16 was higher in the evening. However, post- and 1h post-trial was higher in the morning compared to the evening in both studies, albeit not a significant difference. Morning trial in the cold condition has greater effect on lung injury with slow recovery compared to the evening trial. Possible explanations for high increase in plasma CC16 during the morning trial could be due to the leakage through increased permeability in the airway epithelium, which increases the production of CC16 in the lung or postpones (reduces) renal clearance (Tufvesson et al., 2013), and this phenomena was more pronounced in the morning trial in both different climatic conditions. These changes in CC16 after exercise compared to at rest in the morning should be taken into consideration in clinical research or when looking to the effect of any exogenous substances on changes

in CC16. It is known that environmental conditions impact on the degree of airway epithelial disruption during high-level exercise (Bolger et al., 2011). Moreover, in the recent study the degree of leakage of CC16 can be affected more in cold environment compared to hot and humid environment; this corresponds with Bolger et al. (2011) where urinary excretion of CC16 in a hot and humid environment remained constant (did not change dramatically) compared to a cold environment (250% higher at 4°C and 37% relative humidity compared to at 25°C and 94% relative humidity). This, higher morning phase response in CC16 recorded (more noticed in cold study) could be due to the morning higher physical demand during the trial compared to the evening which resulted in an increase in the rate of ventilation (cold air causes epithelial damage) which lead to an increase of the level of epithelial damage. However, warm humid air inhalation limits the airway injury and epithelial cell perturbation compared to cold air (Bolger et al., 2011). Furthermore, it can be speculated that high intensity exercise in hot and humid conditions may be more effective to decrease the risk of URTI or asthma in athletes.

IL-6 and HSP70 (measured only in hot and humid condition study) showed no changes and a decrease, respectively, 1h post-trial compared to post-trial, however these findings of IL-6 and HSP70 were unexpected. This result may represent athlete training adaptation or the body's recruitment of these two components to the inflamed area caused by exercise, and therefore lower concentrations in the circulating blood. HSP70 and IL6 circadian patterns were disturbed (masked) by zeitgeber (exercise and hot and humid condition), where these variables at post- and 1h post-trial showed a different pattern than at rest. This may lead to better interpretation when considering this variable for clinical research or medical intervention. The changed pattern of IL-6 immediately and 1 h post-trial suggests that the combination of exercise in the evening time as well as the hot and humid environment elicited greater circulating numbers of IL-6 compared to the morning. This elevated evening IL-6 concentration could be due to an increase in total white blood cell counts (including neutrophil and lymphocyte) in the evening as these cells are known to express IL-6 (Febbrai and Pedersen, 2002). There was an inverse relationship between HSP70 and neutrophils that occurred at post-trial, particularly for the evening trial. However, this increase of neutrophils occurring at post- and 1-post-trial may lead to the

degradation of HSP70 (loss potential beneficial effects), this process could make the cell surfaces more fragile and more prone to rupture (Martin-Ventura et al., 2007).

Nevertheless, this study result (Chapter 6) suggests that well-trained athletes are prepared to cope with exercise and the environmental stress at any time of the day without causing IL-6 and HSP70 production, in other words muscle contraction rate did not differ between both times of the day (contractile muscle appears to be the main source of IL-6 in the blood as a response to exercise).

Both studies showed a diurnal variation in most white blood cells measured including neutrophils and lymphocytes, with higher values recorded at all measured time points in the evening compared to the morning. The higher evening time white blood cell concentrations may be due to the increases in daily physical and mental demand that accumulate through the daytime activities. Furthermore, in well trained athletes white blood cells rhythmicity is not masked by zeitgebers in either environmental condition, as the resting level of most white cells are higher in the evening and remained higher at post- and 1h post-trial. In addition, exercise in a cold condition (Chapter 5) produced greater disturbances in immunity in athletes compared to exercise in hot and humid condition (Chapter 6). However, it is known that exercise in hot conditions will produce more physiological stress than in cold conditions (McFarlin et al. 2003). Furthermore, exercising in a cold condition will elicit a blunted stress hormone response than exercising in a hot condition. Thus, plasma catecholamines increase and plasma cortisol is abolished as a response to exercise in cold conditions and could be responsible for neutrophilia after exercising in this condition (Rhind et al., 1999).

The CC16 result in both studies (Chapter 5 and Chapter 6) supported the hypothesis that URTI severity in athletes' peak in the morning with greater predominance in cold conditions. The lung function parameters analysed in both studies showed no significant diurnal variations or differences in the time-points. Nevertheless, PEF a marker of airway inflammation (Dente et al., 2010) showed an identical value between morning and evening for the cold study, whereas for the hot and humid condition morning PEF was higher by 5% than the evening. Furthermore, PEF for the cold study showed a decrease

at post-trial and was more pronounced for the morning trial with a decrease of 6% compared to a decrease of 1% at the evening trial. In the hot and humid condition PEF showed a positive response to exercise at both times of the day with a greater increase recorded in the evening (3%). A small increase in absolute humidity during exercise in a cold environment will help to reduce the rate of water loss from the airways during exercise and consequently reduce the risk of airway injury. The only practical strategy for individual athletes exercising in cold environments to minimise the risk of airway injury is through the use of a moisture exchange device (Beuther and Martin, 2006).

Finally, in either hot or cold environments, exhaustive exercise is followed by acute changes in athlete's immunity; this can be a potential marker of a transient immune-suppression period that can last from immediately following exercise to several days afterwards; with more pronounced changes occurring during exercise in a cold environment. It is difficult for any firm conclusion to be stated in relation to the effect of environmental conditions on performance as the same population of participants were not used for both studied conditions; this is a limitation of this research. However, the transient immune-suppression period after exercise is more pronounced in the cold condition which may formulate an 'open window theory'. Thus, care should be taken to ensure that heavy training is well planned and managed, with adequate volume and intensity variation over time ensuring that athletes are well recovered, to avoid immune-surveillance weakness, that might lead to chronic URTI (Koch, 2010).

An important finding of this research is that exercise in hot and humid conditions can allow for changes to the intensity of airway inflammation and is more effective in the evening were phase response for and recovery period of CC16 and white blood cells are high in the evening. This finding may improve the treatment strategy in athletes suffering from upper airway dysfunction and could even be applied within an unhealthy population with a chronic disease such as asthma. This can be due to adaptive response rather than airway damage.

