DIURNAL PREFERENCE AND SPORTS PERFORMANCE: A SUBJECTIVE AND GENETIC VIEW

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PLAGIARISM DECLARATION

I, **Lovemore Kunorozva**, hereby declare that the work on which this Dissertation/Thesis is based on is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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Date: 30 November 2011

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TERMS AND DEFINITIONS

ANT	Attention network test
BMAL1	Brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1
BMI	Body mass index
bp	Base pair
bpm	Beat per minute
CBT	Core body temperature
cDNA	Complementary deoxyribonucleic acid
CLOCK	Circadian locomoter output cycles kaput
cm	Centimetre
CON	Control group
Cry	Cryptochrome
CYC	Cyclist group
DET	Definite evening-type
dEVE	Designated evening-type group
DMT	Definite morning-type
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSPS	Delayed sleep phase syndrome
EtBr	Ethidium bromide
g	Gram
gDNA	Genomic deoxyribonucleic acid
HÖ-MEQ	Horne-Östberg morningness-eveningness personality questionnaire
HR	Heart rate
HRR	Heart recovery rate
h	Hour
$h \cdot wk^{-1}$	Hours per week
IM	Ironman triathlete group
kg	Kilogram
km	Kilometre
LB	Luria Bertani broth
LSCT	Lamberts and Lambert sub-maximal cycle test
MET	Moderate evening-type
min	Minute
ml	Millilitre
MMT	Moderate morning-type
MORN	Morning-type group
mRNA	Messenger ribonucleic acid
MW	Molecular weight
ng	Nanogram
NT	Neither-type
OBT	Oral body temperature

PER3	Human period 3
PCR	Polymerase chain reaction
PPO	Peak power output
RPE	Rating of perceived exertion
RPM	Revolutions per minute
s	Second
SCN	Suprachiasmatic nuclei
SNP	Single nucleotide polymorphism
UV	Ultra violet
VNTR	Variable number tandem-repeat polymorphism

Note: proteins will be denoted by non-italicised upper-case letters and genes/nucleic acids will be denoted by italics in this thesis.

ABSTRACT

Background: Circadian rhythms are endogenously driven biological variations that oscillate with a period near 24 hours. These rhythms play a fundamental role in sports in terms of optimising training time, performance, adjustment to global time zone changes and scheduling time of events. A link between an individual's diurnal phenotype and *Period3* (*PER3*) variable number tandem-repeat (VNTR) polymorphism has recently been demonstrated. Specifically, the longer (*PER3*^{5}) allele and the shorter (*PER3*^{4}) allele have been associated with morningness and eveningness, respectively. The distribution of this polymorphism in combination with an individual's preference for mornings or evenings has not yet been described in athletic populations. **Aim**: Therefore, the purpose of this study was to describe the distribution of morning- or evening-preferring individuals (measured using the Horne-Östberg morningness-eveningness personality questionnaire) and *PER3* VNTR polymorphism (from genomic DNA products extracted from human buccal cell samples amplified and digested with *Nco*I) within male Caucasian, trained cyclists (CYC, n=138), Ironman triathletes (IM, n=301) and an active, but non-competitive control population of Caucasian males (CON, n=120). In addition, performance was assessed in trained cyclists strongly preferring mornings or evenings at various times of day. **Methods**: Twenty morning-type (MORN) and four evening-type (dEVE) cyclists each completed five laboratory-based Lamberts and Lambert Sub-maximal Cycle Tests (LSCT) at 06h00, 10h00, 14h00, 18h00 and 22h00 in a randomised order. Outcome variables included rating of perceived exertion (RPE), power output, speed, cadence and heart rate recovery, and predicted 40 km time trial time, peak power output and VO_2max were also determined. In addition, oral body temperature of each cyclist was measured during the 24 h period prior to each testing session and the attention network test was performed prior to each LSCT. **Results**: The athletic populations contained significantly more individuals preferring mornings (CYC: 78.9% and IM: 64.7%) than the control group (CON: 41.5%, $p<0.001$). The athletic populations contained significantly more individuals homozygous for the *PER3*$^{5/5}$ allele (CYC: 39.0% and IM: 29.0%) than the control group (CON: 11.0%, $p<0.001$). Furthermore, the prevalence of the longer *PER3*^{5} allele was greater in the athlete groups (CYC: 59.9% and IM: 57.0%) than the control group (CON: 39.0%, $p<0.001$). A significant positive correlation ($p<0.001$, $r=0.659$) between diurnal preference and genotype was observed when all data were pooled together. When cycling at a fixed intensity (i.e. same relative heart rate) morning-types had a lower perception of effort in the morning, while the reverse was true for the evening-type cyclists

($p<0.001$). All other measured variables did not show time-of-day changes and were similar between the two groups. **Conclusion**: South African Caucasian males participating in individual sports have a higher proportion of morning-types based on both genotype and questionnaire data than reported in most other populations world-wide. Results from this study suggest that understanding the relationship between diurnal phenotype and exercise may lead to a better understanding of inter-individual athletic performance and participation in individual sports.

CHAPTER 1
INTRODUCTION

The study of human performance dates back to the ancient Greek times (Brock, 1929, Bowers, 1998), and there has been an increase in interest on issues related to human athletic performance since then. Circadian (Latin: *Circa*= about, *dies*= a day) rhythms research in humans has been under study since the late 1800s (Dijk and von Schantz, 2005, Fuchs and Burgdorf, 2008, Patrick and Gilbert, 1896) and the majority of the research has been focussed on individuals with physiological and psychological disorders (Benedetti et al., 2003, Tafti et al., 2007, Toh et al., 2001), those travelling across time zones (Smith et al., 1997, Waterhouse et al., 2007) and shift workers (Manfredini et al., 1998, Winget et al., 1985, LaDou, 1982). However, studies on human circadian rhythms and exercise only dates back to the 1960s (Klein et al., 1968). The influence of circadian rhythms on human athletic performance has potential implications on both an individual's training efficacy and competition performance, as most of the physiological, neuromuscular and cognitive variables that influence performance have been shown to display a sine wave-like circadian change in relation to the 24 hour day (Baxter and Reilly, 1983, Reilly and Baxter, 1983, Reilly et al., 2007).

Core body temperature (CBT) varies in a circadian manner, peaking in the late afternoon to early evening and reaching the lowest point during sleep, early in the morning (Kerkhof and Van Dongen, 1996, Weinert and Waterhouse, 2007). Furthermore, the circadian rhythms of many of the physiological and neuromuscular markers of performance, for example, muscle strength, peak power output and reaction time, have been shown to follow daily changes in CBT, thus peaking later in the day. Of interest, however, is that there seems to be significant inter-individual differences in the timing of the peaks and troughs of these variables. An individual's diurnal preference (i.e. preference for morning- or evening-activity) may account for some of this variation, although other factors such as an individual's genetic makeup and external factors may also play a role, since diurnal preference may strongly influence an athlete's performance and the efficacy of his/her training. The large inter-individual variation in the circadian timing observed in factors which contribute to performance highlights the need to study diurnal preference in athletes, since diurnal preference may strongly influence the efficacy of training and sports performance. This is particularly important today when: (a) athletes are required to travel across many time zones for matches and competitions, (b)

athletic events are being scheduled outside the traditional times, to accommodate television broadcasting of events, and (c) professional athletes are required to do multiple training sessions per day. I will begin by discussing what circadian rhythms are and how they are generated. I will then discuss the genetic and external factors influencing circadian rhythms and the resultant individual diurnal preference. Finally, I will discuss the subsequent effects of diurnal preference on athletic performance.

1.1. The circadian system

Circadian rhythms are endogenously (internally) driven fluctuations in biological, physiological and behavioural variables that have a period of about 24 hours (Merrow and Brunner, 2011). Importantly, they can be entrained (synchronised) with the temporal environment by photic cues (i.e. light-dark cycles and seasonal changes in day length, also known as photoperiod) and non-photic cues (i.e. physical activity, temperature, sound (Lowrey and Takahashi, 2000, Menaker and Eskin, 1966) and food (Benca et al., 2009). All circadian rhythms are self-sustaining with an intrinsic period length close to 24 h, indicating the presence of an endogenous time measuring system (Hirota and Fukada, 2004). Furthermore, these rhythms persist with a period of about 24 h in the absence of environmental cues (i.e. free running), for example, under conditions of constant dark and temperature. The endogenous nature of mammalian circadian rhythms was demonstrated *in vitro* by the continual generation of neuronal rhythms in cultured rat suprachiasmatic nuclei (SCN) cells that were capable of generating circadian activity up to seven weeks after leaving the body in one of the experiments on the role of the SCN (Welsh et al., 1995). Given that the period of circadian rhythms is not exactly 24 h, entrainment has a vital role in making certain that daily biological rhythms stay in synchronization with the external temporal environment and in this manner, a daily internal temporal order is realized.

There is circadian regulation of physiology, metabolism and behaviour in both prokaryotes and eukaryotes, ranging from cyanobacteria to humans (Merrow and Brunner, 2011). The circadian system consists of three general parts: (1) the input pathway, which transmits environmental signals to (2) the "clock", which in turn generates rhythms of the (3) outputs in each individual cell. In mammals, the "central clock" is located in the SCN, a small bilateral pair of nuclei in the anterior hypothalamus of the mammalian brain (Benloucif et al., 2005). Neural transplantation studies by Ralph et al. (1990) demonstrated that lesions in the SCN

resulted in the abolishment of many circadian rhythms in *tau* hamsters further supporting the evidence that the SCN is the "central" clock. It is only found in mammals and only plays a role in the light entrainment pathway, but output rhythms from the SCN are fundamental to signaling molecules and hormones too. Furthermore, entrainment of the peripheral oscillators for example, the heart and the liver (Figure 1) by food and temperature cues is independent of the light entrainment pathway of the SCN and will only affect cells in those organs. The translation of environmental time signals mainly the light-dark cycles to the SCN, via the retino-hypothalamic tract, and transduction into neural and molecular events is crucial for integrated functioning of the circadian system.

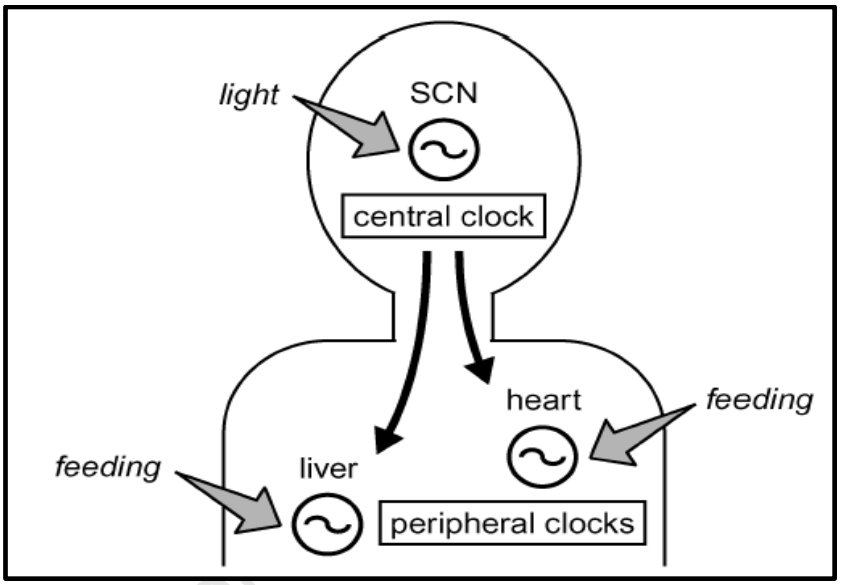

Figure 1: A simplified conceptual framework of the mammalian circadian system showing external stimuli that entrain the central and peripheral clocks. The central clock is entrained mainly through light cues and the peripheral clocks can be affected by food and temperature signals, independent of the light entrainment pathway of the suprachiasmatic nuclei (SCN).
Reproduced from Hirota and Fukada (2004)

The circadian system is chiefly responsible for the phasing (timing) of different physiological events, such that many of the processes occur at specific times of the day (Merrow and Brunner, 2011, Vitaterna et al., 2001). A functional circadian system is important to organisms because it enables them to expect and prepare for cyclic changes associated with day and night in the temporal environment (Merrow et al., 2005). For example, using competition experiments in cyanobacteria, Johnson and Golden (1999) demonstrated that the fitness of cyanobacteria was enhanced when the internal circadian period was matched to that of the external environment. When mixed mutant strains of cyanobacteria (C22a and C28a),

which had point mutations generated by mutagenesis in the *kaiC* gene and free running periods of 23 h and 30 h, respectively, were grown together under differing light-dark conditions, the strain whose period most closely matched that of the external light-dark cycle eliminated the competitor (Ouyang et al., 1998). The adaptive advantage of a circadian oscillation in a 24 h environment has also been demonstrated in plants and chipmunks (Green et al., 2002, DeCoursey et al., 2000, Dodd et al., 2005). Arabidopsis plants over expressing the *CIRCADIAN CLOCK ASSOCIATED 1* gene are arrhythmic with respect to circadian outputs and have lost the ability to expect light-dark changes in their immediate environment. These arrhythmic plants flower later, have less chlorophyll and lower seed count compared to the wild-type plants under long day conditions (Dodd et al., 2005). While in chipmunks living in the wild, a significant number of those that had lesions in their SCN were decimated by weasel predation compared to the intact controls in the first 80 days of the experiment. The SCN-lesioned chipmunks showed no surface activity at night and therefore stayed in their caves where they were easily preyed upon by weasels (Frascina, 2004, DeCoursey et al., 2000). The importance of a functional circadian clock within living organisms is reflected in the abovementioned examples.

1.1.1 Molecular components of the circadian clock

The mammalian circadian clock model has been based mostly on what has been studied in mice. However, since many of the clock genes are conserved across species, the mechanisms (coordination of circadian timing) that bring about circadian rhythmicity in mice are likely to play an important role in regulating human circadian rhythms too. The molecular clockwork of rhythm generation consists of interacting positive and negative autoregulatory transcription-translation feedback loops involving central oscillator genes and proteins (Takahashi et al., 2008). The positive and negative components of the feedback loop have been shown to act through a series of delays, introduced at critical points during the 24 h day (Lowrey and Takahashi, 2000, Benloucif et al., 2005). This ensures regulation of transcriptional activities at appropriate times of the day in order to produce a circadian rhythm. The positive components are two basic helix-loop-helix (bHLH) PAS-domain containing transcription factors: circadian locomoter output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) (Takahashi et al., 2008). During the subjective day, CLOCK interacts with BMAL1 to effect transcription of three Period genes (designated *PER1*, *PER2* and *PER3* in humans), two cryptochrome genes (*hCry1* and *hCry2*) and the orphan nuclear receptor *Rev-erbα* gene (Figure 2), resulting in high transcript levels at

midday. The mouse mCRY and mPER proteins in the SCN exhibit a robust 24 h rhythm of expression with peak levels in the early subjective night (Lowrey et al., 2000). Following a delay imposed by transcription, translation, post-translational modifications, and dimerization, the resulting mPERs and mCRYs heterodimerize and translocate to the nucleus as a heteromultimeric complex. In the nucleus, the mPERs and mCRYs interact with the CLOCK-BMAL1 complex to inhibit the CLOCK-BMAL1 mediated transcription of their own genes (Lee et al., 2001, Lowrey and Takahashi, 2000). Gradually, the transcriptional inhibition is relieved by protein turnover of the mPER-mCRY repressor complex, such that the CLOCK-BMAL1 complex can again initiate a new cycle of transcription (Hirota and Fukada, 2004, Takahashi et al., 2008). The entire cycle takes about 24 h to complete; however the exact stoichiometry and kinetics involved in this autoregulatory feedback loop are unclear.

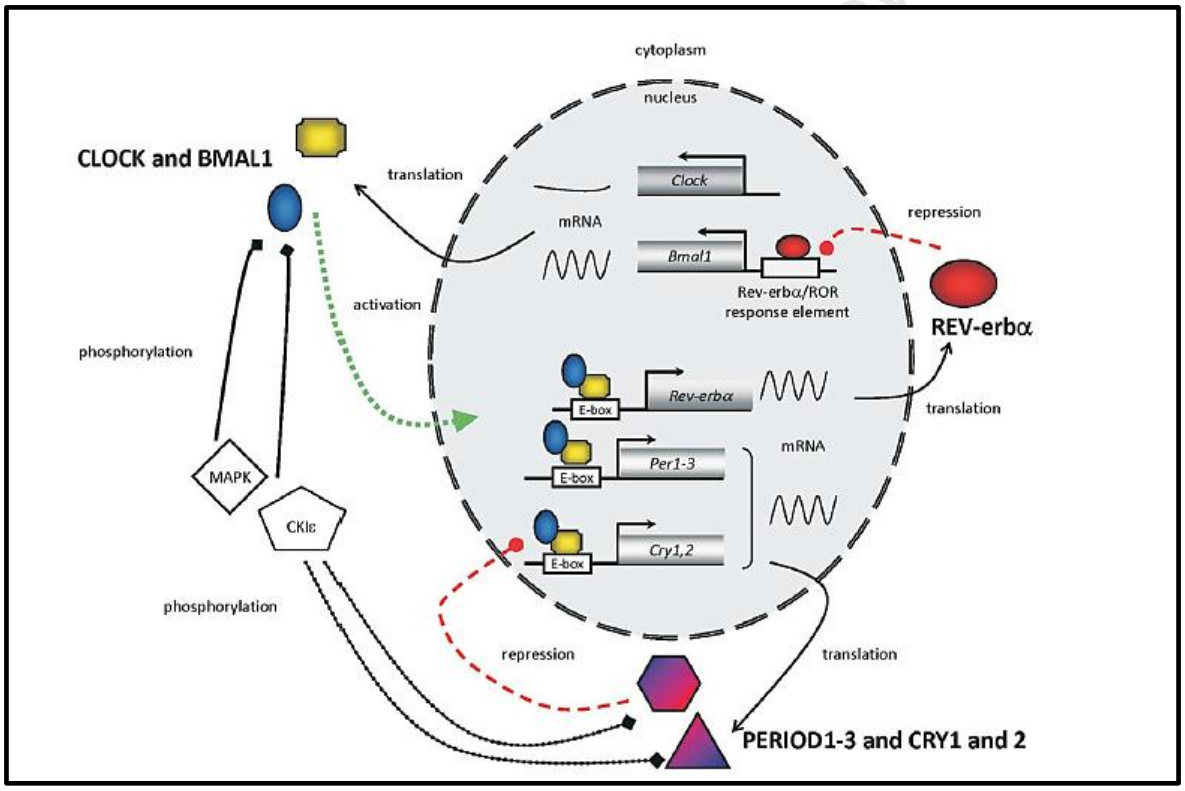

Figure 2: A schematic outline of the molecular clockwork regulating circadian rhythms in mammals. CLOCK and BMAL1 heterodimerize and bind to the E-box promoter elements to activate the transcription of *Per1-3*, *Cry1*, *Cry2* and the *Rev-erbα* gene that encode the negative elements of the loop. The PER and CRY proteins heterodimerize and negatively regulate their own transcription and that of *Rev-erbα*. REV-ERBα represses the transcription of *Bmal1* by binding to REV-ERBα/ROR response element in its promoter. Post-translational modification of clock factors, for example, phosphorylation and sub-cellular localisation play fundamental roles in the regulation of the feedback-loop. CKIε: casein kinase Iε; MAPK: mitogen-activated protein kinase.
Reproduced from Beckett and Roden (2009)

Given the precision of the circadian clock, it is evident that a disruption in any one of the delay mechanisms or clock genes could result in altered circadian rhythms. Gallego and Virshup (2007) demonstrated that post-translational modification and degradation of clock proteins are vital steps towards determining circadian periodicity of the body clock. Using positional syntenic cloning of mutations resulting in period changes in Syrian hamsters, Lowrey and Takahashi (2000) identified the casein kinase 1ε gene. Casein kinase 1ε and the mitogen-activated protein kinase have been shown to play a vital role in phosphorylating the PER, CRY, CLOCK and BMAL1 proteins in mammals, regulating their stability and/or function, nuclear localisation, and degradation (Akashi et al., 2008). Phosphorylation targets clock proteins for polyubiquitination and hence, promotes degradation by the 26S proteosomal pathway.