8.2 Limitations of this research

There are three key limitations in this thesis. The first was that the data was collected only at two times of the day due to resource limitations. Ideally data should have been collected several times within a 24h cycle to enable better understanding of the circadian rhythm variation in sport performance and the parameters related to sport performance measured. The second limitation was the different participants that took part in the two studies of this thesis (Chapters 5 and 6). Recruiting identical participants would strengthen the thesis results as differences could be closer linked to the effect of the two different environmental conditions and diurnal variation rather than the influences of individual athletes. The last limitation is that the sensor pill to measure core body temperature in Chapter 5 was taken one hour prior to the trial. This however was insufficient time for the pill to pass along the digestive tract too deep within the internal organ to give the true temperature reading. It is highly likely that the sensor pill position was much higher in the digestive system throughout the trial, such as in the oesophagus. It is therefore recommended that the sensor pill is taken a minimum of 2 hours prior to testing such as was the strategy adopted for the running trials in Chapter 6 and Chapter 4.

The study design presented the limitation of only investigating plasma IL-6 and HSP70 in the second study only (Chapter 6). This occurred due to financial constraints, but it would have been useful to also analyse samples from nasal lavage. Additionally, it would be better to measure CC16 at more time-points throughout the day, such as 3 h, 6 h, 18 h and 24 h post-exercise, to be able to have a better understanding of the development of the inflammatory process in the upper airway. The research was not able to measure cortisol in either study. In addition, the lack of nasal lavage cell count and differentiation in the first study was also a limitation, but was corrected and measured in the second study. In addition, the number of participants that took part in the cold study (Chapter 5) was a small sample size. This gives less chance of detecting differences, and the trial was conducted on the treadmill which may blind the true performance for the participants (Nummela et al., 2007). Furthermore, the lack of a control group to form a blinded experiment was not possible in this study, therefore results cannot be compared to a control group. Finally, participants did not perform any habitual trial in either condition.

This would allow the athlete's body to adapt to repeated daily exposure to the environmental condition which would reduce the impact of heat on physiological function and exercise performance. However, all participants where based in the U.K. and are habituated with British winter conditions that mimic the 'cold' conditions of Chapter 5. It may be that athletes are therefore acclimated to these conditions. By comparison the hot and humid condition where entirely novel to participants.

8.3 Recommendation for future work

Most chronobiology research demonstrates that circadian rhythms exist in humans and suggests that a shift in this internal clock may be costly in terms of performance and health. When circadian rhythms conflict with environmental conditions, such as following exercise, a flight or sleep disruption, performance may be impaired. Furthermore, several agents, including light, hormones (melatonin), immune system, diet, and exercise, are under investigation for their ability to adjust or shift circadian rhythms. Despite this, the mechanism of how the internal clock works is still unclear, and requires further research, especially over a 24 hour period. Further research is also necessary to substantiate the circadian effects of the other agents that have a direct effect on human health, in general, and on human sport performance, in particular.

An important issue that needs to be addressed is the effect of chronic exposure to hot and humid or cold dry environmental conditions. This would have practical relevance for individuals who live and train in these conditions and would provide information about adaptation processes that might occur in the lungs due to the repeated exposure to this environmental condition. Furthermore, addressing in detail the mechanisms behind variation in plasma CC16, IL-6 and HSP70 is an important aspect to consider for future research in terms of lung and muscle injury. Additionally, there is also scope to investigate if manipulation of circadian rhythmicity, particularly of the body's temperature rhythm, could be used to advantage the travelling athlete who has to compete in a hot and humid environment. That is flying in to race at a time when the body temperature rhythm is at a nadir compared to the local time.

In sport psychology, future research should investigate the mechanisms underlying mood, arousal and alertness, changes at different times of the day to include sleep disruptions and diurnal variation in different environmental conditions. Psychological parameters can also be affected by stress hormones and more research should investigate the link between diurnal variation in stress hormones and psychological changes during the day in highly trained athletes. With these measurements the research will lead to an understanding of the best time that an athlete will experience least stress allowing athletes to train or race with optimal or controlled stress than can occur because of psychological factors.

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APPENDICES

Appendix 1: Statement of informed consent

Circadian effect of 10 km running at an external environment of 28°C and 70% relative humidity on selected physiological and immunological measures

I have read the participant information sheet, detailing the procedure and requirements which are involved with this study and I fully understand what is required of me. I have had an opportunity to ask for further information and clarification of the demands of each of the procedures. I am aware that I have the right to withdraw at any time with no obligation to give reasons for my decision.

I agree to take part in the study.

Name of participant

_____

Phone No.

Contact Address

_____

E-mail _____

Signature of participant _____

Signature of researcher_____

Date_____

Appendix 2: General medical history questionnaire

HEALTH SCREEN FOR STUDY PARTICIPANTS

Name .....

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

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

   (a) on medication, prescribed or otherwise ..... Yes ☐ No ☐

   (b) attending your general practitioner ..... Yes ☐ No ☐

   (c) on a hospital waiting list ..... Yes ☐ No ☐

2. **In the past two years,** have you had any illness which require you to:

   (a) consult your GP ..... Yes ☐ No ☐

(b) attend a hospital outpatient department ..... Yes ☐ No ☐

(c) be admitted to hospital ..... Yes ☐ No ☐

3. **Have you ever had any of the following:**

(a) Convulsions/epilepsy ..... Yes ☐ No ☐

(b) Asthma ..... Yes ☐ No ☐

(c) Eczema ..... Yes ☐ No ☐

(d) Diabetes ..... Yes ☐ No ☐

(e) A blood disorder ..... Yes ☐ No ☐

(f) Head injury ..... Yes ☐ No ☐

(g) Digestive problems ..... Yes ☐ No ☐

(h) Heart problems ..... Yes ☐ No ☐

(i) Problems with bones or joints ..... Yes ☐ No ☐

(j) Disturbance of balance/coordination ..... Yes ☐ No ☐

(k) Numbness in hands or feet..... Yes ☐ No ☐

(l) Disturbance of vision..... Yes ☐ No ☐

(m) Ear / hearing problems..... Yes ☐ No ☐

(n) Thyroid problems..... Yes ☐ No ☐

(o) Kidney or liver problems ..... Yes ☐ No ☐

(p) Any kind of allergy (i.e food) ..... Yes ☐ No ☐

(q) High blood pressure…….... Yes ☐ No ☐

4. **Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise?** Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....

.....

5. **Have you** had a cold or feverish illness in the past month? Yes ☐ No ☐

6. **Are you** accustomed to vigorous exercise (1-3 hours per week)? Yes ☐ No ☐

Thank you for your cooperation

Appendix 3: Daily Health Questionnaire

HEALTH QUESTIONNAIRE

Name: Date:

Trial:

Please complete the following brief questions to confirm your fitness to participate in today's session. At present do you have any problems for which you are:

1) On medication, prescribed or otherwise? Yes/No

2) Seeing your general practitioner? Yes/No

3) Do you have any symptoms of ill health, such as those associated with a cold or other common infection? Yes/No

If you have answered yes to any of the above questions, please give further details below:

…….....

…….....

Would you like to take part in today's experiment? Yes/No

Signature…….

Appendix 4: Blood donation form

EDINBURGH NAPIER UNIVERSITY School of Life Sciences

Subject Declaration for Vene Puncture Blood Donation

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 project entitled:

Circadian effect of 10 km running at an external environment of 28°C on selected physiological and immunological measures; but 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: Respiratory symptoms questionnaire

Post-Trial 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 6: Specific medical history related to this study

Temperature Sensor Pills According to HQ*int wireless sensing system* manufacture safety guideline *(*http://www.hqinc.net*)*.

Is your body weight less than 36.5kg? .....
.....

Do you suffer from any suspected obstructive disease of the gastrointestinal tract?

…….....

……..

Do you suffer from diverticulitis and inflammatory bowel disease?

.....
.....

Do you have a history of disorders or impairment of the gag reflex?.....
.....

Do you have a history of gastrointestinal surgery?.....
.....

Do you have felinization of the esophagus?.....
.....

Do you have to undergo Nuclear Magnetic Resonance (NMR) or MRI scanning during the period that the CorTemp™ Disposable Temperature Sensor is within the body? .....

Do you suffer from hypo motility disorders of the gastrointestinal tract?

.....

Do you have a cardiac pacemaker or other implanted electro medical device?

.....

If you answer yes to any of the above then you cannot be a participant for this study.

Print name:

Signature:

Appendix 7: Arousal and Alertness questionnaire.

The Stanford Sleepiness Scale and sport

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 tired 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.

¦ ---- ¦ ---- ¦ ---- ¦ ---- ¦ ---- ¦ ---- ¦ ---- ¦ ---- ¦ ---- ¦ ---- ¦

0 1 2 3 4 5 6 7 8 9 10

Appendix 8: Morningness and 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.

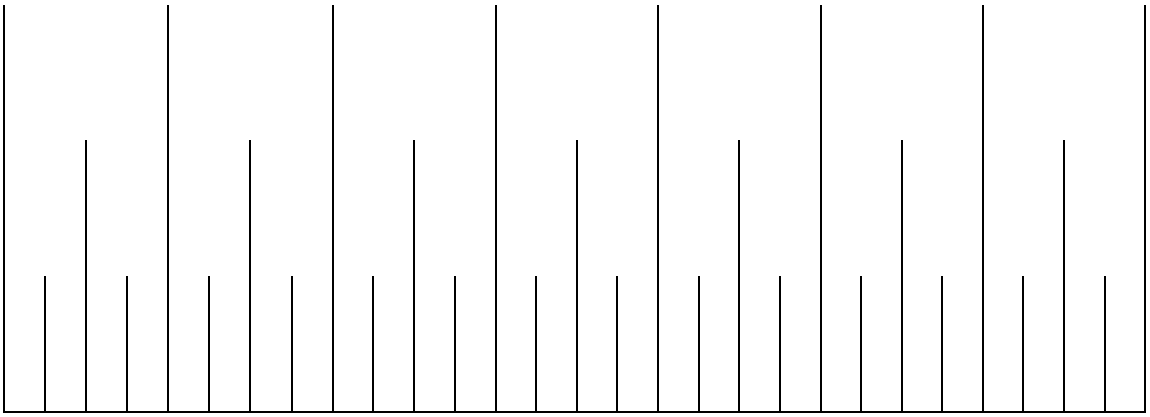
5. All questions have a selection of answers. For each question place a cross alongside ONE answer only. Some questions have a scale instead of a selection of answers. Place a cross at the appropriate point along the scale.

6. Please answer each question as honestly as possible. Both your answers and the results will be kept, in strict confidence.

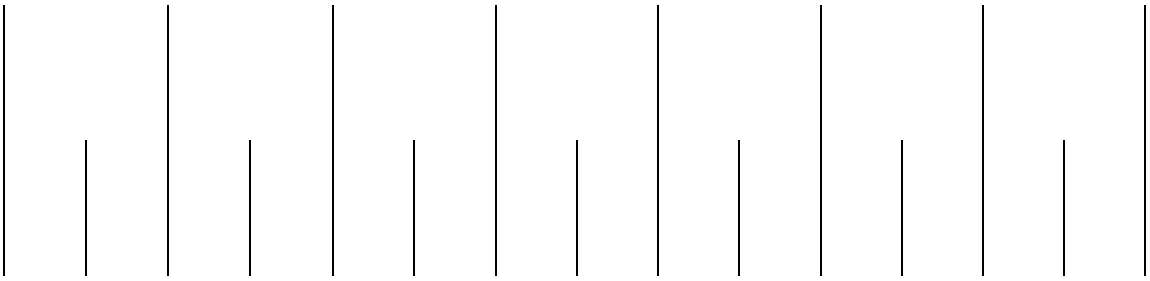
7. Please feel free to make any comments in the section provided below each question.