1.1.2 Entrainment of the circadian clock

The process of entrainment is an important aspect of circadian rhythmicity, whereby the phase and period of the body clock are synchronised to the phase and period of the external temporal environment by zeitgebers (from the German, "time giver") (Hirota and Fukada, 2004, Menaker and Eskin, 1966, Roenneberg et al., 2003a). There are numerous zeitgebers that can entrain or reset the circadian clock in mammals. However, light and temperature cues are the most potent entraining stimuli for many organisms including humans; although other factors such as feeding time, hormone levels (e.g. melatonin) and physical activity exist (Ciarleglio et al., 2008, Baehr et al., 1999). Circadian rhythms frequently take the form of sinusoidal waves (Figure 3) that can be described in terms of period, phase and amplitude.

The universal importance of light and temperature cues reflects the evolutionary significance of solar energy to life on earth. The range of entrainment largely depends on: (1) the free running period of the oscillator (Tau or τ); (2) photoperiod; (3) the strength of the zeitgeber and (4) the amplitude of the zeitgeber (Roenneberg et al., 2003a). The underlying molecular mechanisms of the body clock display an exceptional amount of evolutionary conservation in organisms as diverse as cyanobacteria, plants and mammals (King and Takahashi, 2000). However, the individual molecular components of these clocks vary among different organisms. In mammals, the circadian photoreceptor responsible for light entrainment of the circadian clock is located within the retina of the eye (Hirota and Fukada, 2004).

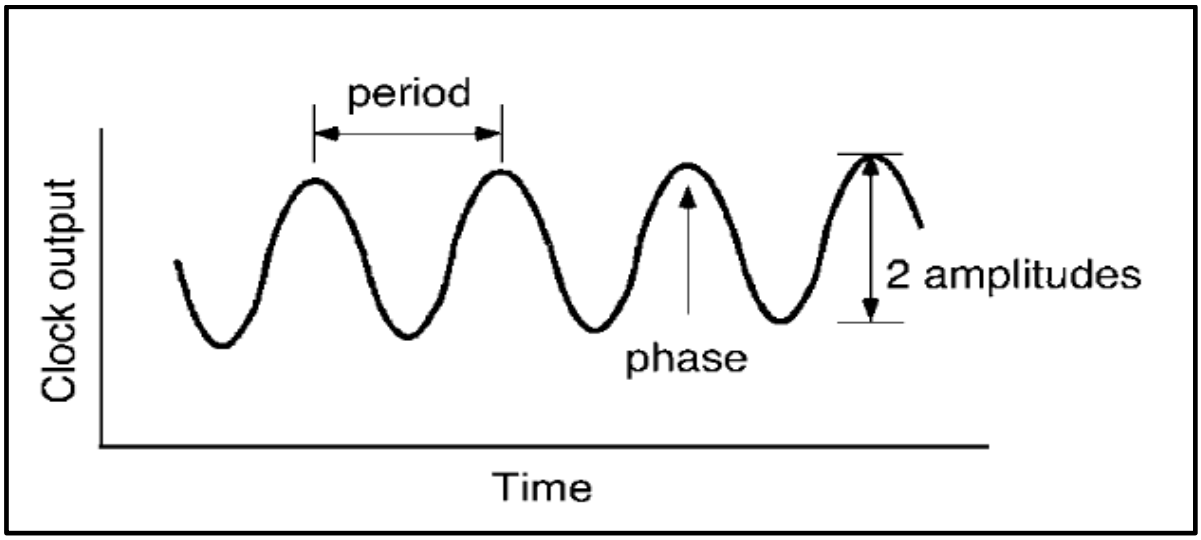

Figure 3: Sinusoidal waves showing critical terms used to define circadian rhythms. Period: the time during which physiological processes complete one circadian cycle. Phase: a stage (time-of-day) in the circadian cycle relative to a marker, and Amplitude: one-half the distances from peak to trough during one period of an oscillation.

Reproduced from McClung (2006)

In humans, the visual system functions best under day light or adequate artificial light (~1000 lux), which is why our vision is impaired in the dark or under poor lighting conditions. Melanopsin, retinal ganglion cells projecting to the SCN (i.e. the "body clock"), via the retino-hypothalamic tract, play a vital role in relaying light information from the retina to the SCN (Lowrey and Takahashi, 2000, Roenneberg et al., 2007). The neural outputs (~20 000 neurons) from the SCN ultimately drive oscillations in all other biological and behavioural rhythms. As the site of light input in mammals, the retina is vital for image-forming and non-image-forming tasks (Provencio et al., 2002). The pathway relevant to light-entrainment relies chiefly on information regarding the irradiance and duration of exposure of the light signal. Depending on when in the 24 h cycle the light signal is presented, it will phase advance, phase delay or result in no shift at all in circadian rhythm. Light perception by the melanopsin results in chromatin remodelling (Crosio et al., 2000) and the induction of various genes (Roenneberg and Merrow, 2005, Cermakian et al., 2003), leading to the transcription of central clock genes. Since the same clock genes are expressed in the SCN and retina as in the cells of the rest of the body, it is easy to observe the circadian oscillation at the transcription level of these genes.

1.1.3 Disruption of the circadian clock

1.1.3.1 Transmeridian travel and jetlag

The rapid crossing of several time zones, such as experienced with air travel often results in a condition known as jetlag. This is a physiological condition, which results from the temporary disruption of the body's normal biological rhythms as a consequence of the misalignment between the intrinsic body clock and the environment (Manfredini et al., 1998, Waterhouse et al., 2007, Reilly et al., 1997a). This transient desynchronisation of physiological and behavioural rhythms lasts until the body clock rhythms are adjusted to the new environmental conditions. The effects of this shift on the body clock are widespread, ranging from sleep disorders, troubles with concentration, loss of appetite to digestive disturbances (Reilly et al., 2009). For example, one study has shown that sleep disturbances occur in 60-70% of cases after the first night of crossing a time zone, which is equivalent to the loss of 3 h of sleep (Manfredini et al., 1998). The time required for an individual to recover from the effects of jetlag varies and depends on the number of time zones crossed; thus the more time zones crossed the longer it will take for an individual to recover (Reilly et al., 2005, Reilly and Edwards, 2007). It is thus easy to see that the effects of temporal misalignment mentioned above can have significant behavioural and performance related consequences for sports people.

1.1.3.2 Other factors that disrupt the circadian clock

Numerous other factors result in the disruption of the circadian clock and these include: shift work, melatonin secretion, medications and CBT, just to name a few. I will focus mainly on CBT and melatonin secretion in this section, as these act as internal zeitgebers.

1.1.4 The circadian rhythms of melatonin and core body temperature

There is a wide range of human physiological, behavioural and endocrine variables that exhibit distinct 24 h rhythms (Scheer and Czeisler, 2005). Melatonin and CBT are the two most commonly measured markers of the circadian phase because of their strong internal presence. They act as internal zeitgebers for mammals, including humans, entraining various circadian functions particularly in the peripheral oscillators (Weinert and Waterhouse, 2007, Taillard et al., 2003). Melatonin is a hormone derived from the neurotransmitter serotonin, and is produced by the pineal gland. Melatonin levels in the blood are highest at night and are very low during the day, so that its primary role appears to be the entrainment of the sleep-wake cycle. In mammals, including humans, body temperature is controlled within narrow

limits by an intricate feedback system, mainly due to rhythmic output from the SCN (Weinert, 2010). This is realized by complex homeostatic mechanisms of heat generation and heat loss when temperature starts to fall and rise, respectively. In addition, melatonin onset at dusk appears to cue the rise in blood flow in distal skin regions leading to heat loss. The 24 h fluctuations in CBT (Figure 4) also appear to be involved in the regulation of the circadian sleep-wake cycle (Van Someren et al., 2002, Sinnerton and Reilly, 1992). An individual usually falls asleep when CBT is decreasing, and awakens on the rising part of the circadian temperature curve. The rise in CBT in the early morning, largely due to the circadian clock, is amplified by the change from sleep to waking activity (Cagnacci et al., 1997).

In the evening, the clock-mediated drop in body temperature is highlighted by the reduction in activity and change in posture on retiring to bed and most importantly the onset of melatonin secretion (Weinert and Waterhouse, 2007, Aizawa and Cabanac, 2002). Many human physiological variables display 24 h rhythms closely in phase with the variation in CBT. For example, the anaerobic threshold, strength, and power output appear to peak later in the day coinciding with the peak in CBT. The notable exception is the peak in mental performance, which may occur earlier than the peak in CBT. One explanation for this is that mental performance may be strongly influenced by other factors such as the neuro-endocrine system (Wright et al., 2002)

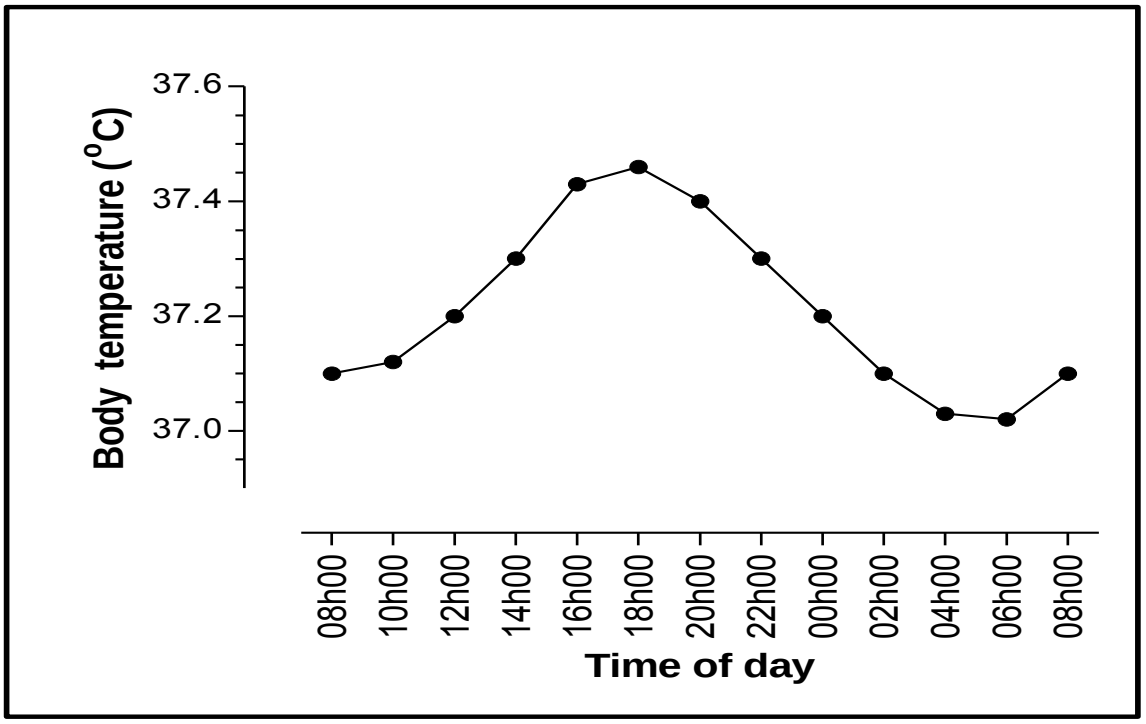

Figure 4: A schematic diagram showing diurnal variation in human body temperature. The peak in body temperature occurs between 17h00-20h00, while the nadir or lowest point occurs between 04h00-06h00 when individuals are sleeping.

1.1.5 Diurnal preference

There are considerable inter-individual differences in the temporal organisation of physiological and behavioural rhythms (Roenneberg and Merrow, 2007, Roenneberg et al., 2007, Dijk and Archer, 2010). These differences have a genetic basis and are mostly due to polymorphisms in the central clock genes (Randler, 2008b, Duffy et al., 2001). Alternatively, they may be due to differences in entrainment of the body clock, brought about by differences in conditioning or differences in exposure to phase-resetting stimuli, such as the light-dark cycle (Archer et al., 2008). Diurnal preference can be considered as one of the most striking individual differences in circadian rhythms. Individuals are characterized along a spectrum of preference for mornings or evenings, ranging from extreme morning-types (also called "larks''), who typically go to bed and awake early, to extreme evening-types (also called ''owls''), who have later bedtime and wake-up times (Natale and Cicogna, 2002). A number of studies have reported varying frequencies of morning- and evening-type individuals. For example, Paine et al. (2006) reported differing proportions of evening-types (6%), neither-types (45%), and morning-types (49%) in a Caucasian population, while the majority of studies conducted to date report more individuals in the neither-type category and very few in

the extremes. The middle of the spectrum encompasses the intermediate or neither-type individuals. The greater part (~60-70%) of the populations tested so far has shown patterns of behaviour belonging to this intermediate area between the two extremes of the spectrum (Tonetti et al., 2010, Racy, 1969, Chelminski et al., 1997, Zavada et al., 2005). Several lines of evidence suggest that diurnal preference is directly controlled by the internal body clock, as indicated by differences in the timing of circadian rhythms and the internal free-running period between diurnal phenotypes (Archer et al., 2008, Baehr et al., 2000, Groeger et al., 2008). Diurnal preference is determined subjectively using the Horne-Östberg morningness-eveningness personality questionnaire (HÖ-MEQ), which is a 19-item questionnaire used to categorize individuals into different diurnal phenotypes (Horne and Östberg, 1976). The scoring scale ranges from 16 to 86 with lower scores indicating a greater preference for evenings, and higher scores indicating a greater preference for mornings (see Appendix A3). There is some evidence to suggest that diurnal preference is influenced by developmental changes and ageing (Bliwise et al., 2005, Adan and Natale, 2002).

A preference for evenings is more prevalent in adolescents and young adults, whereas morning-types are more common in young children and older adults (Koskenvuo et al., 2007, Roenneberg et al., 2004). The change to morning preference later in adulthood typically begins to be apparent around the age of 50 years (Bliwise et al., 2005, Taillard et al., 2004). Confounding this relationship is the strong synchronising influence of societal demands, in particular work schedules, in modifying the natural diurnal preference (Adan and Natale, 2002). While there are other validated questionnaires available to determine diurnal preference, such as the Munich chronotype questionnaire (Roenneberg et al., 2003b), the HÖ-MEQ which has been translated into many languages (Randler, 2008b, Punduk et al., 2005), remains the most commonly used subjective tool (Adan and Natale, 2002, Zavada et al., 2005). Furthermore, psychometric evaluation of this questionnaire confirms that it is made up of a fairly homogenous set of items with respectable internal consistency and is reliable among non-shift workers (Taillard et al., 2004, Randler, 2008b). The HÖ-MEQ has also been shown to correlate well with numerous mechanisms involved in the regulation and timing of the sleep-wake cycle (Randler, 2008a, Laberge et al., 2000), including the secretion of melatonin (Taillard et al., 2003), providing further validation of its use.

1.1.6 Circadian phase differences between diurnal phenotypes

There are notable circadian phase differences between morning-type and evening-type individuals. For example, the daily rhythms of CBT and melatonin secretion have been used to distinguish the timing of circadian rhythms between individuals preferring mornings compared to those preferring evenings (Scheer and Czeisler, 2005, Weinert and Waterhouse, 2007). Baehr et al. (2000) demonstrated that the HÖ-score consistently illustrates a contrasting relationship with the timing of CBT minima, such that higher scores on the questionnaire are linked with an earlier temperature rhythm (Figure 5). Morning-type individuals exhibit an earlier CBT peak and minima (Taillard et al., 2003, Kerkhof and Van Dongen, 1996), while the CBT of evening-type individuals peaks later (Bailey and Heitkemper, 2001, Baehr et al., 2000).