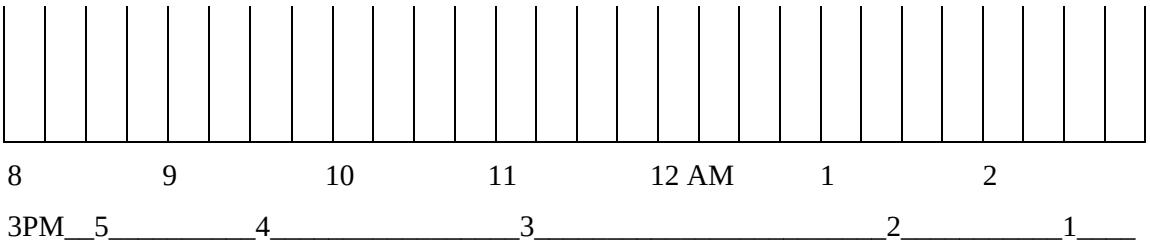
The Questionnaire, with scores for each choice

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



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





2. 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 dependent	4
Slightly dependent	3
Fairly dependent	2
Very dependent	1

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

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

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

Not at all alert	1

Not very alert	2
Fairly alert	3
Very alert	4

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

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

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

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

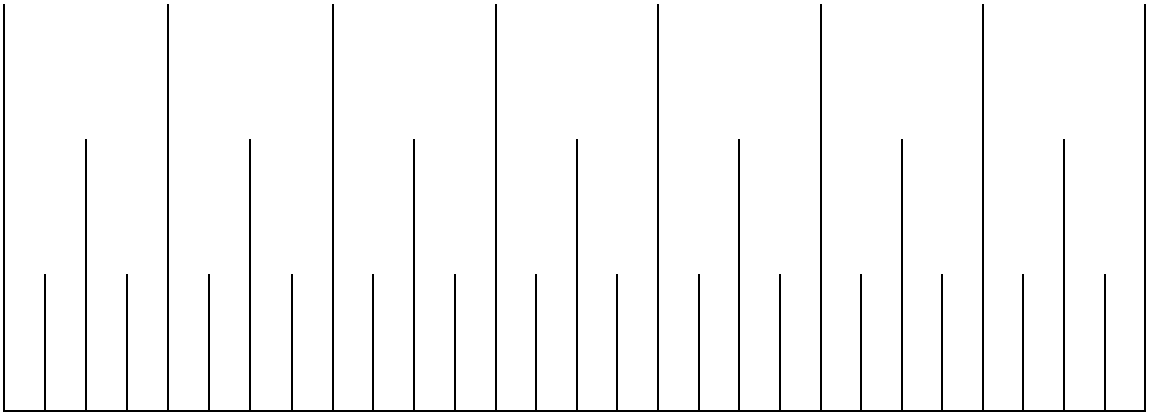
7. When you have no commitments the next day, at what time do you go to bed compared to your usual bedtime?

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

8. 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

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



8 9 10 11 12 AM 1 2 3

PM__5_____4_____3_____2_____1_____

10. 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?

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

11. 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

12. 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 doze thereafter	3

Will wake up at usual time and will fall asleep again	2
Will not wake up until later than usual	1

13. 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 is over	1
Would take a nap before and sleep after	2
Would take a good sleep before and a nap after	3
Would take all sleep before watch	4

14. 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?

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

15. 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 findit very difficult	4

16. 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_____

_____

17. 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_____

18. 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 9: Testing Sheet

Testing sheet

Date: .....Participant name: .....

Age: …… $\dot{V}O_{2max}$: ..... $mlO_2 \cdot kg^{-1} \cdot min^{-1}$.

	Pre-test	Post-Test	1h post-test
BP			
Blood collections			
Height			
Weight			
Lung function			
Flexibility (Sit and Reach Box)			
Power (Vertical Jump Mat)			

Strength (Strength dynamometer)									
Grip strength									
Saline amount collected									
Nutrition status (last 24 h)									

KM	temperature	HR	Speed	RPE	Time
Rest					
1					
2					
3					
4					

5					
6					
7					
8					
9					
10					
Post- test					
1h post-test					

RPE: Ratings of Perceived Exertion. BP: blood pressure. HR: heart rate.

Signature of the researcher ……..

Signature of the participant ……

Signature of the supervisor ……

Signature of the supervisor ……

General comment

	CBT	Ear temperature	Skin temperature	Comment
Rest				
1km				
2km				
3km				
4km				
5km				
6km				
7km				
8km				
9km				
10km				
Post-				
1H				

Appendix 10: The Brunel Mood Scale (BRUMS)

Edinburgh Napier UNIVERSITY

Description

- The Brunel Mood Scale was developed to serve as a brief measure of mood states among adolescent and adult populations. Derived from the Profile of Mood States (McNair, Lorr, & Droppleman, 1992), the BRUMS[1] contains 24 simple mood descriptors, such as angry, energetic, nervous, and unhappy. Respondents indicate whether they have experienced such feelings on a 5-point scale (0 = not at all, 1 = a little, 2 = moderately, 3 = quite a bit, 4 = extremely). The standard response timeframe is "How you feel right now" although other timeframes, such as "How you have felt during the past week including today" or "How you normally feel" can be used. The BRUMS takes about 1-2 minutes to complete.

Scoring

- The 24 items comprise the following six subscales: anger, confusion, depression, fatigue, tension and vigour. Each subscale contains four items. When responses from the four items in each subscale are summed, a subscale score in the range 0-16 is obtained. The items in each subscale are:

Anger: annoyed, bitter, angry, bad tempered (items 7, 11, 19, 22)

Confusion: confused, mixed up, muddled, uncertain (items 3, 9, 17, 24)

Depression[2]: depressed, downhearted, unhappy, miserable (items 5, 6, 12, 16)

Fatigue: worn out, exhausted, sleepy, tired (items 4, 8, 10, 21)

[1] The BRUMS is also known as the Profile of Mood States – Adolescents (POMS-A).

[2] The depression scale is an indicator of depressed mood not clinical depression.

Tension: panicky, anxious, worried, nervous (items 1, 13, 14, 18)

Vigour: lively, energetic, active, alert (items 2, 15, 20, 23)

- Details of the development and validation processes for the BRUMS can be found in Terry, Lane, Lane, and Keohane (1999) and Terry, Lane, and Fogarty (2003). The BRUMS was initially developed for use with adolescents (see Terry et al., 1999) but it is equally valid for use with adults (see Terry et al., 2003). Further information and permission to use the BRUMS can be obtained from the authors (terryp@usq.edu.au or a.m.lane2@wlv.ac.uk).

The Brunel Mood Scale

Below is a list of words that describe feelings. Please read each one carefully. Then cross the box that best describes HOW YOU FEEL RIGHT NOW. Make sure you answer every question.

	Not at all	A little	Moderately	Quite a bit	Extremely
1. Panicky……	❑	❑	❑	❑	❑
2. Lively..……	❑	❑	❑	❑	❑
3. Confused..……	❑	❑	❑	❑	❑
4. Worn out..……	❑	❑	❑	❑	❑
5. Depressed……	❑	❑	❑	❑	❑
6. Downhearted……	❑	❑	❑	❑	❑
7. Annoyed……...……	❑	❑	❑	❑	❑
8. Exhausted……	❑	❑	❑	❑	❑
9. Mixed-up..……	❑	❑	❑	❑	❑
10. Sleepy……	❑	❑	❑	❑	❑
11. Bitter..……	❑	❑	❑	❑	❑
12. Unhappy……	❑	❑	❑	❑	❑

13. Anxious..... ❑ ❑ ❑ ❑ ❑

14. Worried..... ❑ ❑ ❑ ❑ ❑

15. Energetic..... ❑ ❑ ❑ ❑ ❑

16. Miserable..... ❑ ❑ ❑ ❑ ❑

17. Muddled..... ❑ ❑ ❑ ❑ ❑

18. Nervous..... ❑ ❑ ❑ ❑ ❑

19. Angry..... ❑ ❑ ❑ ❑ ❑

20. Active..... ❑ ❑ ❑ ❑ ❑

21. Tired..... ❑ ❑ ❑ ❑ ❑

22. Bad tempered..... ❑ ❑ ❑ ❑ ❑

23. Alert..... ❑ ❑ ❑ ❑ ❑

24. Uncertain..... ❑ ❑ ❑ ❑ ❑

For official use only:

Ang: ____ Con: ____ Dep: ____ Fat: ____ Ten:____ Vig: ____

Conversion of Raw Scores to Standard Scores

- The following tables of normative data can be used to convert raw scores to standard scores (T-scores).

- Raw scores are shown in the left hand column and the equivalent T-score is shown for each subscale.

- There are separate norms for each of four populations: adult students (aged >18 yr.), adult athletes (aged >18 yr.), schoolchildren (aged 12-17 yr.), and young athletes (aged 12-17 yr.).

- For example, for an adult student, a raw score of 6 for vigour converts to a T-score of 50 (i.e., the mean, or 50th percentile, for vigour), whereas a raw score of 5 for depression converts to a T-score of 60 (i.e., the 60th percentile, or one standard deviation above the mean, for depression).

- **Adult Students (*N* = 656)**

	Anger	Confusion	Depression	Fatigue	Tension	Vigour
0	44	42	43	38	42	34
1	48	45	47	40	46	36
2	51	48	50	42	49	39

3	54	51	54	45	53	42
4	58	55	57	47	57	45
5	61	58	60	49	61	47
6	64	61	64	51	65	50
7	67	64	67	54	69	53
8	71	68	71	56	72	55
9	74	71	74	58	76	58
10	77	74	77	61	80	61
11	81	77	81	63	84	64
12	84	81	84	65	88	66
13	87	84	88	67	92	69
14	90	87	91	70	96	72
15	94	91	94	72	99	75
16	97	94	98	74	103	77

Adult athletes (*N* = 621)

	Anger	Confusion	Depression	Fatigue	Tension	Vigour
0	45	42	45	40	37	29
1	52	46	52	44	40	32
2	58	50	58	47	43	34
3	65	54	64	51	46	37
4	71	58	70	54	49	39
5	78	62	77	58	52	42
6	84	66	83	61	55	44
7	91	70	89	65	58	47
8	98	74	95	68	61	49
9	104	77	102	72	64	52
10	111	82	108	75	67	55

11	117	86	114	79	70	57
12	124	90	120	82	72	60
13	130	94	127	86	75	62
14	137	98	133	89	78	65
15	143	102	139	93	81	67
16	150	106	145	96	84	70

- **Schoolchildren (*N* = 596)**

	Anger	Confusion	Depression	Fatigue	Tension	Vigour
0	44	43	44	37	42	33
1	48	46	45	39	45	35
2	52	50	50	42	48	38
3	56	53	54	45	50	40
4	60	57	57	47	53	43

5	64	60	60	50	56	45
6	68	64	64	52	59	48
7	72	67	67	55	62	50
8	76	71	70	58	65	53
9	80	74	74	60	68	55
10	84	78	77	63	71	58
11	87	81	81	66	74	60
12	91	85	84	68	76	63
13	95	88	87	71	79	65
14	99	92	91	74	82	68
15	103	95	94	76	85	70
16	107	99	97	79	88	72

Young Athletes (*N* = 676)

	Anger	Confusion	Depression	Fatigue	Tension	Vigour
0	45	43	45	40	39	29
1	49	47	49	43	42	31
2	53	51	52	46	45	34
3	58	55	56	49	48	36
4	62	59	60	52	51	39
5	66	63	64	56	54	41
6	71	67	68	59	57	43
7	75	71	72	62	61	46
8	79	75	76	65	64	48
9	84	79	80	68	67	51
10	88	83	83	71	70	53

11	92	87	87	75	73	55
12	97	91	91	78	76	58
13	101	95	95	81	80	60
14	105	99	99	84	82	63
15	110	103	103	87	86	65
16	114	107	107	90	89	67

- All norms were generated from data collected using the response timeframe, "How do you feel right now?" and may not be relevant to data collected using other response timeframes.

- Norms for students and schoolchildren were generated from data collected in a classroom setting and may not be relevant to data collected in other settings.