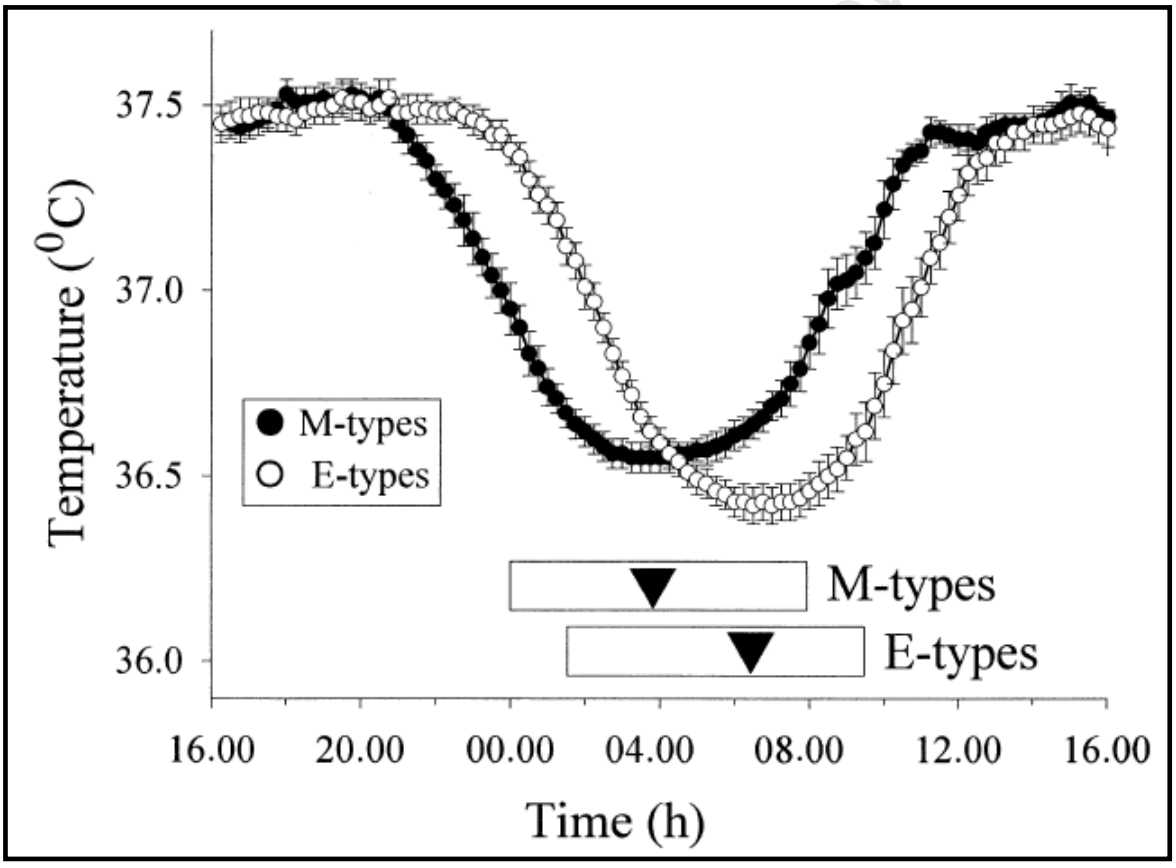

Figure 5: A schematic diagram showing diurnal variation in core body temperature between morning-type and evening-type individuals. Black circle represents morning-type individuals (M-types), and the open circle represents evening-type individuals (E-types). The black and open circles show 15-min averages with standard error bars. Rectangles represent scheduled sleep times while triangles represent minimum temperature averages.

Reproduced from Baehr et al. (2000)

1.1.7 Clock genes and diurnal preference

Polymorphisms in a number of clock genes are associated with mood disorders such as bipolar and depression (McClung, 2007, Toh, 2008), increased and decreased risk for cancer (Wood et al., 2009, Zhu et al., 2005) and sleep disorders, such as familial advanced sleep phase syndrome (FASPS) and delayed sleep phase syndrome (DSPS). Of interest for the purposes of this thesis, are polymorphisms in genes associated with diurnal preference which are summarized in Table 1.

Table 1: Clock gene polymorphisms and their associated phenotypes in humans

Gene	Polymorphism	Associated phenotype	Reference
PER2	A2106G	FASPS	(Carpen et al., 2005, Carpen et al., 2006)
	C111G	Morning preference	(Mishima et al., 2005, Choub et al., 2011)
	G3853A	Morning preference	(Lee et al., 2011, Choub et al., 2011)
PER3	VNTR (4-repeat allele)	Evening preference and DSPS	(Archer et al., 2003, Pereira et al., 2005, Archer et al., 2010)
	VNTR (5-repeat allele)	Morning preference	(Ebisawa et al., 2001)
CLOCK	C3111T	Evening preference	(Mishima et al., 2005, Katzenberg et al., 1998)

DSPS: delayed sleep phase syndrome, FASPS: familial advanced sleep phase syndrome, *PER2*: human Period 2 gene, *PER3*: human Period 3 gene, and VNTR: variable number tandem-repeat polymorphism.

Some researchers have found associations between polymorphisms in the central clock genes and diurnal preference. For example, the C3111T polymorphism of the *CLOCK* gene (Table 1) has been associated with evening preference (Katzenberg et al., 1998, Mishima et al., 2005), and the C111G and G3853A polymorphisms of the *PER2* gene have been associated with extreme morning preference (Lee et al., 2011, Choub et al., 2011). However, there is a lot of controversy surrounding the *CLOCK* C3111T polymorphism as some studies have failed to find a link between this polymorphism and diurnal preference (Robilliard et al., 2002, Pedrazzoli et al., 2007). Possible reasons for the conflicting results in these studies vary and range from, the use of different ethnicities, differences in sample sizes to differences in

latitude. For example, the sample size used in the Japanese population (Mishima et al., 2005) consisted of two small pedigrees, and Katzenberg and co-workers (1998) used participants from different Caucasian ethnic groups (German, British, Scandinavian, Central and Southern European), which may have influenced the outcome of these studies. To date, the *PER3* gene, which plays a fundamental role in resetting the phase of the circadian clock, seems to be the most consistent and highly correlated with diurnal preference. Specifically, the variable number tandem-repeat (VNTR) polymorphism within the coding region of the *PER3* gene has been linked with extreme diurnal preference (Groeger et al., 2008, Archer et al., 2003). This polymorphism is a 54 base pair repeat sequence on exon 18 of the *PER3* gene, which is repeated four (*PER3*^{4}) or five (*PER3*^{5}) times (Figure 6), encoding proteins of different lengths (Ebisawa et al., 2001).

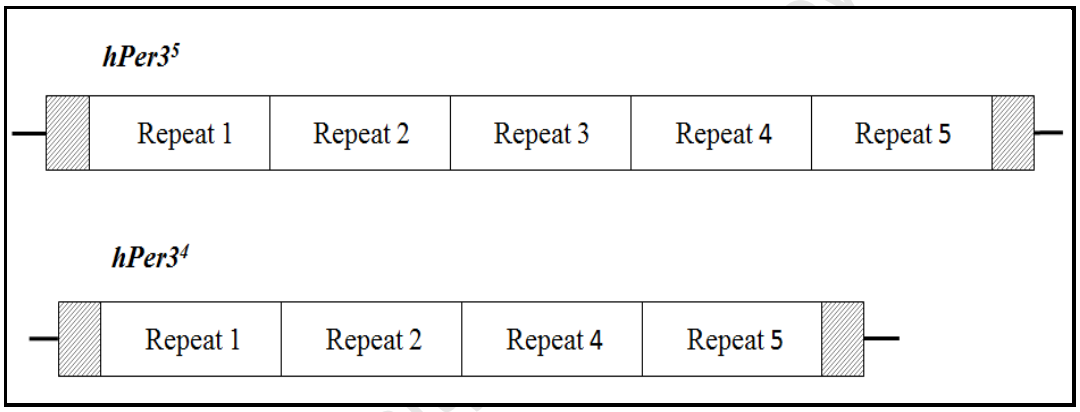

Figure 6: A schematic diagram showing the *PER3* VNTR polymorphism located on exon 18. Grey boxes with diagonal lines indicate the flanking regions. White boxes indicate the 54 base pair (bp) repeats, which are repeated four (*PER3*^{4}) or five (*PER3*^{5}) times, encoding proteins of various lengths.

The longer variant of the gene, *PER3*^{5}, has been associated with morningness in a Caucasian British population, while the shorter variant of the gene, *PER3*^{4}, with eveningness (Archer et al., 2003), a result that was replicated in a Caucasian Brazilian population (Pereira et al., 2005). The global distribution of the *PER3* VNTR polymorphism varies greatly around the world (Ciarleglio et al., 2008, Nadkarni et al., 2005) as indicated by the differences in allele frequencies in Papua New Guinea, Han Chinese, Ghanaians, European American and African American populations in these studies. For example, the frequency of the 4-repeat allele in European American (59%), Han Chinese (81%) and Papua New Guinea (41%) populations varied greatly (Ciarleglio et al., 2008). At first glance one may think that the differences in allele frequencies may be due to differences in environmental parameters of relevance to the

circadian clock (e.g. seasonal fluctuations in photoperiod and temperature) that vary greatly as a function of latitude, however this has been refuted in Nadkarni et al. (2005). The exact functional role of *PER3* in the central clock is still unclear, yet it has this distinct effect on circadian phenotype. The other two Period genes *PER1* and *PER2* have been shown to have a direct effect in maintaining circadian rhythms. A single knockout mutation in the mouse *mPer1* gene was shown to result in shortening of the circadian period length, which in due course resulted in the abolishment of circadian rhythmicity (arrhythmicity) under constant dark conditions (Zheng et al., 2001). Similarly, a single knockout mutation in *mPer2* resulted in a shortened free-running period, rapidly damping to result in arrhythmicity under constant dark conditions (Zheng et al., 1999, Ripperger et al., 2011). The role of the *PER3* gene in the central clock is less clear. Shearman and co-workers (2000) suggested that *PER3* may be involved in buffering other clock components or may even be a central clock gene.

1.2. Sports performance

Sports performance is a complex phenotype, which has been studied since the 1800s (Schneckloth, 2008, Galton, 1979). Performance is multifactorial and is influenced by a number of factors such as genetics, the environment (Baker et al., 2003, Peiser and Reilly, 2004), behaviour, personality, training, nutrition (Maughan, 2002), and a sense of occasion, to name a few. Current knowledge of the relative contributions of genetic and external factors suggests that these interact rather than act independently to affect performance (Tucker and Colins, 2011). However, a substantial percentage of the ability found in sportsmen/women has been attributed to heredity (Reilly and Waterhouse, 2009, Bray, 2000, Plomin et al., 1994). For example, a recent study conducted by Wilmore et al. (2001) demonstrated the link between genetic factors and physical characteristics such as heart rate, blood pressure and aerobic capacity. Thus, the level of improvement is mainly constrained by genetic factors, although other factors such as motivation, self-confidence and other opponents play a role. Lewontin (2000) devised the metaphor "empty bucket" to describe the relative contribution of genetics and environmental factors to athletic performance. They proposed that genetic factors are crucial in determining the capacity of the "bucket" i.e. the athlete, while the environmental factors determine the contents and the extent to which the bucket can be filled i.e. the realisation of athletic potential. Of interest to this thesis are the genetic and environmental factors relating to circadian rhythms and their subsequent effects on athletic performance.

1.3 Sports performance and circadian rhythms

Reilly et al. (2009) proposed that there are three components of circadian rhythms, which may affect performance, namely, the external, internal and lifestyle factors (Figure 7). Examples of external factors include environmental conditions such as ambient temperature and external motivation, while internal factors include the genetics of circadian rhythms and desynchronisation with travelling across time zones and lastly, lifestyle factors include sleep loss and scheduling time of training. An individual may have the potential for being an elite athlete, but if he/she lives a lifestyle of overeating and lacking regular exercising, they may not be able to realize that potential.

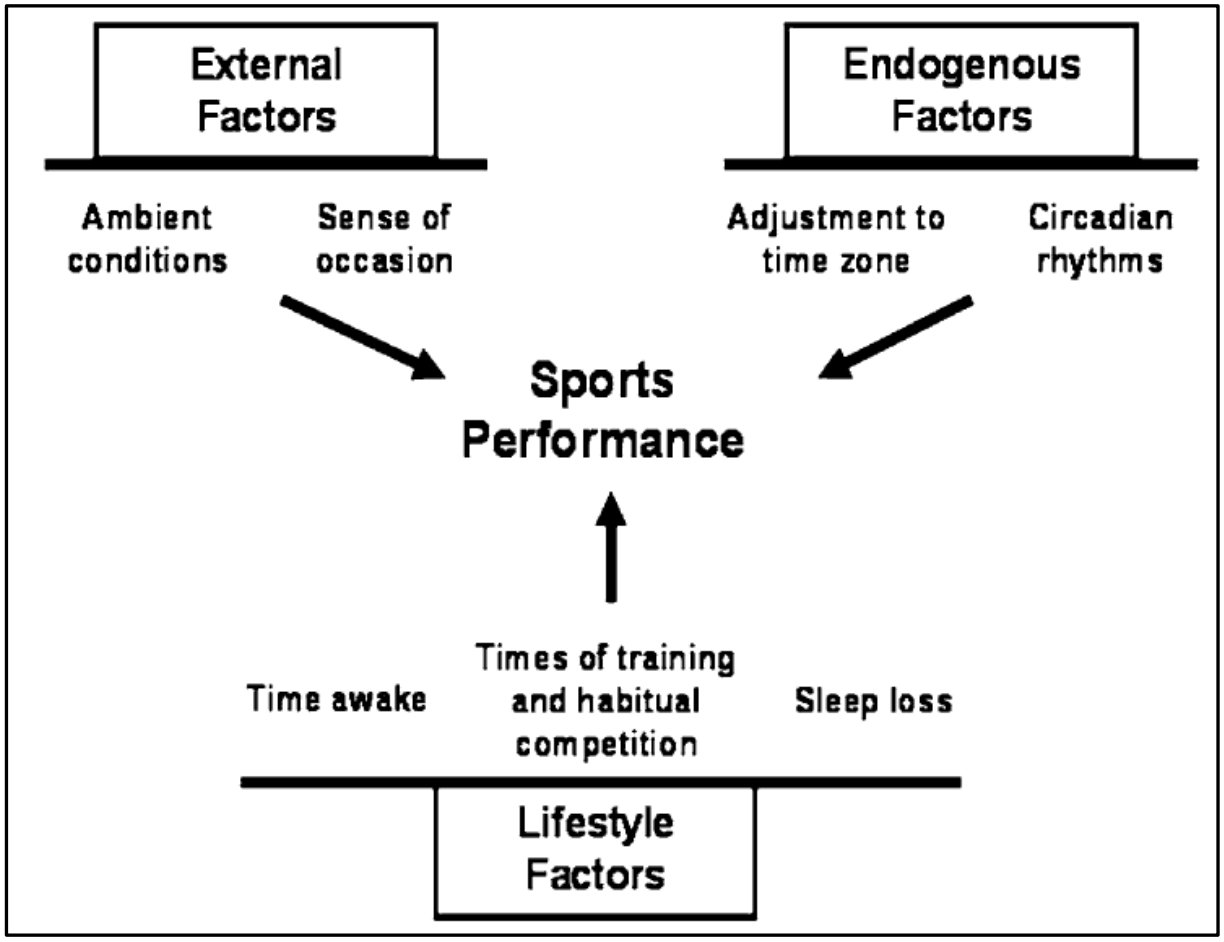

Figure 7: An outline of endogenous and external circadian-related factors that affect sports performance. External, internal and lifestyle factors proposed to interact to influence performance on any given day.

Reproduced from Reilly et al. (2009)

1.3.1 Environmental factors

A number of environmental factors such as light, photoperiod and temperature influence the phase and period of circadian rhythms in humans (Ciarleglio et al., 2008, Nadkarni et al., 2005). These factors fluctuate with the time of year, local meteorological changes and geographical locale. A change in the environment's level of lighting (i.e. in the wavelength of 460-500 nm) during the day has a profound effect on human physiology, since light perception by the retina of the eye elicits a circadian response (Gaddy et al., 1993). For example, exposure to blue light in the morning, or during the day enhances an individual's health and well-being, alertness and productivity (Roberts, 2000, Cutolo et al., 2005, Van Someren, 2000). Furthermore, changes in the environment's photoperiod may significantly affect an individual's predisposition for physical activities and consequently athletic performance (Baker and Horton, 2004). Poor lighting conditions in the immediate environment may affect visibility, which in turn may lead to poor athletic performance or the athlete terminating training prematurely.

When training sessions or competitions must take place at night under floodlights melatonin secretion may be suppressed (Lewy et al., 1980, Escames et al., 1996). Increased levels of melatonin secretion causes the body to feel drowsy, as it essentially prepares for sleep relaxation (Roberts, 2000, Wehr et al., 2001). In comparison, decreased levels of melatonin lead to un-refreshing sleep, high blood pressure, depression and fatigue all of which influence an individual's performance (Reilly and Edwards, 2007, Roberts, 1995). Suppression of melatonin secretion might have consequences for athletes training under high light intensities (~2500 lux) in the blue region (400-500 nm) of the visible light spectrum, since melatonin shows a rather large amplitude circadian rhythm, which is greatly associated with rhythms in sleepiness and alertness (Atkinson et al., 2005a, Akerstedt and Gillberg, 1982). Many athletes may eventually fail to produce optimum performance or find the inner motivation when they train late at night under floodlights three days a week for a longer period. Besides the levels of lighting in the immediate environment, ambient temperature may also affect an individual's circadian rhythms and subsequently athletic performance.

The circadian system generates circadian rhythmicity and helps maintain the CBT around a set point (Weinert and Waterhouse, 2007). During cold weather, the CBT falls slightly below the set point (~37.5^{o}C) and the body has to constantly employ heat generation mechanisms to maintain the CBT (Meigal et al., 1998). Low ambient temperatures (-4^{o}C) may result in a

slightly decreased CBT, which may affect an individual's mental ability and manual dexterity (Palinkas, 2001, Daanen, 1993). Manual dexterity has been shown to vary with time-of-day and displays distinct peaks in morning-type and evening-type individuals (Cappaert, 1999, Monk and Leng, 1986). Furthermore, hot weather results in a raised resting metabolic rate and an elevated heart rate, which may negatively influence physical performance if CBT goes above 40^{o}C (Maughan et al., 2007). A high ambient temperature in the environment causes thermal strain, which in turn negatively affects physical performance (Borresen, 2008). All this taken together suggests that, athletes have to constantly adapt to changing environmental conditions in order to excel in their respective sports.