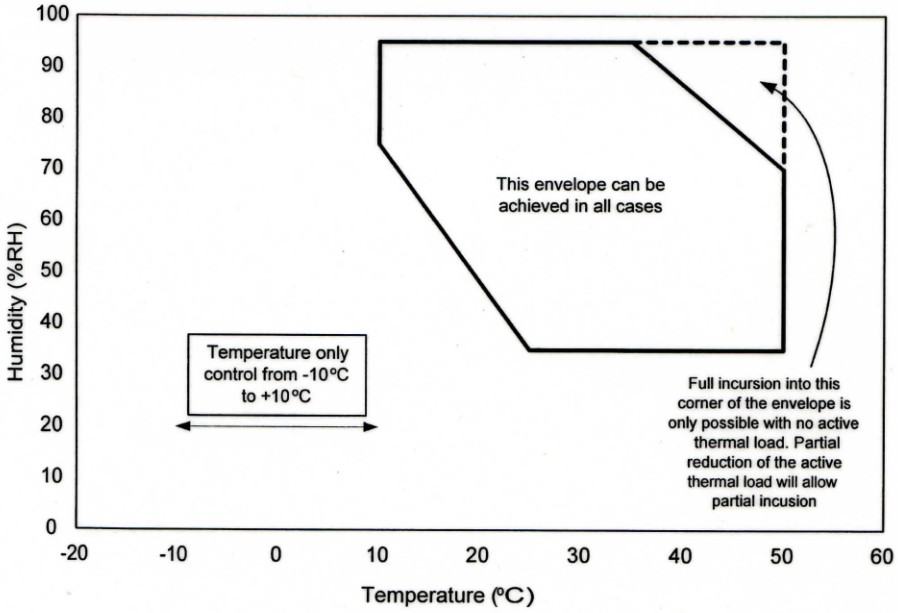
- Norms for athletes were generated from data collected approximately one hour prior to a competition and may not be relevant to data collected at other times or in other settings.

- Raw scores can also be plotted on the profile sheets that follow. There are different profile sheets for adult students, adult athletes, schoolchildren, and young athletes. To use these profile sheets, circle the raw score for each subscale. The equivalent T-score is shown in the far left- and right-hand columns.

Appendix 11: Environmental envelope

APPENDIX 3 - ENVIRONMENTAL ENVELOPE

The room is capable of achieving the environmental conditions as depicted in the graph below.



Appendix 12: Research Ethics Approval Application Form

Faculty of Health, Life & Social Sciences Research Ethics Committee

Research Ethics Approval Application Form

This application form must be completed by all staff and students conducting research which involves the gathering and processing of primary data concerning human participants, and where the outcomes will be disseminated beyond the individual who originally collected or processed the information.

Exceptions: (1) research involving NHS staff or patients, or therapeutic interventions in healthy normal populations are covered by separate arrangements: in these cases, a COREC form should be filled in and submitted to the FHLSS Ethics Committee before submission to the appropriate committee; (2) research concerning non-human animals and environmental issues are covered by separate arrangements, in accordance with relevant Home Office regulations; (3) research which involves the analysis of documents or material in non-print media which are freely available for public access does NOT need Ethics Committee approval; (4) external applicants. Please discuss the appropriateness of using a COREC submission with your Supervisor before sending this form in to the Committee Clerk.

Section one: General

1. Applicant *(All correspondence will be sent to this address unless otherwise stated)*

Full Name and Title:

MR BOUKHEMISS BOUKELIA

School (if applicable):

SCHOOL OF LIFE, SPORT AND SOCIAL SCIENCES

Postal Address (if external):

Please enter your postal address

Sighthill Campus (Room 2.B.46), Sighthill Court, Edinburgh, UK

EH11 4BN

Affiliation (please tick): ☐ Napier Staff

☐ Napier Student (fill out details below)

Matriculation Number: 060198257

Name of Programme:

Master by research /sport and exercise science Level of Study: UG1 UG2 UG3 UG4 MA/MSc **MPhil/PhD**

Please underline the level of this course

☐ Non-Napier external applicant (Please include a copy of your home institution ethics application form along with the letter of approval.)

Email Address (University, work or employer): Z.Boukemis@napier.ac.uk……

This will be used to contact you after the initial decision has been made.

Contact Telephone Number:

01314552350

2. Title of research project

Diurnal variation in physiological and immune response to intense exercise performed in hot and humid environment.

3. Start and end dates of research project

Start 01/03/13. Finish 01/05/13

4. Details (amount and source) of any financial support from outside Napier University.

If you are a **Non-Napier external applicant** and have included a copy of your home institution ethics application form, along with the letter of approval please go to **Section 4**.

5. Other researchers involved, together with role (e.g. PI / Director of Studies/ Supervisor) and affiliation (e.g. School of Health and Social Sciences, University of Napier).

Dr Elisa Gouto Gomes – Supervisor

Dr Geraint Florida-James - DOS.

6. Name of Independent Advisor (where applicable)

Remember that the independent advisor should be someone who knows about the project but is not directly involved in it therefore your supervisor(s) or Director of Studies would not be permissible.

Dr Kevin Smith Reader – School of Life Sport and Social Sciences

Section 2: Details of project

Supporting documentation should be attached where detailed below.

7. Aims and research questions of the project *(maximum 5).*

Athletes can be repeatedly exposed to a hot humid environment during daily training and racing as well as to many inhalant irritants and allergens (Helenius et al., 2002). This

exposure to hot and humid air and other inhalants often causes upper and lower airway dysfunction, moreover this exposure can cause airway epithelial injury (Gomes et al., 2010). The array of immunological and physiological data analysed offers an important overview on the effect of exercising at a distinct time of day under these stressful environmental conditions. It is important for coaches and athletes to be aware of how the time of day can affect various physiological and immunological responses, and running performance. This knowledge can allow for improved tailoring of training programs and specific activities to the time of day that generates maximum effectiveness. Furthermore, understanding the diurnal cycle of athlete performance could also be applied to testing sessions and competition times.

The main aim of this study is to investigate the diurnal variation in different physiological, psychological and immunological parameters during a 10km run in a hot humid environment: the same conditions as would be expected at the Olympic games 2016 in Rio de Janiero. Thus the main research question is: Do athletes perform better at running a timed 10 km trial at 9 am or 4 pm when exposed to hot and humid conditions?

The specific aims to be tested are:

1) Diurnal rhythms of the immune system will be measured in blood serum (neutrophil, lymphocytes, monocytes, eosinophil, and basophil, DNF, IL6, IL10, CC16 and HSP70); to investigate if time of day effects the immune response to a running time trial in a hot and humid environment.
2) The diurnal variation in airway inflammation in a hot and humid environment, will be measured using biomarkers of the immune system (as listed above) and spirometer test; to investigate if time of day has an effect on the occurrence of airway inflammation during a running trial.
3) To investigate at which time of the day athletes perform better under hot and humid conditions.
4) To investigate the time of the day that mental strength is greatest, (if at all), in athletes running a timed trial in a hot and humid environment.