1.3.2 Transmeridian flights and sport

Today, travelling across time zones prior to a competition or between competitions has become a necessity for international athletes and poses several difficulties on the travelling athlete. One such difficulty is jetlag (discussed in detail in section 1.1.3.1 above). Effects of jetlag on performance have been well documented in the travelling American football teams, whose performance is affected depending on how close the game time corresponds to the usual afternoon circadian-related peaks in performance (Smith et al., 1997). Recht and co-workers (1995) analysed the performance of 19 North American football league teams based in the cities of Eastern and Pacific Time zones for three consecutive seasons (1991-1993). Their results indicate that the teams had significantly more victories when they had a home advantage than when they played away (54% *vs.* 46%). Furthermore, the probability of winning by the visiting team relied on whether they travelled eastward. The West coast teams appeared to be at an advantage over East coast and Central teams for night games since they played at a time-of-day, which was close to the proposed athletic peak performance time with games starting at 21h00 Eastern Standard Time (Smith et al., 1997).

Jetlag studies by Klein and Wegmann (1974) further strengthen this notion by stating that athletes travelling westwards (from Germany to United States) need a longer time period to resynchronize their psychomotor performance rhythms than those travelling eastwards, when only a day is required to resynchronize. It is worth mentioning that there is considerable inter-individual variability in the time required to recover for a given rhythm in a given individual. Several factors are associated with differing rates of rhythm re-adaptation, namely, an individual's diurnal preference, sleep habits, direction of the flight and behavioural traits (Reilly and Edwards, 2007). Jetlag also affects other aspects of physiology such as CBT, heart

rate, and blood pressure (Reilly et al., 2009), each of which may in turn affect performance. As far as diurnal preference is concerned evening preferring individuals are less affected by jetlag than those preferring mornings for both directions of travel (Manfredini et al., 1998, Winget et al., 1985). This is because morning-type individuals take longer to resynchronise following jetlag compared to evening-type individuals. In addition, the CBT of morning-type individuals is phase advanced, thus it peaks earlier following a westward transmeridian flight (Colquhoun, 1979). This has adverse effects on performance, since many competitions take place late in the afternoon to early evening and many physiological and neuromuscular variables of performance follow peaks in CBT. Evening-type individuals adapt more easily to a phase delay than to an advanced shift in circadian rhythms, and thus find it easier to cope and perform after a westward transmeridian flight (Winget et al., 1985).

1.3.3 Sleep

Sleep is crucial to many organisms, particularly humans, because it provides the necessary recovery functions for the brain following the effects of wakefulness (Hobson, 2005). The master pacemaker in the SCN (Moore and Eichler, 1972) regulates the circadian rhythms of the sleep-wake cycle. The central clock genes are integral to the sleep-wake balance, and polymorphisms within these genes may lead to disorders, such as DSPS and FASPS (Ebisawa et al., 2001, Toh, 2008), depression and bipolar disorders (Benedetti et al., 2003). The harmonious co-existence of circadian rhythms is compromised when the normal sleep-wake cycle is upset (Winget et al., 1985). Many factors contribute towards upsetting the normal sleep-wake cycle such as shift work, travelling across time zones, sleep deprivation and other circumstances that interrupt normal sleep, for example, anxiety (Reilly and Edwards, 2007), just to name a few. All these factors result in the suppression of melatonin secretion, thus causing fatigue and staleness in athletic performance (Winget et al., 1985).

Chronic sleep disturbance is experienced in physical activities such as long distance sailing (Bennet, 1973), military operations and multi-day adventure races, for example, the Race Across America (4640 km), where participants often sleep for only 1 or 2 h per night for a number of consecutive days (Smith et al., 1998). Total and chronic sleep deprivation, which disturb the natural circadian rhythm of the sleep-wake cycle, have been shown to greatly influence athletic performance. For example, members of a five-a-side indoor soccer team underwent 96 h of continuous exercise at moderate intensity and continued wakefulness (Reilly and Walsh, 1981). The observed effects related to acute sleep-deprivation ranged

from, impaired visual reaction time and mental agility to a decreased work and heart rate. In addition, athletes tended to hallucinate and exhibit disturbed behaviour, and these factors worsened with each night spent awake, which the authors conclude is indicative of the stress posed by the lack of sleep (Reilly and Walsh, 1981). In addition to total and chronic sleep deprivation, partial sleep disruption, also affect performance through desynchronisation of the sleep-wake cycle. A study conducted on swimmers revealed that partial sleep disruption resulted in fatigue and confusion, which in turn adversely influenced athletic performance (Sinnerton and Reilly, 1992). This is mainly because, like chronic and total sleep deprivation, partial sleep loss was shown to significantly affect mood state, increase depression, confusion and fatigue (Sinnerton and Reilly, 1992). In partial sleep disruption, these symptoms can be restored to normal after one full night of sleep. In contrast, resynchronisation following total and chronic sleep deprivation generally depends on the number of days that sleep was deprived or disturbed and may take longer to restore sleep to normal (Manfredini et al., 1998, Reilly and Edwards, 2007). In general, athletes should have adequate sleep daily and prior to races or competitions for essential human health and in order to maximize their chances for optimal performance (Hobson, 2005).

1.3.4 Social factors

Social factors are a significant and mostly overlooked part of the external equation in athletic performance. For example, for some people it may be difficult for them to go to bed early enough to get sufficient sleep because of their work, family and other social obligations. These individuals may suffer from circadian rhythm-related sleep disorders, such as insomnia, mood swings and other effects already mentioned in the section above. Other social factors that might affect scheduling time of events include television audiences and broadcasting times. For example, a competition or game of soccer may take place in the morning (10h00) or at night (21h00) so that the live broadcasting time of the event on television is suitable for audiences across a range of time zones. These are not optimal times for many physiological, cognitive and neuromuscular variables, thus athletes may perform with less than optimal efficiency, compared to when these events are scheduled late in the afternoon at the peak of CBT (Reilly et al., 2007). In addition to scheduling time of event, different cultures value different sporting activities and as such may provide community resources to promote the involvement and development of skill in that particular sport (Baker et al., 2003, Bloom, 1985). This allows athletes to be full-time sportsmen/women thereby allowing them to choose their preferred time for training. Furthermore, athletes may not have to worry about training

after day time or shift work and at non-preferred times of day. Failure of a sporting activity to receive societal backing may result in it not receiving the same quality of available resources and support, thus making the path to elite performance very difficult for athletes (Racy, 1969, Baker et al., 2003). This is not ideal as athletes may have to train after day or shift work which is not at their preferred times, and fatigue in the case of shift work may negatively influence performance during training or competitions.

1.3.5 Training and training time-of-day

Not surprisingly, high levels of training are generally required to attain high levels of performance. Choosing the time of training in a day is crucial, since a number of physiological, psychological (Cappaert, 1999, Burgoon et al., 1992) and environmental variables influence physical and mental performance. These variables, which ultimately contribute to performance, display peaks and troughs at specific times of the day. For example, a study conducted by Baxter and Reilly (1983), demonstrated that swimming performance peaks later in the afternoon when CBT is high. They showed that swimmers' time-trial times (100 m and 400 m) improved towards the early evening and peaked between 20h00-22h00. These findings are consistent with those conducted by Rodahl et al. (1976) who showed that swimmers produced faster times in 100 m time trials early in the evening at 17h00 compared with morning time trials at 07h00 in three out of the four strokes studied. While it is clear that performance in some sports may peak in the afternoon, what was yet to be established is whether adaptation to a training session varies at different times of the day. Optimising adaptation to training by varying training time-of-day would be advantageous to amateur and professional athletes alike.

Preparation time-of-day in sport has only been given attention in approximately the last two decades (Hill et al., 1989, Reilly and Greeves, 2000, Reilly and Baxter, 1983). A study conducted by Reilly and Greeves. (2000) suggests that time-of-day may be important for strength training adaptations. Habitually active males were divided into two groups (group I and group II) and followed a supervised strength training programme at 09h00 and 21h00, respectively, for four days a week over a four-week period (Reilly and Greeves, 2000). At the end of the four-week period, they showed that the increase in strength in group II was 20% greater than that of group I. One explanation for this may be that more work may be performed when resting CBT is at its highest, thereby increasing the intensity of the evening training sessions. Cycle ergometer performance studies conducted by Hill et al. (1989) on

untrained individuals with moderate levels of activity revealed that time-of-day of endurance training also affects cycling performance. They divided individuals into two groups that underwent supervised laboratory based training in either the morning (between 06h00-08h00) or the evening (between 15h00-18h00) for six weeks. Interval work was performed at 90-100% of VO_2max, while continuous exercise was conducted at the maximum sustainable intensity for 30-35 minutes. After the six weeks of training, individuals were pooled together and randomly assigned to either the group that tested in the morning or in the evening. It was observed that individuals who underwent morning training (interval and continuous cycling and/or running) had low rating of perceived exertion (RPE) scores, reduced heart rates and increased time to exhaustion in the morning compared to those who performed training in the evening and were randomly assigned to the morning session.

Similarly, individuals who trained in the evening, had low RPE scores, reduced heart rates and increased time to exhaustion in the evening compared to those who underwent morning training. This led Hill and co-workers (1989) to suggest that athletes should train at the same time-of-day that an event is scheduled to take place if they are to maximize their chances of performing better. The reduced RPE observation is interesting as it suggests that there may be a psychological component to training time-of-day that can be manipulated. Training at a preferred time when a session feels easier may enable or encourage an athlete to accumulate more hours of practice (Baker and Horton, 2004), ultimately resulting in better athletic performance. The results documented by Hill et al. (1989) are consistent with findings by Reilly and Baxter (1983) who demonstrated that RPE and heart rate were reduced in an endurance exercise, while VO_2max and time to exhaustion were increased.

Eight volunteers performed cycling tests at 40% and 95% of VO_2max at 06h30 and 22h00, respectively, until they could not pedal anymore. It was observed that time to exhaustion was 68% longer in the evening than in the morning and a positive correlation was found between RPE at 95% and the time to exhaustion for both morning and evening sessions. Despite the evidence that freedom to train at preferred times is important, the reality is that both professional and recreational athletes may be constrained as to when they can train, as a result of work and family commitments or team training schedules. Therefore, their training sessions may not always be at their preferred time-of-day or even the time-of-day at which the event will occur.

1.4 Effects of circadian rhythms on sports performance

Most physiological aspects of performance show sinusoidal patterns of change over a 24 h period, typically peaking in the late afternoon to early evening, with a few exceptions (Atkinson and Reilly, 1996, Wright et al., 2002). Most of these rhythms tend to follow the rhythm of CBT which peaks between 16h00-20h00 (Drust et al., 2005, Baxter and Reilly, 1983). Table 2 summarizes the time-of-day peaks for a number of performance-related physiological variables and skills. Different sporting activities are influenced by different physiological components, hence it may be important for athletes to consider physiological factors that influence a particular sport when scheduling training time-of-day (Cappaert, 1999).

Table 2: Time-of-day changes in physiological variables and skills that influence sports performance

Variable	Method of testing	Type of sport	Peak time	Reference
Flexibility	Sit and reach test	Soccer	No time of day	(Reilly et al., 2007)
Dribbling	Dribbling time	Soccer	No time of day	(Reilly et al., 2007)
Manual dexterity	Timed maze task	All sports	09h00-11h00	(Lundeen et al., 1990)
Neuromuscular coordination	Seesaw board balance test	All sports	12h00	(Ilmarinen et al., 1980)
Grip strength	Hand dynamometer	Soccer	14h00-15h00	(Lundeen et al., 1990)
Juggling	Juggling touches	Soccer	16h00	(Reilly et al., 2007)
Reaction time	Electronic time device	Soccer	16h00	(Reilly et al., 2007)
Leg strength	Portable dynamometer	Soccer	18h00	(Coldwells et al., 1994)
Back strength	Portable dynamometer	Soccer	18h00	(Coldwells et al., 1994)
Alertness	Visual analogue scale	Soccer	20h00	(Reilly et al., 2007)
Rating of perceived exertion	Borg's RPE scale	Cycling	06h00-09h00	(Hill et al., 1989)
Heart rate recovery	Heart rate monitor	Cycling	15h00	(Reilly et al., 1984)
Heart rate	Heart rate monitor	Cycling	15h00-17h00	(Crockford and Davies, 1969)
Anaerobic capacity	Wingate anaerobic test	Cycling	16h00	(Reilly and Down, 1992)
Peak power output	Wingate cycle test	Cycling	16h00-22h00	(Hill and Smith, 1991)
Maximal aerobic capacity	VO_2max test	Cycling	18h00	(Brisswalter et al., 2007)
Aerobic capacity	Time to exhaustion	Cycling	22h00	(Reilly and Down, 1992)
Power output	Margaria stair run test	Running	17h00-1800	(Reilly and Down, 1992)
Broad jump distance	Margaria stair run test	Running	17h00-18h00	(Reilly and Down, 1992)
Swim performance	200 m time trial	Swimming	16h00-18h00	(Kline et al., 2007)
Tennis serve accuracy	Average error	Tennis	19h00-21h00	(Atkinson and Speirs, 1998)

1.4.1 Performance of morning- and evening-type individuals

In addition to the time-of-day peaks in these variables, there is considerable inter-individual variation, which may be explained in part by an individual's diurnal preference. Among other things, the genetics of the circadian system helps determine an individual's diurnal phenotype, which in turn influences diurnal preference. An individual's preference for morning or evening activities may have an impact on his/her acute response to exercise. For example, a study conducted by Hill et al. (1988) demonstrated that in a group of 32 athletes who performed two maximal progressive cycling tests in the morning and evening, respectively, individuals who preferred evenings had higher VO_2max in the evening compared to their morning counterparts. These authors concluded that exercise response is a function of time-of-day, and that evening or morning preferring individuals should be considered when testing VO_2max at different times of day.

Of the studies that have looked at performance at different times of the day of morning- and evening-type individuals, the results have been conflicting. For example, in a cycling study conducted by Atkinson and co-workers (2005b) performance was shown to peak in the evening even in morning preferring individuals. Some studies have shown no significant difference between the performance of morning and evening preferring individuals with time-of-day (Cappaert, 1999, Burgoon et al., 1992). In contrast, a study conducted on 16 collegiate rowers (Brown et al., 2008) showed that morning-type individuals who habitually trained in the morning rowed significantly faster in a morning 1000 m time trial. Strikingly, the evening-type rowers who habitually trained in the morning were still fastest in the evening 1000 m time trial. In order to fully understand the basis for the physiological variation between morning- and evening-type individuals it is necessary to consider the mechanisms that drive this oscillation which have been discussed in section 1.1.

1.5. Summary and conclusions

Much research has investigated the effects of circadian rhythms on mental (Waterhouse et al., 2005, Frascina, 2004, Fan et al., 2002, Palinkas, 2001, Wright et al., 2002) and physical (Reilly and Waterhouse, 2009, Reilly et al., 2007, Reilly and Baxter, 1983) performance. These studies have shown large inter-individual variation in the peak times for each variable studied, therefore differences in diurnal preference need to be considered. Exactly how morning- or evening-type personalities vary with respect to their capacity to perform tasks at

different times of day is, however, still unclear. Since there are physiological differences between individuals who prefer mornings compared to those who prefer evenings, it seems logical that morning- and evening-type individuals should have different optimal times of the day for optimal mental and physical performance. To date, only a few studies (Brown et al., 2008, Burgoon et al., 1992, Hill et al., 1988) have compared the performance of athletes preferring either morning- or evening-activity. While it has been well established that certain aspects of performance in athletes are affected by circadian rhythm cycles, the extent to which these disruptions affect individuals preferring mornings compared to those preferring evenings has not yet been established. There are polymorphisms in clock genes that lead to differences in circadian phenotypes such as sleep-wake cycles, mood disorders and diurnal preference. Therefore, understanding the molecular factors driving diurnal preference is important, particularly since recent studies have shown an association between diurnal preference and a polymorphism in the *PER3* gene (Archer et al., 2003, Ebisawa et al., 2001).

There have been no published studies to date describing diurnal phenotype and genotype interaction in either sedentary or sporting populations in South Africa. Furthermore, the effect of this phenotype and genotype interaction on sports performance has not been explored elsewhere. While much has been studied of the influence of circadian rhythms on specific skills or physical outputs and physiological processes that affect sports performance, the impact, particularly clock gene polymorphisms and an individual's ability to perform at a particular time-of-day, has not been studied. One aspect of this study is to describe regularly active individuals and recreationally well-trained Ironman triathletes and cyclists in terms of their subjective preferences for a particular time-of-day as well as their *PER3* genotypes for "morningness" or "eveningness". We hypothesised that trained populations of athletes participating in individual endurance events may contain higher proportions of individuals preferring the morning compared to the general population. Furthermore, we hypothesised that athletes exercising closer to their preferred time-of-day will perform better compared to athletes exercising at their non-preferred time-of-day.

1.6. Aims of this study

The aims of this study were:

1. To describe the distribution of individuals preferring either mornings or evenings in trained Caucasian, male cyclists and Ironman triathletes, and a control population of healthy, regularly active but non-competitive Caucasian males.
2. To genotype the same three populations for the VNTR polymorphism within the *PER3* gene and,
3. To compare the performance of trained cyclists strongly preferring either mornings or evenings at different times of the day.

CHAPTER 2
METHODS AND MATERIALS

2.1 Study 1

2.1.1 Participants

Trained, recreational, Caucasian (self-reported European descent) male cyclists (CYC, n=138) and Ironman triathletes (IM, n=301), were compared to a matched population of active, but non-competitive males (CON, n=120). Only male participants were included in these studies because the menstrual cycle of females may have affected studies on performance and body temperature. All participants were between the ages of 25-50 years, in self-reported good mental and physical health, with no chronic medical conditions. The CYC and IM participants had trained for their respective sports at least three times a week for the three months prior to participation in the study. The CON group exercised 2-3 times a week. Cyclists were included if they had completed the Cape Argus Pick 'n Pay Cycle Tour (109 km) or similar event in less than 3 hours 45 minutes in the previous two years. Similarly, only triathletes who had completed a full Ironman triathlon (3.8 km swim, 180 km cycle and 42.2 km run) in less than 12 h in the past two years were included. We chose participants between 25-50 years of age, as we wanted to avoid the possible age dependent effects on diurnal phenotype of adolescence and older adults (Bliwise et al., 2005). We only chose to study males of self-reported European descent to avoid variation in *PER3* VNTR allele frequency due to ethnic origins (Barbosa et al., 2010, Ciarleglio et al., 2008, Nadkarni et al., 2005). Participants who had self-reported to have taken any of the following substances: amphetamines, modafinil, soporific drugs, hypnotics or melatonin three months prior to the study were excluded as these drugs affect the sleep-wake cycle and/or circadian system.

2.1.2 Study design

Cyclists were recruited through advertisement and at cycling clubs and races. Of the 301 Ironman triathletes, 249 DNA samples were from a previous study (REC No. **425/2005** and REC No. **002/2005** (Collins et al., 2004) - for which the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee approved the appropriate protocol recommendations for this study) and 52 samples were from triathletes recruited during the Ironman triathlon in Port Elizabeth (April, 2010). Active participants were recruited at gyms at all times of the day to avoid bias in sampling that could occur by recruiting only in the

mornings or evenings. Participants were informed of the purpose of the study (see Appendix A1) and gave written informed consent to participate (see Appendix A2). Participants completed the HÖ-MEQ as well as a brief personal, health, training and competition-history questionnaire. The buccal cell samples were obtained from participants for DNA extraction by scraping the inside of both cheeks using Epicentre Catch-All^{TM} Sample Swabs (Epicentre Biotechnologies, Madison, USA). The swabs were stored at 4^{o}C, until further analysis. Genomic DNA was extracted from these samples and used to genotype each individual for the VNTR polymorphism within the *PER3* gene (Archer et al., 2003). The study was approved by the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee (**Ref: 412/2009**), and performed in accordance with the principles of the Declaration of Helsinki (October 2008, Seoul), ICH and South African Good Clinical Practice (GCP) guidelines.

2.1.3 Detailed testing procedures

2.1.3.1 Horne-Östberg morningness-eveningness personality questionnaire

All CYC and CON participants completed the HÖ-MEQ. Only a sub-group of the IM (n=52) completed the HÖ-MEQ, as the majority of the IM group (n=245) was contacted post-event to participate in this particular study and most did not choose to complete the questionnaire. Of the individuals contacted post-event (n=10) chose to respond to the email follow-up. The HÖ-MEQ is the most widely used questionnaire to describe diurnal preference, and is highly correlated to the timing of melatonin and body temperature, the two most important indicators of diurnal preference (Horne and Östberg, 1976). The questionnaire consists of 19 questions relating to sleep-wake behaviour and schedules (see Appendix A3). It yields scores ranging from 16-86, with low scores indicating "eveningness" and high scores indicating "morningness". Based on their score, individuals were placed into one of five diurnal preference categories: definite evening-type (DET score, 16-30), moderate evening-type (MET, 31-41), neither-type (NT, 42-58), moderate morning-type (MMT, 59-69), and definite morning-type (DMT, 70-86).

2.1.3.2 Genomic DNA extraction

A simple salting out protocol was used to extract total human genomic DNA (gDNA) from the buccal cell swabs (Aljanabi and Martinez, 1997), with some modifications. The swab tubes were incubated in lysis buffer for 1 h in a waterbath at 25°C to allow the cells to lyse. Samples were centrifuged for 10 minutes at 9200 g at room temperature using a bench

centrifuge (Labnet international, Edison, USA). After extraction and purification samples were dried for 8 minutes at 37°C. The pellet was resuspended in 20-30 µl of sterile distilled water and DNA quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, USA) and the quality assessed after electrophoresis through a 1% (w/v) agarose gel in 1 X Tris Acetate EDTA (TAE; 40 mM Tris, 1 mM EDTA and 0.11% (v/v) glacial acetic acid) buffer. DNA samples were stored at -20°C until further analysis.

2.1.3.3. PER3 VNTR amplification and confirmation

In order to genotype individuals for the *PER3* VNTR polymorphism, exon 18 of *PER3* and its flanking regions were amplified by PCR from 80-100 ng of gDNA using the primers described and according to Ebisawa et al. (2001) and Archer et al. (2003). Amplified gDNA was separated by electrophoresis on a 2% (w/v) agarose gel and visualised under ultra-violet (UV) light after ethidium bromide staining (Figure 8). In order to confirm that the amplified fragments corresponded to *PER3* and to confirm that I could distinguish between $PER3^4$ and $PER3^5$, products were cloned and sequenced as described in section 2.1.3.4.

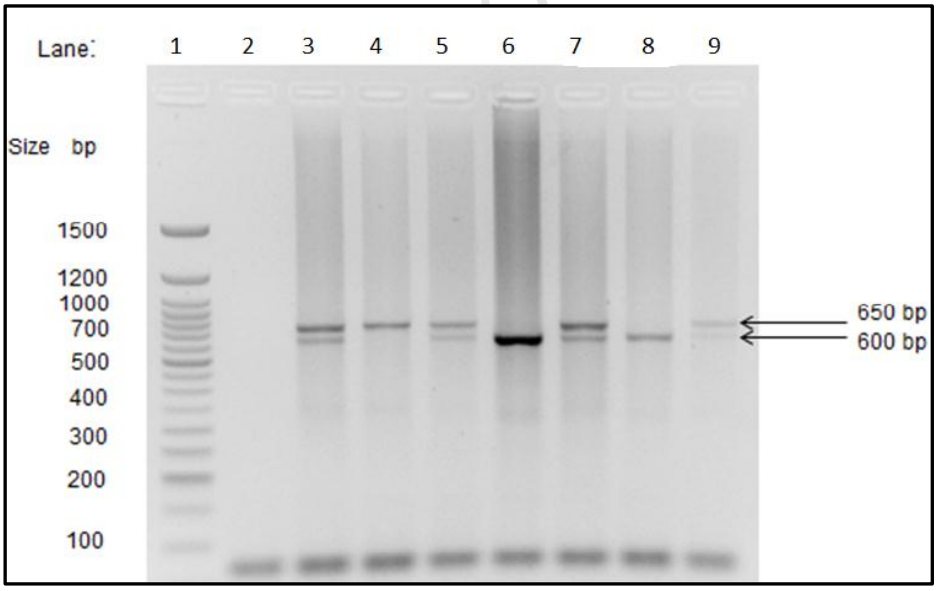

Figure 8: Polymerase chain reaction (PCR) products from amplification of the *PER3* VNTR polymorphic region from genomic DNA according to Ebisawa et al. (2001). Exon 18 of the *PER3* gene was amplified from genomic DNA and resolved on a 2% (w/v) agarose gel and visualised under ultra violet (UV) light using ethidium bromide staining. Lane 1: 50 bp DNA ladder, (O'GeneRuler, Fermentas, USA), Lane 2: negative control, Lanes 3-9: PCR products from seven of the participants. Lanes 3, 5, 7 and 9: individuals with both the $PER3^5$ (top band 650 bp) and $PER3^4$ (bottom band 600 bp) alleles; Lane 4: an individual homozygous for the $PER3^5$ allele and Lanes 6 & 8: individuals homozygous for the $PER3^4$ allele.

2.1.3.4. DNA cloning and transformation

Competent *Escherichia coli* (*E. coli*) DH5α cells were prepared and transformed according to the calcium chloride protocols described in Sambrook and Russell. (2006) and were used for all cloning studies. Luria Bertani (LB) broth was used for *E. coli* growth. Amplified gDNA was separated by electrophoresis, excised from the 2% (w/v) agarose gels (Figure 8) and eluted using HiBind® DNA Spin Columns from the DNA Gel Extraction Kit (PEQLAB Biotechnologies, Washington, USA). Cloning was performed according to the sticky-end protocol (CloneJET® kit, Fermentas, Maryland, USA) with a few modifications. Transformed DH5α cells were plated on LB plates containing ampicillin (80 $\mu g \cdot ml^{-1}$) and incubated overnight at 37°C (Sambrook and Russell, 2006). Colony PCR using pJet 1.2 primers and restriction enzyme digests with *Nco*I to drop out inserts were performed to select for transformants that had the insert of interest. Purified plasmid DNA was sequenced by Macrogen (Macrogen, Seoul, South Korea) using T7 promoter primers. Sequences were edited up using the Chromas programme (Chromas, Version 2.3.3) and homology searches were performed using BLAST (Altschul et al., 1997).

2.1.3.5. PER3 VNTR and M1037T polymorphism

Since the resultant fragments of the *PER3*^{4} and *PER3*^{5} were so similar in size (approx. 650 bp and 600 bp, respectively), the PCR products were restricted with *Nco*I (Promega, Madison, USA) for 16 h at 37°C and the resulting fragments were resolved on a 2% (w/v) agarose gel and visualized under UV light after ethidium bromide staining (Figure 9). *Nco*I recognizes and cleaves a CCATGG sequence. However, in the 4-repeat allele of the *PER3* VNTR there is a 3110 (T→C) nucleotide substitution, which results in an amino acid change from methionine to threonine and this is not cleaved by *Nco*I. Digestion with *Nco*I is thus able to distinguish six possible M1037T genotype combinations: homozygous 5M/5M, 4M/4M, 4M/4T and 4T/4T or heterozygous 5M/4M and 5M/4T (Table.3). The 4 and 5 represent the 4- and 5-repeats of the VNTR polymorphism respectively, and the M and T represent the methionine and threonine amino acid residues of the M1037T polymorphism.

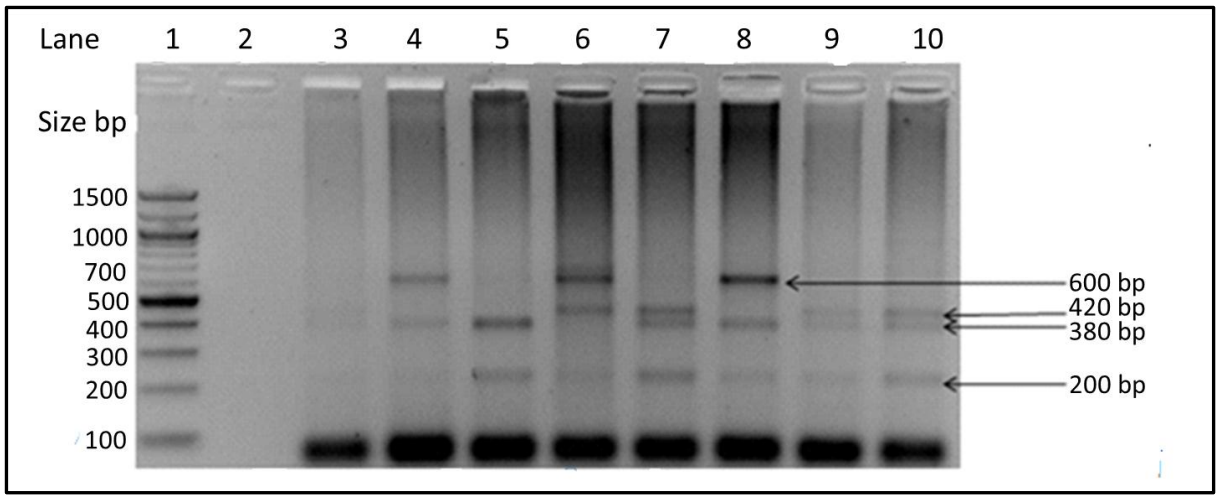

Figure 9: Genotyping of *PER3* M1037T polymorphism using *Nco*I restricted PCR products. Fragments were resolved by electrophoresis on a 2% (w/v) agarose gel and visualized under ultra-violet (UV) light using ethidium bromide staining. Lane 1: 100 bp DNA ladder, (O'GeneRuler, Fermentas, USA), Lane 2: negative control, Lanes 3-10: Fragments from 8 of the IM participants. Lanes 3, 4 and 6-10: individuals with both the $PER3^5$ and $PER3^4$ alleles; Lane 5: an individual homozygous for the $PER3^4$ allele.

Table 3: Expected fragment sizes of *PER3* VNTR and M1037T polymorphisms.

VNTR genotype	M1037T genotype	Band 1 (bp)	Band 2 (bp)	Band 3 (bp)	Band 4 (bp)
$PER3^{5/5}$	5M/5M		420	-	200
$PER3^{4/5}$	4M/5M		420	380	200
$PER3^{4/5}$	4T/5M	600	420	-	200
$PER3^{4/4}$	4T/4M	600	-	380	200
$PER3^{4/4}$	4M/4M		-	380	200
$PER3^{4/4}$	4T/4T	600	-	-	-

2.2 Study 2

2.2.1 Participants

Based on the results of study 1 morning-type (MT) and evening-type (ET) cyclists were invited to participate in the follow-up study (study 2). Twenty cyclists who scored as MT (59-86) on the HÖ-MEQ and were genotyped as $PER3^{5/5}$ or $PER3^{4/5}$ formed the morning cyclist group (MORN). Of the three cyclists who scored as ET (16-41) on the HÖ-MEQ and were genotyped as $PER3^{4/4}$, only one volunteered to take part in study 2. To increase the sample size of the evening cyclist group, cyclists who scored as NT (41-58) on the HÖ-MEQ and

were genotyped as *PER3*$^{4/4}$ (n=3) were also invited to participate making the total number of participants in the evening cyclist group four. For the purposes of this study, the evening cyclist group shall be referred to as the designated evening-type group (dEVE) since it comprised individuals who were genotyped as *PER3*$^{4/4}$, but scored as either ET or NT using the HÖ-MEQ.

2.2.2. Study design

The study was conducted over six consecutive months during which all participants (n=24) visited the laboratory six times over a two week period. The initial visit took place at 10h00 and was used to familiarize the cyclists with the testing protocol to be used on subsequent visits, as well as for the investigator to take anthropometrical measurements and for the cyclists to perform a peak power output (PPO) test (including respiratory gas analysis). In the subsequent five visits, cyclists repeated the same protocol - measurements of weight, oral body temperature (OBT), Attention Network Test (ANT) and the Lamberts and Lambert Sub-maximal Cycle Test (LSCT) at different times of the day. Visit two took place between two and five days after visit one, and visits two to six took place between 24 h and 48 h after the previous visit to minimize the influence of fatigue on subsequent visits, but to simultaneously minimize the number of days over which testing spanned (see Appendix B1). Visits two to six took place at 06h00, 10h00, 14h00, 18h00 and 22h00, and the order in which participants completed these visits was randomized to reduce the chance of a learning effect influencing the results.

Valid reasons for postponement of a visit included: illness, injury, not being able to start at the required time due to unforeseen events (e.g. work and traffic), or inadequate sleep prior to the test. Each participant was allowed one postponement before being asked to restart the entire study. Participants were asked to keep training, sleep and eating habits as close to normal as possible for the duration of the study with the following exceptions: no caffeine three hours before each test, no racing 48 h prior to any visit and no hard training session 24 h prior to any visit. Cyclists were also advised to wear the same or similar cycling outfit during the testing period and no changes to the bicycle set-up were allowed. For the 24 h period prior to visits two to six, cyclists were asked to keep a diary logging training, time of sleep, diet and oral body temperature (see Appendix B2). This was done to monitor sleep patterns, dietary and training status of the cyclists to assist the investigator in determining whether, possible confounding factors may have influenced performance on a given day and to record the 24 h

fluctuations in body temperature throughout the testing period respectively. All participants provided written informed consent, before taking part in the study, which was approved by the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee (**Ref: 412/2009**), and performed in accordance with the principles of the Declaration of Helsinki (October 2008, Seoul) ICH and South African Good Clinical Practice (GCP) guidelines.

2.2.3 Detailed testing procedures

2.2.3.1 Training, Sleep and Temperature diary

For the 24 h period prior to visits two to six participants were required to provide information regarding their training, sleep, temperature and diet. This was achieved by completing a diary that consisted of four sections, the training, sleep, OBT and diet sections. The training section was broken down into exercise session type, time-of-day a session was conducted, duration, distance covered and intensity rating on a scale of 1-10, 1 being very easy and 10 being extremely hard (see Appendix B3). In the sleep section, participants were required to report the time-of-day when they went to sleep and time-of-day they woke up. In the temperature section, participants were required to record their OBT hourly for the 24 h period prior to testing. Lastly, participants were required to log all items of food they either ate or drank (excluding water), the time-of-day they ate or took a beverage, including the amount taken during the 24 h period, as well as any supplements or medication they may have taken.

2.2.3.2. Anthropometry

Height (m), weight (kg) and skinfolds (mm) at seven sites (triceps, biceps, supra-iliac, subscapular, calf, thigh and abdomen) were measured during visit one (Ross et al., 1988). Body Mass Index (BMI) was calculated from weight and height ($kg \cdot m^{-2}$) and the percentage body fat was estimated as described by Durnin and Womersley (1974).

2.2.3.3. Cognitive performance test

Cognitive performance was assessed using the Attention Network Test (ANT) (Fan et al., 2002) as previously described by Matchock and Mordkoff (2009). The ANT was administered before the cycling test on each visit and lasted 20 minutes. Briefly, the aim of the test was to measure any circadian-related changes in mental performance for each of the five testing sessions. This was important to aid our interpretation of any potential change in the cyclists' physical performance over the 24 h period as well as to detect differences

between the MORN and dEVE cyclist groups. Many protocols exist to assess changes in performance of trained cyclists namely: the simulated 20 km time trial (TT) time test, 40 km TT time test and the shuttle walk test (Revill et al., 2010, Laursen et al., 2003, Sporer and McKenzie, 2007), to mention a few. A field based 40 km TT time would have been the best in monitoring changes in the performance of trained cyclists. However, the time required to recover between TT to ensure that there is no effect of cumulative fatigue is great (up to a week) and this would mean that the testing duration would span two months per person instead of two weeks; hence this could not be used. In this study a laboratory based sub-maximal test, the Lamberts and Lambert Sub-maximal Cycle Test (LSCT) (Lamberts et al., 2009a) was used to predict cycling performance differences between the MORN and dEVE cyclist groups. The LSCT test has been shown to be reliable and good in detecting small significant differences (<1%) in everyday performance of trained cyclists, particularly when it is performed under well-controlled conditions (Lamberts et al., 2009a).