8. Background of research project *(300 words maximum)*

The daily light and dark cycle governs rhythmic changes in the behaviour and physiology of most species (Atkinson and Reilly 1996). These changes are due to a biological clock, which is located in the brain, in an area called the suprachiasmatic nucleus (Inouye and kawamura, 1979). Studies have found that the internal clock consists of an array of genes and the protein products they encode, which regulate various physiological processes throughout the body (Vitaterna *et al*., 2001).

There are numerous behavioural and biological functions that could influence athletic performance, such as pulmonary function (Spengler and Shea 2000), core body temperature (Waterhouse *et al*., 2005), mood (Boivin *et al*., 1997), reaction time (Wright et *al*., 2002), memory and alertness (Johnson *et al*., 1992), and cognitive functioning (Dijk *et al*., 1992): all of these factors have been shown to exhibit circadian rhythmicity. Moreover, generally peak performance has been found to occur in the early evening, at approximately the peak time of body temperature; with the worst performance found to occur in the morning (Drust *et al*., 2005). Smolensky and Halberg (1977) showed that circadian periodic changes in bronchial motility and responsiveness characterise lung function in clinically healthy participants.

Challenging environmental conditions, including heat, pose particular risks to the health of the athlete. Athletes control on the environment such as cold and heat in which they compete and train is limited. Nonetheless, there is strong evidence that elite athletes in different types of sport express an increased risk of airway epithelium injury (Kippelen et al., 2012). However, this risk varies across sports depending on the environment conditions (Kippelen et al., 2012).

With the Olympic Games to be held in Rio de Janeiro in 2016, where mean temperatures are approximately 28°C, this study will investigate the risk of airway epithelium injury and both fatigue and diurnal variation during prolonged exercise in hot conditions, as often endurance can be impaired in hot conditions compared with temperate climates (Febbraio et al., 1994). Many athletes, particularly those based at higher latitudes will not

be accustomed to the hot and humid conditions of Rio de Janeiro; this research will highlight the effect, (if any), of these two factors on running performance.

9. Brief outline of project and study method *(approx 500 words)*

Participants will be required to go through a familiarisation trial where they will perform a $\dot{V}O_2$max test (Gomes *et al*., 2010). Selection criteria will be set at a $\dot{V}O_2$max of 70ml/kg/min to ensure that the participants are a homogenous group in terms of aerobic fitness and hence this will not be a confounding variable. During the $\dot{V}O_2$max test participants run on the treadmill (Woodway, ergo ELG55, Germany) at a speed of $10km \cdot h^{-1}$and 0% gradient. Treadmill speed will increase by $3km \cdot h^{-1}$ every 3 minutes until achieving a maximum speed of $19km \cdot h^{-1}$, after running for 3 minutes at this speed, the gradient of the treadmill will increase by 2.5% every one minute until the runners reached volitional fatigue. Oxygen uptake (VO_2) will be measured using online gas analysis (CPX MedGraphics, Oldham, UK).

<u>Research protocol</u>

The exercise will consist of an 10 km time trial run at 2 different times of the day (9am and 4pm) on a treadmill (Woodway, ergo ELG55, Germany). The trials will be performed in an environmental chamber (Weis-Gallenkamp, UK) where the temperature and humidity will be controlled at 28°C and 70%, respectively. Participants will be required to complete the trial as fast as they are able. For safety reasons core temperature will be measured using an ingestible telemetric temperature sensor (hereafter referred to as "the sensor pill" THermodot, USA); every 1 km ran and then 1h after the completion of the test.

During the time trial, subjective ratings of perceived exertion – RPE – on a scale of 6-20 (Borg, 1998) and heart rate (Polar Electro, Finland) will be recorded at the end of each 1 km run. In addition, the athlete's running speed will be recorded every 1 km; participants will have free control of the speed at which they run but without having access to the

value of the speed. An assessment of Respiratory Symptoms questionnaire will be completed by the athletes on cessation of the exercise protocol (Gomes *et al*., 2010).

Lung Function Tests.

Lung function of each participant will be measured pre- and post- each 10km time trial. This test will use the previously published procedure of *Gomes et al*., (2010). In a standing position the participants will inhale filling their lungs maximally and then blow into the mouthpiece of the spirometer (Compact II: Type C, Vitalograph Ltd., UK) as hard and as long as they can until all air is expired. Forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), forced expiratory flow in the middle half of expiration (FEF_{25-75}) and peak expiratory flow (PEF) will be measured. The participants will perform the test three times and the best values will be recorded.

Blood Sample Collection

A trained phlebotomist (the researcher obtained certification on the 17 November 2011 from GNSC) will collect a total of 18 ml of whole blood samples, 6 ml will be collected at each stage (pre-, post- and 1hour post – exercise), in vacuum tubes containing EDTA or sodium heparin as an anticoagulant (Becton-Dickinson, Oxford, UK). Blood will be collected by venepuncture from the anticubital vein. The tubes will be centrifuged for 10 min at 1000g at room temperature (Mistral 2000R, Sanyo, Leicester, UK). The plasma will be removed, aliquoted into eppendorfs and immediately stored at -80°C until further analysis. Blood sample collection will be carried out by trained individuals.