2.2.3.4. Lamberts and Lambert Sub-maximal Cycle Test (LSCT)

The LSCT is a 17-minute sub-maximal bicycle test, which has been developed to monitor changes in training status and detect the symptoms of overreaching, but is also able to accurately predict 40 km time trial performance and PPO of cyclists (Lamberts et al., 2010). While, the LSCT was used as a warm-up protocol before the PPO test during the first visit, it was used to measure changes in performance (absolute power, speed, cadence, and predicted 40 km TT time and PPO) at different times of the day during visits two to six. Prior to the test, the rear wheel tyre of the participant's own road bicycle was inflated to 110 pounds per square inch (PSI) and the bicycle was mounted, by a rear axle quick release mechanism to a cycle ergometer (Computrainer Pro 3D, RacerMate, Seattle, Washington, USA). A cadence sensor was attached to the bicycle and plugged into the computrainer box (Computrainer Pro 3D, RacerMate, USA), which relayed the power, speed and cadence to the computer. Before the start of each test, the contact pressure of the load generator against the rear wheel was calibrated to 0.95-1.05 kg as recommended by Davison et al. (2007) and described by Lamberts and co-workers (2009a). After six minutes, when the tyre had warmed-up, the load generator was recalibrated to 2.35-2.40 kg. The LSCT was performed on a simulated flat course, with three different exercise intensities defined by different target heart rates of 60%, 80% and 90% of the maximum heart rate achieved during the PPO test for stages 1, 2 and 3, respectively (Figure 10). The target heart rates (HRs) for the LSCT before the PPO test on the initial visit were based on predicted maximal heart rate (HR_{max}) defined as (220 minus age)

(Tanaka et al., 2001). HR was measured and stored every two seconds using a Suunto t6d heart rate monitor (Version 2.1.0.3, Suunto, Oy, Vantaa, Finland). During the test, cyclists were free to manipulate their cadence, speed and gear choice in order to elicit and maintain their HRs within 2 beats·min^{-1} (bpm) of the target for stage 1 and within 1 bpm of the target for stages 2 and 3. When the HR deviated from the required rate, participants pedalled either faster or slower to correct for the change in HR. During the LSCT, power output, speed and cadence were measured continuously, and the rating of perceived exertion (RPE) was measured using the 20-point Borg-scale (Borg, 1973), 30 seconds before the end of each stage (5:30 stage 1, 12:30 stage 2 and 15:00 stage 3).

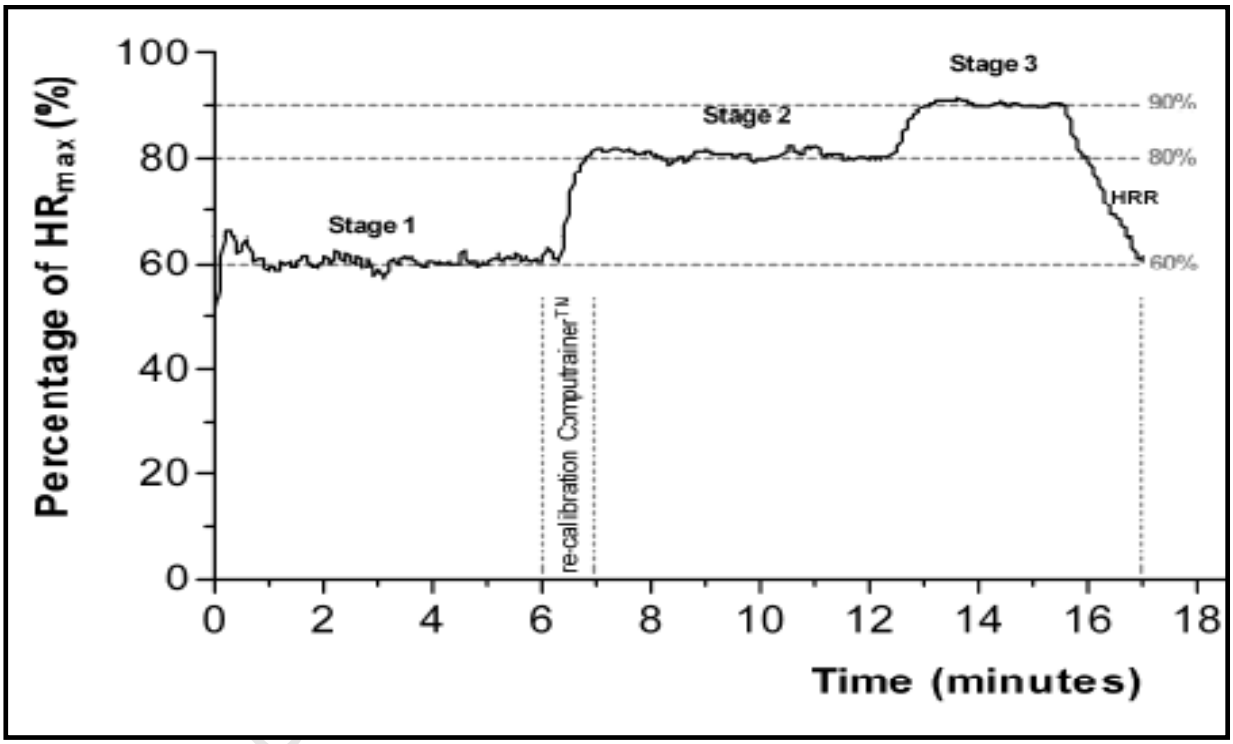

Figure 10: An arbitrary heart rate response expressed as a percentage of maximal heart rate (HRmax) during the Lamberts and Lambert Sub-maximal cycle test (LSCT). The diagram shows a 17-minute profile and duration of the cyclist's workload. Two recalibrations were performed the first one before the start of stage 1 and the second at the end of stage 1. The heart recovery rate period started immediately at the end of stage three, lasting 90 seconds. HR_{max}: maximal heart rate, LSCT: Lamberts and Lambert Sub-maximal Cycle Test (Reproduced from Lamberts et al., 2009)

Since cyclists were constantly adjusting their workload in order to reach targeted HRs during LSCT, the power, speed and cadence data in the first minute for each stage was excluded from analysis. Thus data values for each of the variables were calculated over a five minute period (from minute 1.0 to 6.0 and from minutes 7.5 to 12.5) for stages 1 and 2, respectively, and for a two minute period (from minutes 13.5 to 15.5) for stage 3. Heart recovery rate (HRR) was measured over a period of 90 seconds, during which cyclists where asked to sit straight and stay calm.

2.2.3.5. Peak power output (PPO) test

The PPO test, which included respiratory gas analysis, was performed during visit one as previously described by Lamberts et al. (2009a) and started exactly 8 minutes after the end of the LSCT performed as a familiarisation test. The PPO test was performed at a starting workload of 2.5 $W \cdot kg^{-1}$ of body mass after which the load was steadily increased by 20 watts (W) each minute until the cyclist could not maintain a cadence ≥70 revolutions per minute (rpm) or when the participant voluntarily stopped. The participant was asked to give feedback on RPE every minute during the PPO test. Breath-by-breath oxygen uptake (VO_2) and carbon dioxide production (VCO_2) were measured using an online breath-by-breath gas analyser and pneumotach (Oxycon, Viasis, Hoechberg, Germany) and final data values were the average of those measured over a 15 seconds interval (Lamberts et al., 2009a). The gas analyser was calibrated prior to each testing session using a 3 L calibration syringe (Hans Rudolf, Germany) and a standard gas mixture of 5% carbon dioxide in nitrogen (Afrox, South Africa). Power (W), speed ($km \cdot h^{-1}$) and cadence (rpm) were measured continuously using the computrainer. Cyclists were verbally encouraged to perform at the maximum of their ability. Maximum PPO (W) was determined as the average power output of the last 20 seconds during the final minute of the test, while VO_2max ($ml \cdot kg^{-1} \cdot min^{-1}$) was determined as the highest recorded reading for 15 seconds. Maximum HR was determined as the highest recorded reading for 20 seconds during the PPO test. This HR_{max} was used in the LSCT in subsequent visits.

2.3 Statistical analysis

All data are expressed as the mean ± standard deviation. Participant characteristics were compared using a one-way analysis of variance (ANOVA). Chi-squared analysis was used to compare diurnal preference, genotype and allele frequency distributions between the groups.

Correlations were performed using Pearson's product moment correlation test. A Friedman ANOVA test was performed to compare performance parameters at different times of the day, while a Man Whitney-U test was performed to check for significant time-by-group interaction effects between MORN and dEVE cyclist groups. A repeated-measures ANOVA was performed to determine if there was a significant variation in OBT values at different times of the day. Hardy Weinberg exact tests were performed to determine if the percentage frequencies of the 4- and 5-repeat alleles and the Horne-Östberg scores were normally distributed (http://genepop.curtin.edu.au/genepop_op1.html). The data were analysed using STATISTICA version 10 (Soft Inc., Tulsa Oklahoma, USA) and Microsoft Excel®. Significance was assumed when $p<0.05$.

CHAPTER 3
RESULTS

3.1 Study 1

3.1.1 Participant characteristics

Participant characteristics of the CYC, IM and CON groups for study 1 are presented in Table 4 below. There were significant differences in all the variables except for height between the CON and athletic populations. Tukey's post-hoc analysis showed that the CON group was younger ($p<0.001$) and heavier ($p<0.001$) than the CYC and IM athletic groups. The CON group had a higher body mass index compared to the IM and CYC groups ($p<0.049$). The athlete populations had more years training experience compared to the CON group (CYC, $p=0.049$ and IM, $p<0.001$, respectively). The IM group trained on more days of the week compared to the CYC and CON groups ($p<0.001$). Furthermore, the IM group spent more hours training compared to the CON and CYC groups ($p<0.001$).

Table 4: General characteristics of the CYC, IM and CON groups

		CYC (n=138)	IM (n=301)	CON (n=120)	p-value
	Age (years)	38.0±7.9	38.7±7.1	33.9±8.4[a]	<**0.001**
	Height (m)	1.79±0.06	1.80±0.07	1.79±0.07	0.730
	Weight (kg)	77.6±7.8	77.80±8.7	81.0±10.0[a]	<**0.001**
	BMI (kg·m^{-2})	24.1±1.6	24.0±2.1	25.8±2.7[a]	**0.049**
Training History	**Years**	9.7±7.3	8.9±7.2	5.8±4.9[a]	<**0.001**
	Days·week^{-1}	4.0±1.4	6.0±0.9[a]	4.0±1.3	<**0.001**
	Hours·week^{-1}	8.40±4.00	15.50±5.40[a]	5.20±3.20	<**0.001**

The data are represented as the mean ± standard deviation. CYC: Cyclist group, IM: Ironman triathlete group, CON: Control group and BMI: Body mass index. [a] indicates statistically significant difference from the other groups. The p-values represent significance as determined by a one-way ANOVA.

3.1.2 Actual and preferred training times

Only a sub-group of the IM group (n=52) reported actual and preferred training times. Furthermore, the IM sub-group represented the larger IM group with respect to the characteristics described in Table 4. Significantly, more of the athlete populations (CYC, 78.2% and IM, 85.3%) trained in the mornings compared to the control group (28.0%, $p<0.001$). Most individuals, 89.8% of the CYC, 90.4% of the IM and 89.2% of the CON

group, indicated that their current training time-of-day was their time of choice. Reasons given by those who did not train at their preferred time-of-day included work and family commitments. Most individuals, 63.7% of the CYC and 67.3% of the IM group who trained in the morning indicated that this was their preferred time (Table 5). On the other hand, 54.2% of the CON group who trained in the evening indicated that this was their preferred time. In the athletic populations (CYC, 15.3% and IM, 13.5%) and control group (CON, 16.6%) indicated that they trained in the afternoon and preferred afternoons as their training times. A significant difference between the athletic populations and the control group in the preferred training times existed (Table 5).

Table 5: Preferred training time-of-day

	CYC (n=138)	IM (n=52)	CON (n=120)
Morning (04h00-12h00)	88 (63.7%)	35 (67.3%)	22 (18.4%)*
Afternoon (12h00-17h00)	21 (15.3%)	7 (13.5%)	20 (16.6%)
Evening (17h00-22h00)	15 (10.8%)	5 (9.6%)	65 (54.2%)**
No answer/preference	14 (10.2%)	5 (9.6%)	13 (10.8%)

Data are presented as actual numbers with percentages in parentheses. CYC: Cyclist group, IM: Ironman triathlete group and CON: Control group. A p-value of ($p<0.001$) was obtained when a 3x4 chi-squared test was performed. *, ** indicates significant differences between the CON and the athletic populations with respect to morning and evening training time, respectively.

3.1.3 Horne-Östberg classification of diurnal phenotype

The IM sub-group (n=52) completed the HÖ-MEQ questionnaire. There were diurnal preference differences between the athletic populations compared to the CON group (Figure 11). The mean HÖ-MEQ score differed significantly between groups (CYC: 63.2±10.1, IM: 59.6±10.6 and CON: 52.5±10.7, $p<0.001$). Tukey's post-hoc analysis showed that the CYC group had a higher mean HÖ-MEQ score compared to the CON group ($p<0.001$). Figure 11 shows the frequencies of morning-types (MT), neither-types (NT), and evening-types (ET) in all groups. For simplicity, individuals classified as definite and moderate morning-types were grouped together as MT, and similarly, definite and moderate evening-types were grouped together as ET. The athletic groups contained significantly more MT individuals (CYC: 78.9%, n=109 and IM: 64.7%, n=34; $p<0.001$) than the control group (CON: 41.5%, n=50). Significantly more NT individuals were observed in the CON group (48.5%, n=58) than the CYC (19%, n=26; $p=0.019$) and IM (27%, n=14; $p=0.047$) groups.

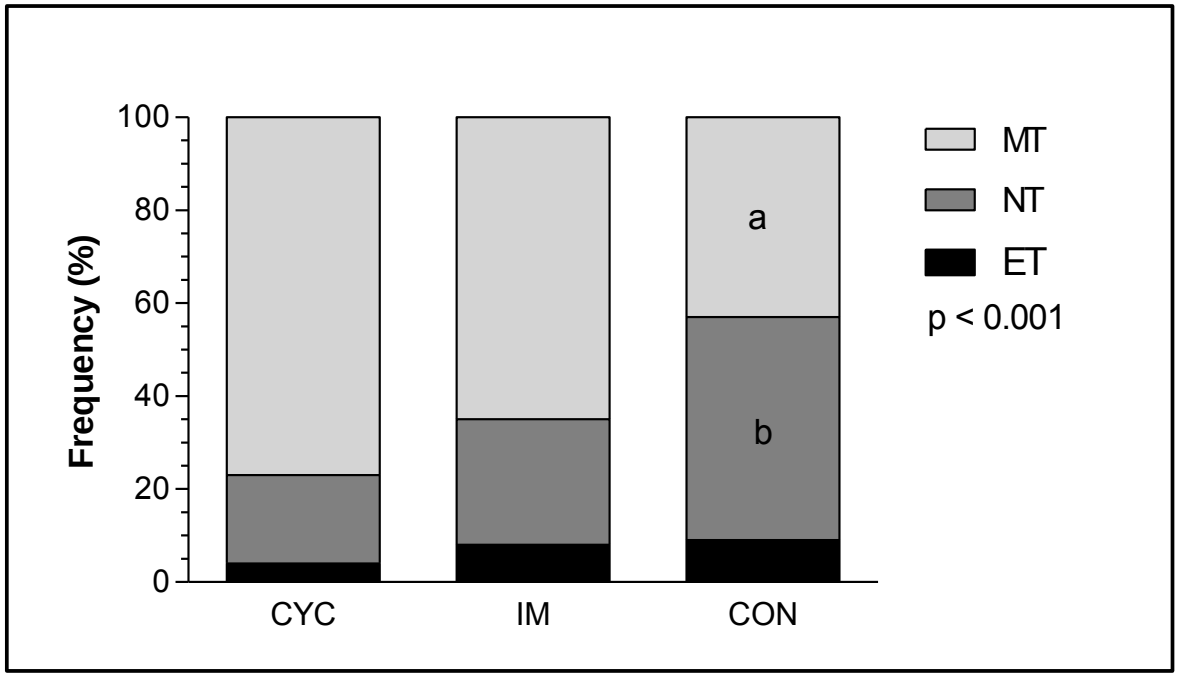

Figure 11: Frequency of diurnal preference categories in the CYC (n=138), IM (n=52) and CON (n=120) groups. MT: morning-type, NT: neither-type, ET: evening-type, CYC: Cyclist group, IM: Ironman triathlete group, CON: Control group. The p-value represents the frequency analysis significance determined using the Chi-squared test. [a] indicates a significant difference between the CON and athlete groups with respect to MT frequency. [b] indicates a significant difference between the CON and athlete groups with respect to NT frequency.

3.1.4 Amplification of the *PER3* gene

To confirm that the products amplified from gDNA using the primers described in Ebisawa et al. (2001), the target fragments from *PER3* were electrophoresed on a 2% agarose gel, excised (Figure 7 in Materials and Methods), cloned and sequenced. The sequences of the 600 bp and 650 bp products were identical to the portion of the *PER3* gene (Genbank Accession NM_016831.1) corresponding to 2027507-2027932 bp in a BLAST alignment. Furthermore, a BLAST using somewhat similar sequences and an mRNA sequence (Genbank accession AL157954) as a query sequence confirmed the identity of the 4- and 5-repeat alleles. The 5-repeat allele (top band in Figure 7) was 54 bp longer than the 4-repeat allele.

3.1.5 The *PER3* VNTR polymorphism frequency

There was a significant difference in the *PER3* genotype (Figure 12A) and allele (Figure 12B) frequency distribution between the CYC, IM and CON groups. The athletic groups contained significantly more individuals homozygous for the $PER3^{5/5}$ allele (5/5) (CYC: 39%, n=54, IM: 29%, n=15) than the control group (11%, n=13; $p<0.001$) and the CON group contained more

individuals homozygous for the *PER3*$^{4/4}$ allele (4/4) (CON: 33%, n=40) than the athletic groups (CYC: 18%, n=25 and IM: 15%, n=8; p<0.001) (Figure 12A). Furthermore, the prevalence of the longer *PER3*^{5} allele was greater in the athlete groups (CYC: 59.9%, n=83 and IM: 57.0%, n=30) than the CON group (39.0%, n=47; p<0.001) (Figure 12B). In contrast, the shorter *PER3*^{4} allele was more prevalent in the CON group (65.0%, n=78), compared to the athletic populations (CYC: 39.0%, n=54 and IM: 42.8%, n=22; p<0.001).

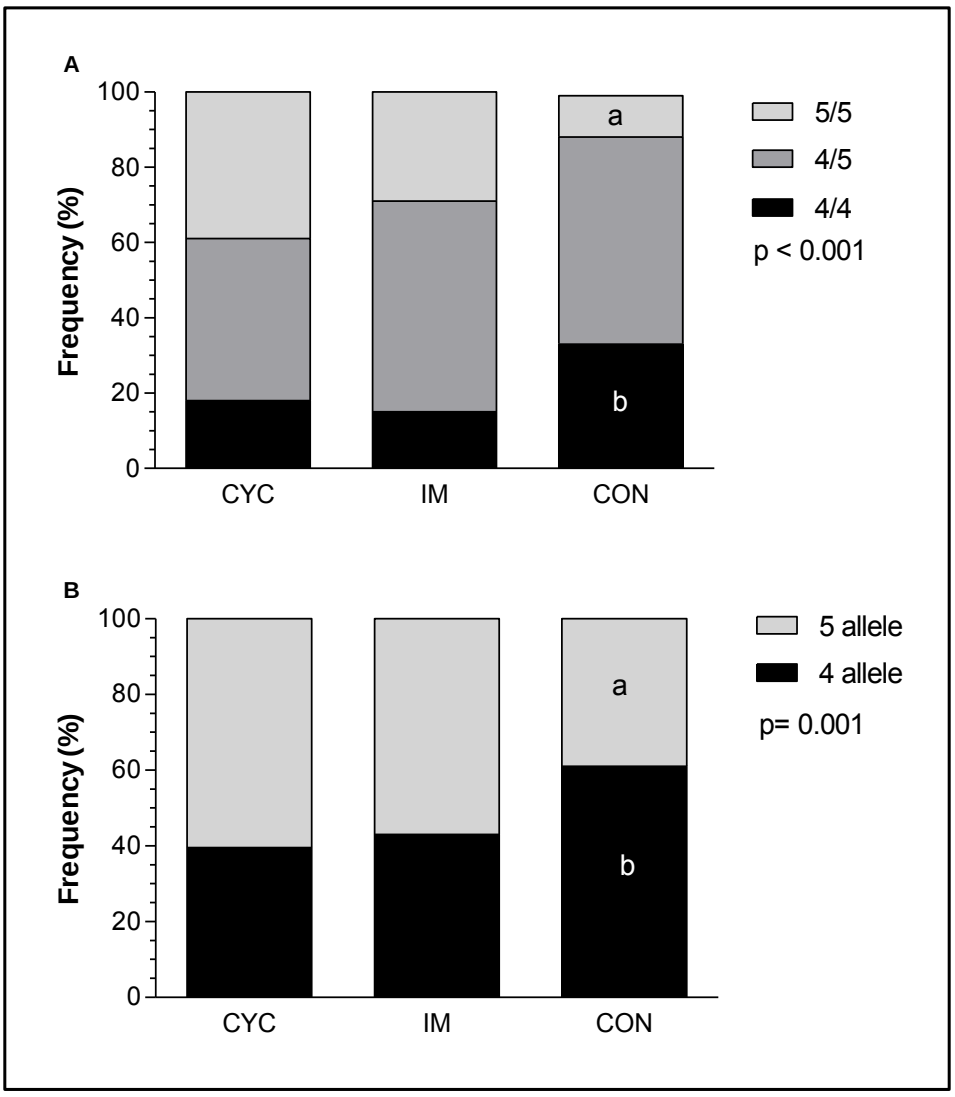

Figure 12: Frequencies of *PER3* VNTR (A) genotypes and (B) 4- and 5-repeat alleles in the CYC (n=138), IM (n=301) and CON (n=120) groups. 5/5: *PER3*$^{5/5}$, 4/5: *PER3*$^{4/5}$, 4/4: *PER 3*$^{4/4}$. CYC: cyclist group, IM: Ironman triathlete group, CON: control group. The p-values represent the frequency analysis significance as determined using a Chi-squared test. [a] indicates a significant difference between the CON and athlete groups with respect to the 5-repeat allele. [b] indicates a significant difference between CON and athlete groups with respect to the 4-repeat allele.

3.1.6 Relationship between diurnal preference and *PER3* genotype

The association between diurnal preference and the *PER3* VNTR polymorphism in the athletic and CON populations is presented in Figure 13. A Pearson's correlation test showed a significant positive correlation ($p<0.001$, $r=0.659$) between diurnal preference and genotype when all data were pooled together. Significantly, the majority (92.0%) of individuals genotyped as $PER3^{5/5}$ scored highly on the HÖ-MEQ (i.e. were classified as either MMT or DMT). The remaining 8.0% were classified as NT, with not a single $PER3^{5/5}$ individual being classified as MET or DET. A notable proportion (67.6%) of the individuals genotyped as $PER3^{4/5}$ were classified as DMT or MMT. The remaining 32.4% were classified as NT, with not a single $PER3^{4/5}$ individual being classified as MET or DET. 97.3% of the individuals genotyped as $PER3^{4/4}$ were classified as MET (21.4%), DET (6.2%) or NT (69.7%). Of the 310 individuals, there were two outliers (2.7%): two $PER3^{4/4}$ individuals were classified as MMT.

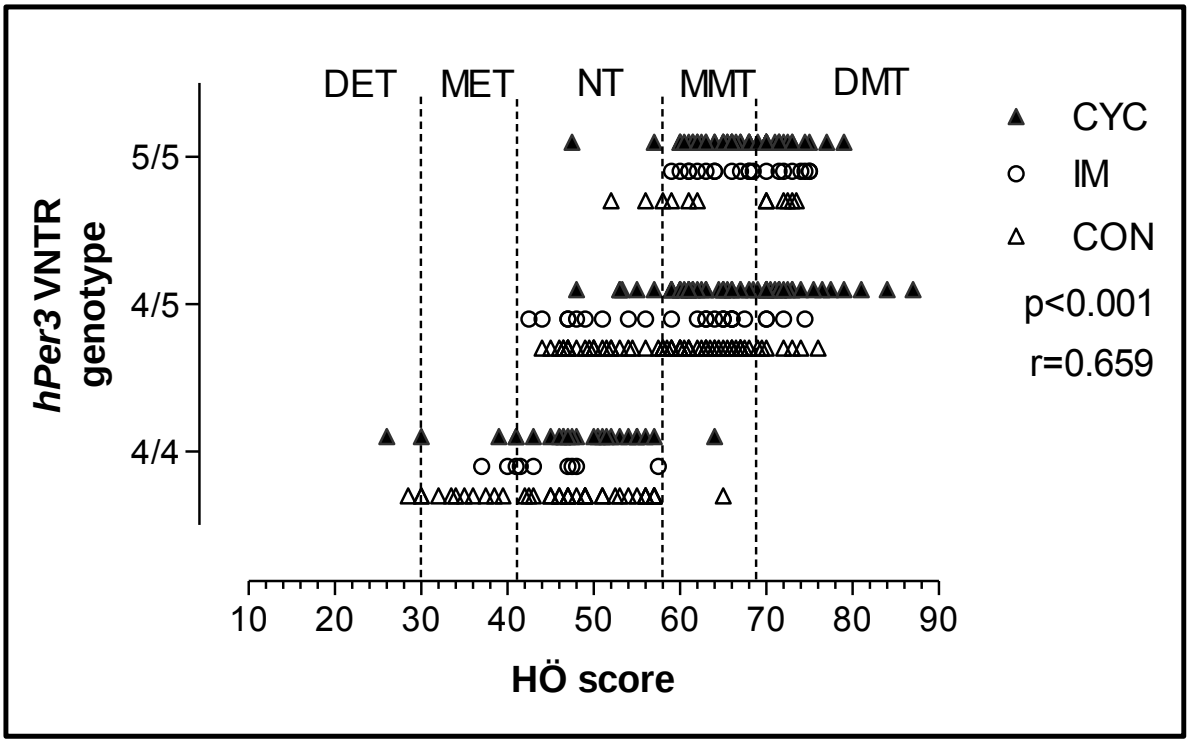

Figure 13: Relationship between *PER3* VNTR genotype and the HÖ-MEQ score in the CYC (n=138), IM (n=52) and CON (n=120) groups. DET: definite evening-type, MET: moderate evening-type, NT: neither-type, MMT: moderate morning-type, DMT: definite morning-type, VNTR: variable number tandem-repeat, 5/5: $PER3^{5/5}$, 4/5: $PER3^{4/5}$, 4/4: $PER3^{4/4}$, CYC: Cyclist group, IM: Ironman triathlete group, CON: Control group and HÖ: Horne-Östberg. The p-value represents the correlation significance determined by a Pearson's correlation test.

3.2 Study 2

3.2.1 Participant characteristics

Based on results from study 1, trained cyclists were invited to participate in this follow-up study to compare performance characteristics of morning- and evening-type cyclists at different times of the day. Specifically, we were able to recruit 20 cyclists for the morning group (MORN) who were classified as DMT or MMT based on the HÖ-MEQ score, and who were genotyped as either homozygous for the 5-repeat allele (*PER3*$^{5/5}$) or heterozygous (*PER3*$^{4/5}$) for the VNTR polymorphism (Figure 14). Using the same criteria for the evening group, only three cyclists qualified and only one of the three agreed to participate in this follow-up study. Thus NT cyclists homozygous for the 4-repeat allele (*PER3*$^{4/4}$) were invited to participate based on their genotype and the final sample size for this group was four (Figure 14). This is not ideal since, although a strong correlation has been noted between the HÖ-MEQ score and *PER3* VNTR polymorphism (Archer et al., 2003), we acknowledge that genotype alone, especially of a single gene, does not necessarily define the behavioural phenotype. This group was named the designated evening-type group (dEVE), since not all were categorised as ET based on their HÖ-MEQ score.

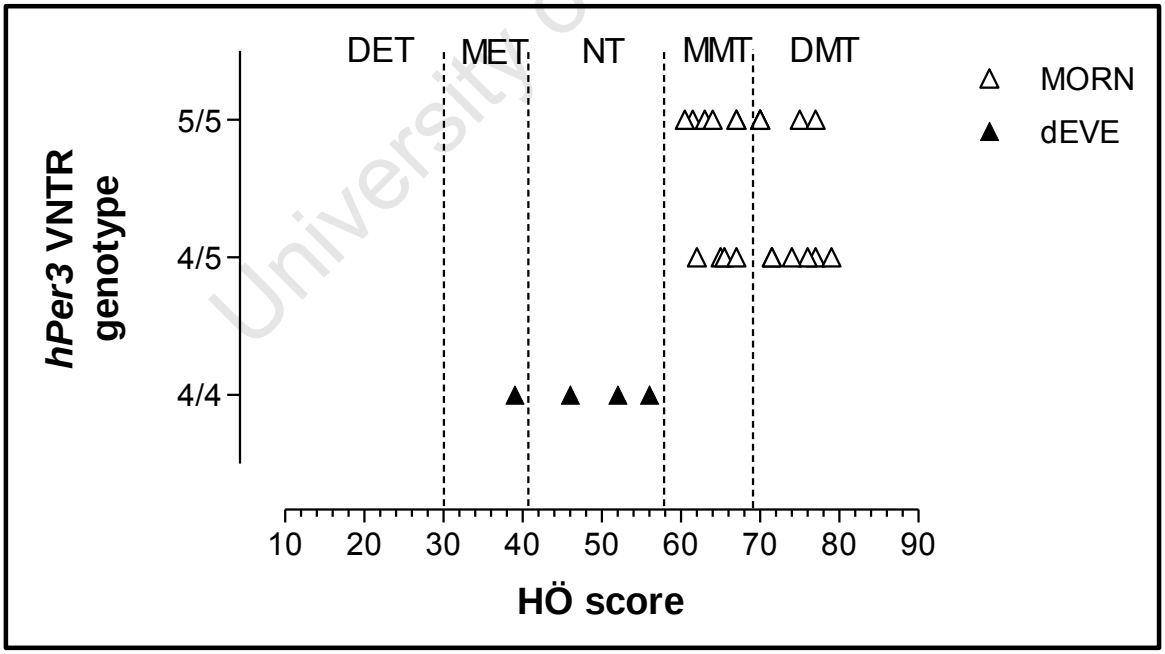

Figure 14: Relationship between *PER3* VNTR genotype and the HÖ-MEQ score of the MORN (n=20) and dEVE (n=4) cyclist groups. DET: definite evening-type, MET: moderate evening-type, NT: neither-type, MMT: moderate morning-type, DMT: definite morning-type, 5/5: *PER3*$^{5/5}$, 4/5: *PER3*$^{4/5}$, 4/4: *PER3*$^{4/4}$, MORN: morning-type group, dEVE: designated evening-type group and VNTR: variable number tandem-repeat.

Individual characteristics for cyclists who took part in study 2 are shown in Table 6. By design the MORN group had a significantly higher HÖ-MEQ score compared to the dEVE group (p=0.002). The two groups were matched for all other general characteristics.

Table 6: General characteristics of the cyclists who participated in the study comparing the performance of trained cyclists preferring either mornings (MORN) or evenings (dEVE) at different times of the day.

	MORN (n=20)	dEVE (n=4)	p-value
HÖ-score	69.2±5.6	48.3±7.4	**0.002**
Age (years)	39.5±8.1	37.0±8.1	0.651
Weight (kg)	81.0±12.5	81.4±8.6	0.974
Height (m)	1.80±0.08	1.80±0.05	0.450
BMI ($kg \cdot m^{-2}$)	25.0±2.7	26.0±3.0	0.590
Body fat (%)	20.6±9.2	21.0±5.1	0.852
Sum of 7 skinfolds (mm)	73.9±21.9	75.7±23.3	0.861
Absolute PPO (W)	354.0±51.1	342.0±30.0	0.684
Relative PPO ($W \cdot kg^{-1}$)	4.42±0.58	4.30±0.20	0.642
VO_2max ($ml \cdot kg^{-1} \cdot min^{-1}$)	50.5±7.6	50.4±4.4	0.910
HR_{max} (bpm)	181.9±9.0	187.8±7.8	0.394

The data are represented as mean ± standard deviation. MORN: morning-type group, dEVE: designated evening type-group, HÖ: Horne-Östberg, BMI: body mass index, PPO: peak power output, VO_2max: maximal oxygen uptake and HR_{max}: maximal heart rate. The p-values indicate significance as determined by a Mann Whitney-U test.

3.2.2 Participant racing and training history

Training and racing history data of the MORN and dEVE cyclist groups are presented in Table 7. Despite the big range in the number of years training, the cyclists in both groups had trained for a similar number of years, and did not differ in current (i.e. three months prior to participation) training frequency, duration and distance. Similarly, the number of years that the MORN and dEVE cyclists had participated in recreational level racing was not significantly different, and their current race frequency and personal best time for a 110 km race were also similar.

Table 7: Training and racing history of the MORN and dEVE cyclists

		MORN (n=20)	dEVE (n=4)	p-value
Training	**No. of years**	13.7±7.9 (5.8-21.6)	12.0±6.5 (5.5-18.5)	0.281
	Frequency (d·wk^{-1})	4.0±1.4	4.0 ±1.3	0.984
	Duration (h·wk^{-1})	9.65±2.50	7.40±1.14	0.523
	Distance (km·wk^{-1})	255.0±67.0	200.0±38.0	0.221
Racing	**No. of years**	9.6±7.1	6.0±4.0	0.413
	No. of races (Past 2 yrs)	14.3±7.7	12.8±10.0	0.820
	PB time (h)	3.20±0.31	3.17±0.20	0.932

The data are represented as mean ± standard deviation with the range in parentheses. MORN: morning-type group, dEVE: designated evening-type group, PB: personal best time for a 110 km cycle race. The p-values represent significance between the MORN and dEVE cyclist groups as determined by a Mann Whitney-U test.

3.2.3 Training, food and sleep monitoring 24 hours prior testing

Participants were asked to avoid vigorous training sessions for the 24 h period prior to each testing session (06h00, 10h00, 14h00, 18h00 and 22h00). This was monitored by asking them to log training done within this period and to rate the intensity of session using a scale of 1-10, where 1 was very light intensity and 10 was very high intensity. Therefore, each cyclist's training intensity was the average of the five readings given before each of the five testing sessions. Both groups trained at similar, low-to-moderate intensities prior to testing (MORN: 4.4±0.8 and dEVE: 4.0±0.8, p=0.456). Furthermore, the diary indicated that all participants maintained a similar dietary pattern for the 24 h period prior to each testing session and that they avoided caffeine three hours prior to testing. Finally the MORN and dEVE cyclists had equal amounts of sleep the night before each testing session (MORN: 7.17±0.95 h and dEVE: 7.00±1.02 h, p=0.477).

3.2.4 Diurnal variation in body temperature

Oral body temperature (OBT) was measured as a proxy for core body temperature at hourly intervals in the waking hours of the 24 h period prior to each of the five (06h00, 10h00, 14h00, 18h00 and 22h00) testing sessions using a digital thermometer. Therefore, each cyclist's OBT for a given time point was the average of five readings. Hourly OBT for the MORN and dEVE groups are shown in Figure 15. A repeated-measures ANOVA showed no significant time-by-group interaction effect in the MORN and dEVE cyclist groups (p= 0.136). However, using a two-way ANOVA a significant time-by-group interaction effect in

the peak OBT phases of the two groups ($p<0.001$) was found. The OBT peak for the MORN cyclist group is significantly earlier compared to that of the dEVE cyclist group. We were unable to determine the OBT nadir of participants as they were sleeping during this period.