Nasal Lavage Proceedure

A nasal lavage procedure will be carried out on each participant pre- and post- the 10km time trial following the procedures detailed in Gomes et al. (2010). It will follow the lung function tests. The Participant will sit with their head tilted backwards. Participants will be asked to elevate the palate to close the nasopharynx; 4 ml of sterile pre-warmed (37°C)

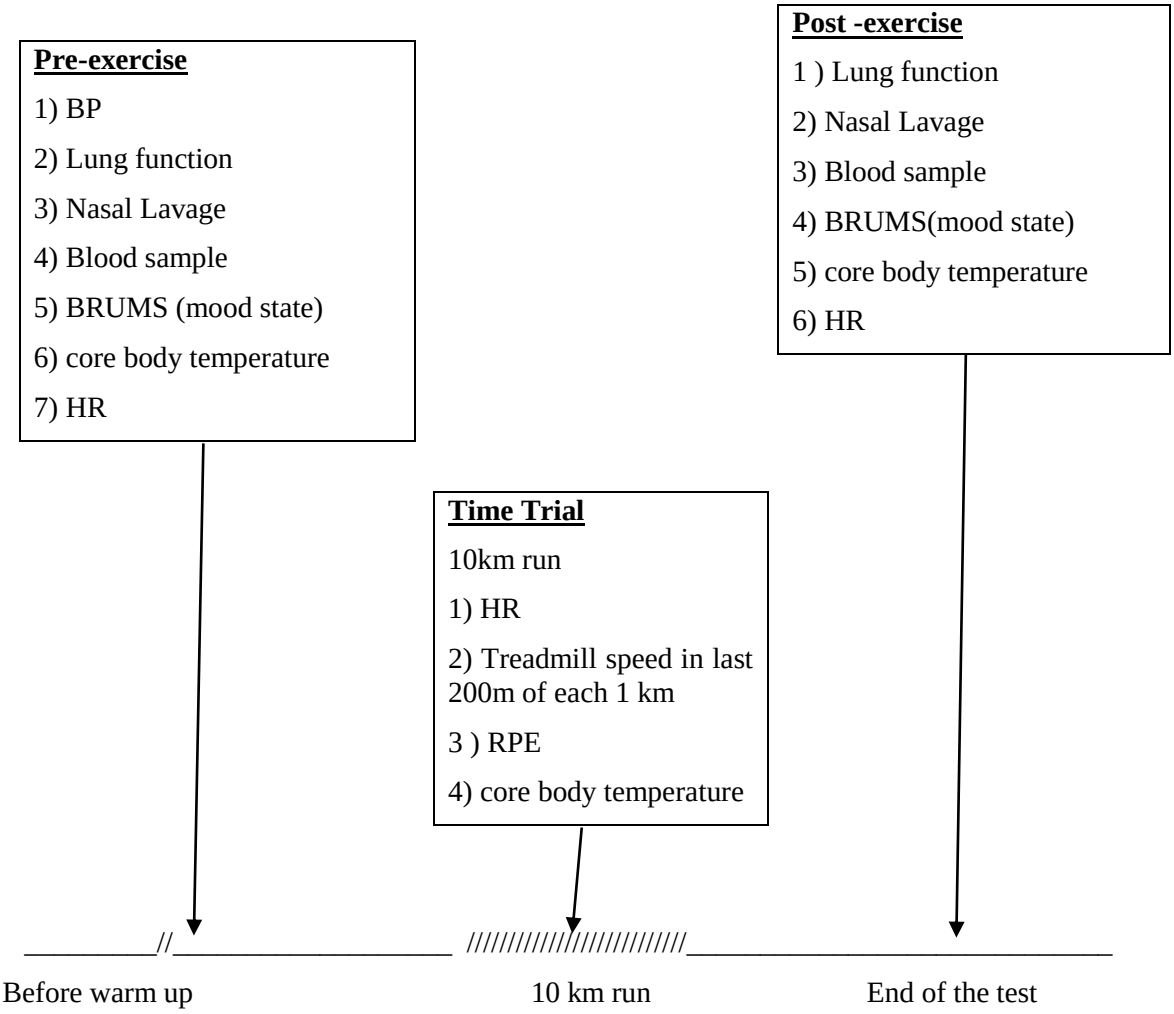
saline will then be inserted into one nostril with a 10ml sterile pipette. After 10 seconds has elapsed the participant will put his head forward and expire the lavage into a 15ml centrifuge tube via a polyamide gauze-filtered funnel (100 meshes) to separate the mucus. This procedure will be repeated for the other nostril. Total volume collected will be recorded and the tube will immediately be placed on ice. Samples will be centrifuged at 600g for 10 min (Mistral 2000R, Sanyo, Leicester, UK) at a temperature of 4°C. The supernatant will be aliquoted into eppendorfs (400µl) and immediately stored at -80°C until further analysis (Gomes *et al*., 2010).

The remaining cell pallet (200µl) will be re-suspended with 100µl of saline. In an eppendorf tube, 25µl of the nasal lavage suspension will be mixed with 10µl of trypan blue. From this mixture 20µl will be placed into a Neubauer haemocytometer (Assistant, Germany) and the cell count will be performed immediately with the microscope set at x10 magnification. On each side of the haemocytometer, four 1 mm^2 squares will be counted and then averaged. The amount found will be multiplied by 1 x 10^4 to determine the number of cells per ml. From the remaining re-suspended volume, 150µl will be used for the cytospin (Cytospin3, Shandon, England), 1000 rpm for 5 min. After this procedure, the slide will be left to dry and then stained with Romanowsky stain (Raymond A. Lamb, London, UK). The cells will be counted and differentiated according to their morphological appearance. This procedure will be conducted in a blinded manner to eliminate the possibility of examiner bias. The nasal lavage sampling technique and the processing of the samples will be performed in a consistent and tightly regulated manner to ensure reliability of the results. The disposal of nasal lavage fluid and blood will be according to human tissue authority guidelines.

Psychological effect (BRUMS, mood states):

Mood will be assessed pre-, post- and 1h post- the 10km time trial using the 24-item Brunel Mood scale (BRUMS), (Terry *et al*., 1999). The Brunel Mood Scale assesses anger, confusion, depression, fatigue, tension, and vigour. Items are rated on a 5-point scale anchored by 0 ("not at all") to 4 ("extremely").

Each blood sample at each stage of the testing was labelled with a different colour. The health and safety of this study is in accordance with the Helsinki declaration. Nasal lavage and blood taking from participants are classified as hazardous substances and should be treated as clinical or infectious wastage; the disposal of this human biological tissue will be in accordance to Edinburgh Napier clinical wastage guidelines. The blood and nasal lavage samples will be kept in the freezer at -80°C until analysis and will be destroyed on completion of the research study according to Edinburgh Napier University's disposal guidelines. Only the researcher and supervisor will have access to the data which will be held for the purpose of this research according to Edinburgh Napier University's data storage guidelines.



Note: Blood pressure (BP) needs to be checked before the test starts to make sure that subjects are in good health during the test day.

Figure 1. Timeline showing the protocol of tests.