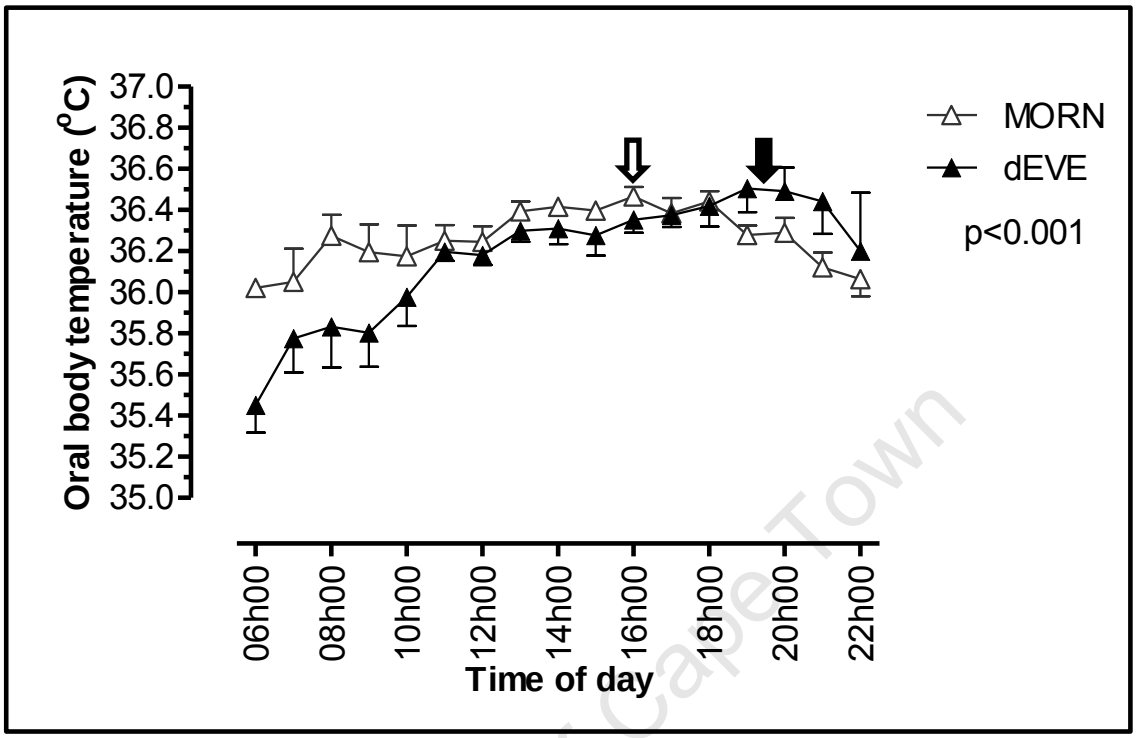

Figure 15: Changes in oral body temperature of the MORN (n=20) and dEVE (n=4) groups during the waking hours of a 24 h period. The arrows on the graph indicate the peaks for each group. MORN: morning-type group (open arrow), dEVE: designated evening-type group (closed arrow). The p-value represents time-by-group interaction effect in the phases as determined by a two-way ANOVA. The error bars indicate standard error of the mean.

3.2.5 Time-of-day effect on attention

Cyclists performed the Attention Network Test (ANT) immediately before the Lamberts and Lambert Sub-maximal Cycle Test (LSCT) at each of the five testing sessions. A Friedman ANOVA test was computed on the MORN and dEVE cyclist groups' four markers of attention (reaction time, accuracy, alerting and orienting) to determine if there was a time-of-day effect on attention (Figure 16). No time-of-day effect was observed in the MORN and dEVE cyclists in any of the variables. The data in Table 8 shows the number of individuals who recorded their fastest and slowest reaction times at a given time point. For example, eight MORN cyclists had their fastest reaction time scores at 06h00, while ten had their slowest time recorded at 22h00. Of the eight whose reaction time was fastest at 06h00, six of them were DMT individuals according to the HÖ-MEQ score and of the seven MORN cyclists whose reaction time was slowest at 06h00 (Table 8), five of them were MMT and three were

DMT. Furthermore, of the ten individuals whose reaction time was slowest at 22h00, seven of them were DMT and three were MMT. No clear pattern in reaction time was observed for the dEVE cyclist group.

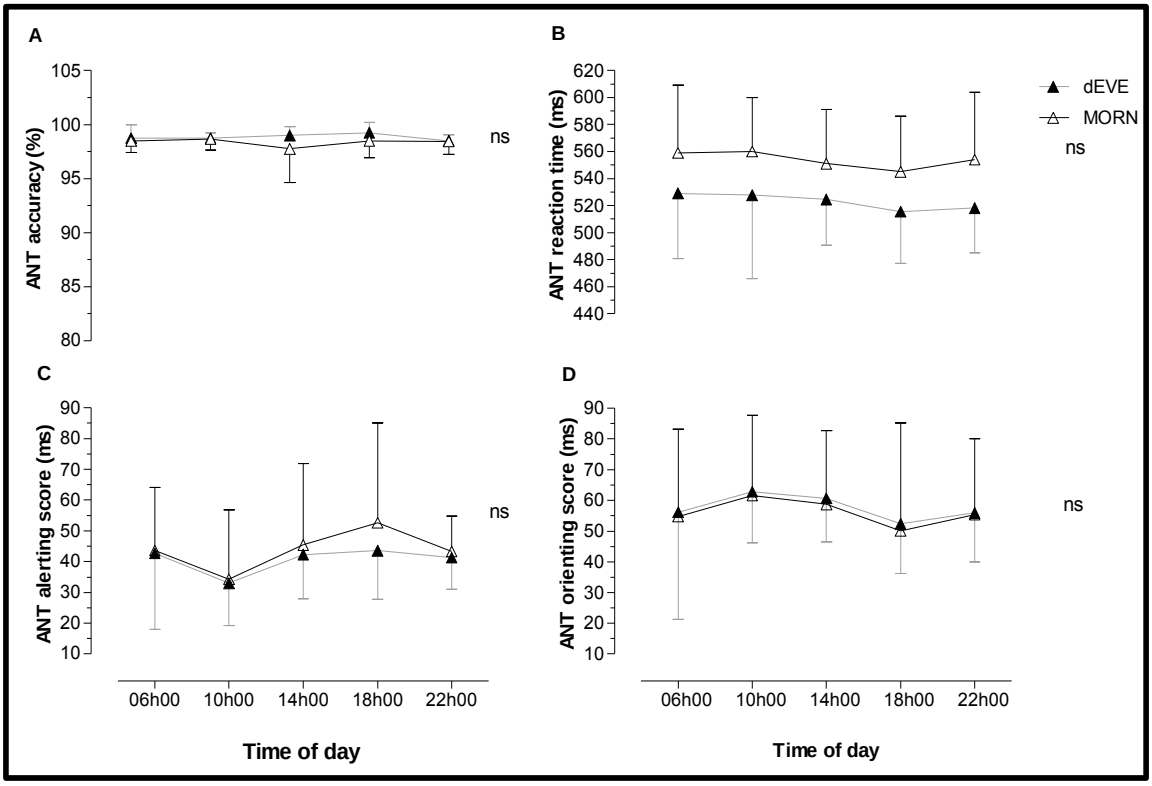

Figure 16: Diurnal variation in markers of attention in the MORN (n=20) and dEVE (n=4) as measured using the Attention Network Test (ANT) in (A) Accuracy, (B) Reaction time, (C) Alerting and (D) Orienting. MORN: morning-type group, dEVE: designated evening-type group, ns: not significant, represents the time-of-day effect as determined by a Friedman ANOVA test.

Table 8: The number of MORN and dEVE cyclists recording their fastest and slowest reaction time scores at different times of the day

	MORN (n=20)		dEVE (n=4)	
	Fastest	**Slowest**	**Fastest**	**Slowest**
06h00	8 (40%)	7 (35%)	1 (25%)	2 (50%)
10h00	5 (25%)	1 (5%)	1 (25%)	
14h00	4 (20%)	1 (5%)		1 (25%)
18h00	3 (15%)	1 (5%)	1 (25%)	
22h00		10 (50%)	1 (25%)	1 (25%)

The data are presented as actual numbers with percentages in parentheses. MORN: morning-type group and dEVE: designated evening-type group.

3.2.6 Lamberts and Lambert Sub-maximal Cycle Test

Cyclists rode at 60% of HR_{max} for six minutes in stage 1 of the test. In stage 2 they rode at 80% of HR_{max} for a further six minutes, and then at 90% of HR_{max} for three minutes in stage 3, while RPE, speed, power and cadence were being measured.

3.2.6.1 Time-of-day effect on rating of perceived exertion (RPE)

The time-of-day effects on RPE during stages 1, 2 and 3 of the LSCT are shown in Figure 17. There was a time-of-day effect for RPE during stage 1 in the MORN cyclist group (p=0.010), but not for the dEVE group (p=0.262). Furthermore, significant time-of-day effects were observed for stages 2 and 3 of the LSCT in both groups. The RPE score of the MORN cyclist group was lower in the morning and increased in the later part of the day. In contrast, that of dEVE participants was higher in the morning and gradually decreased during the day. Specifically, a Mann Whitney-U test indicated that the differences in RPE for the MORN and dEVE groups occurred at 10h00, (p=0.047), 18h00, (p=0.015) and 22h00, (p=0.008) during stage 2, and at 06h00 (p=0.025) for stage 3.

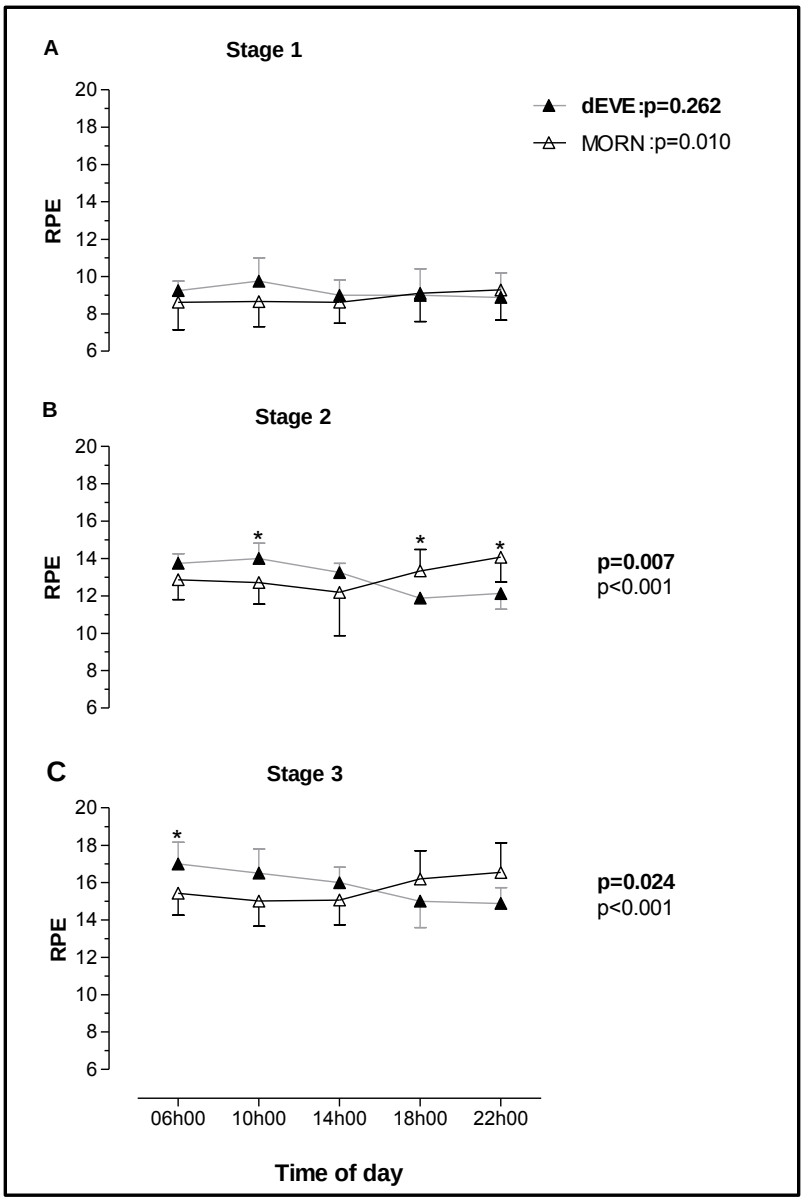

Figure 17: Time-of-day effects on rating of perceived exertion (RPE) during stages 1 (A), 2 (B) and 3 (C) of the Lamberts and Lambert Sub-maximal Cycle Test in the MORN (n=20) and dEVE (n=4) groups. MORN: morning-type group, dEVE: designated evening-type group. The p-values represent time-of-day effects for each group as determined by a Friedman ANOVA test. * indicates the time points at which RPE was significantly different between the MORN and dEVE groups as determined by a Mann Whitney-U test.

3.2.6.2 Time-of-day effect on power output

There was a time-of-day effect for both absolute and relative power output in the MORN cyclist group during stage 1 of the LSCT. However, no time-of-day effects were observed for absolute or relative power output of both groups during stages 2 and 3 of the LSCT (Figure 18).

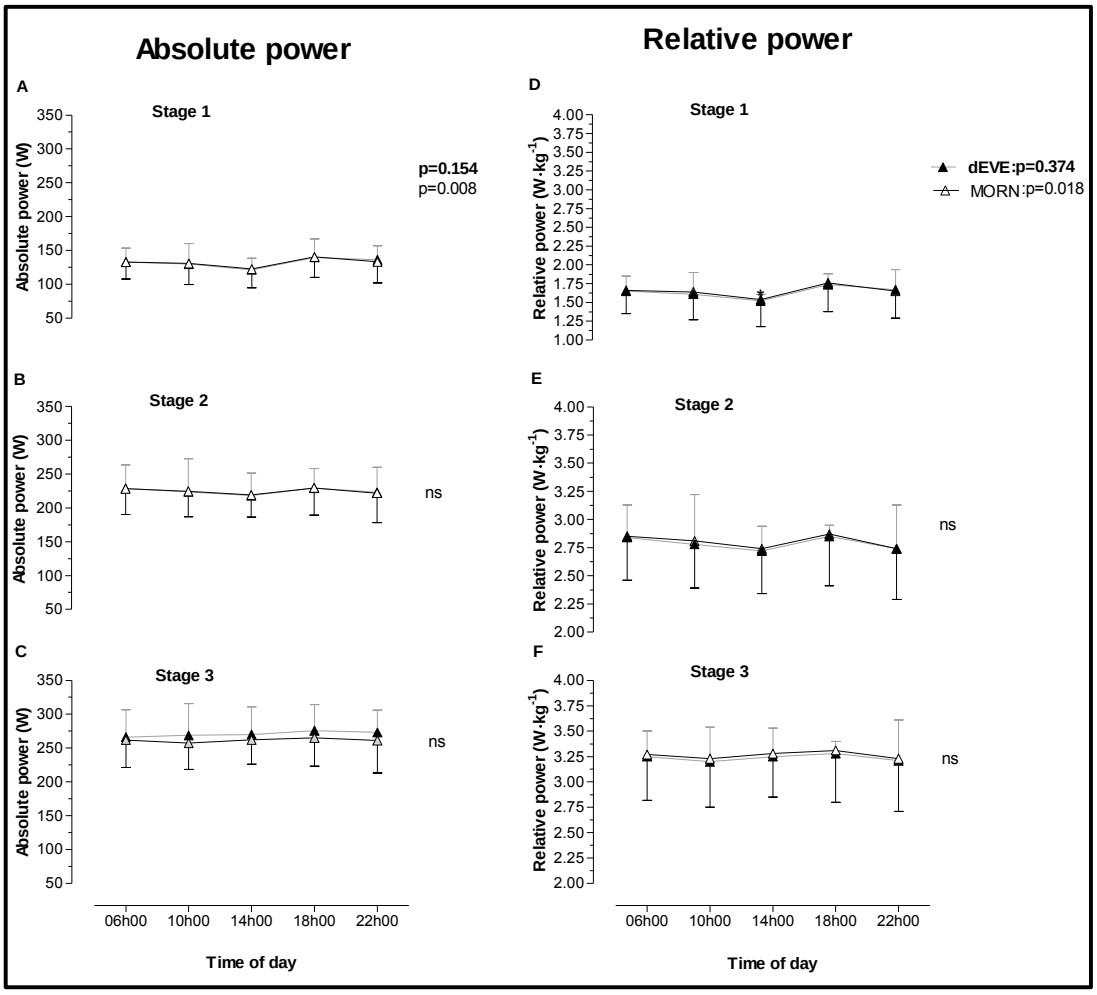

Figure 18: Time-of-day effect on absolute and relative power output during stages 1 (A and D), 2 (B and E) and 3 (C and F) of the Lamberts and Lambert Sub-maximal Cycle Test in the MORN (n=20) and dEVE (n=4) groups. MORN: morning-type group, dEVE: designated evening-type group. ns: not significant, represents time-of-day effect as determined by a Friedman ANOVA test. Note: the graphs are almost superimposed in stages 1 and 2 of the LSCT.

3.2.6.3 Time-of-day effect on normalised OBT, RPE and absolute power

Time-of-day changes in normalised OBT, RPE and absolute power for stage 3 of the LSCT are shown in Figure 19. Data were normalised to a neutrally chosen time point (14h00) for each individual and expressed as a percentage thereof. Figure 19B and 19C show general trends of how RPE and power varies with time-of-day as body temperature increases. The normalised RPE score for the MORN group is lower in the morning than the dEVE group and worsens with time-of-day, while that of the dEVE group improves with time-of-day as OBT increases. For example, the RPE for the MORN group is high at the beginning of the day because their OBT is higher than that of the dEVE group. Furthermore, their RPE appears to decrease with the time-of-day until OBT starts to drop later in the afternoon. In contrast, the

RPE for the dEVE group is higher in the morning because their body temperature is lower and their RPE decreases with time-of-day peaking at about 20h00 coinciding with the peak in OBT. There is an apparent dissociation between normalised OBT and RPE, and there seems to be an association between OBT and power. Specifically, it looks as though normalised power tracks normalised OBT for both groups. Relative power output does not seem to show a time of day effect.

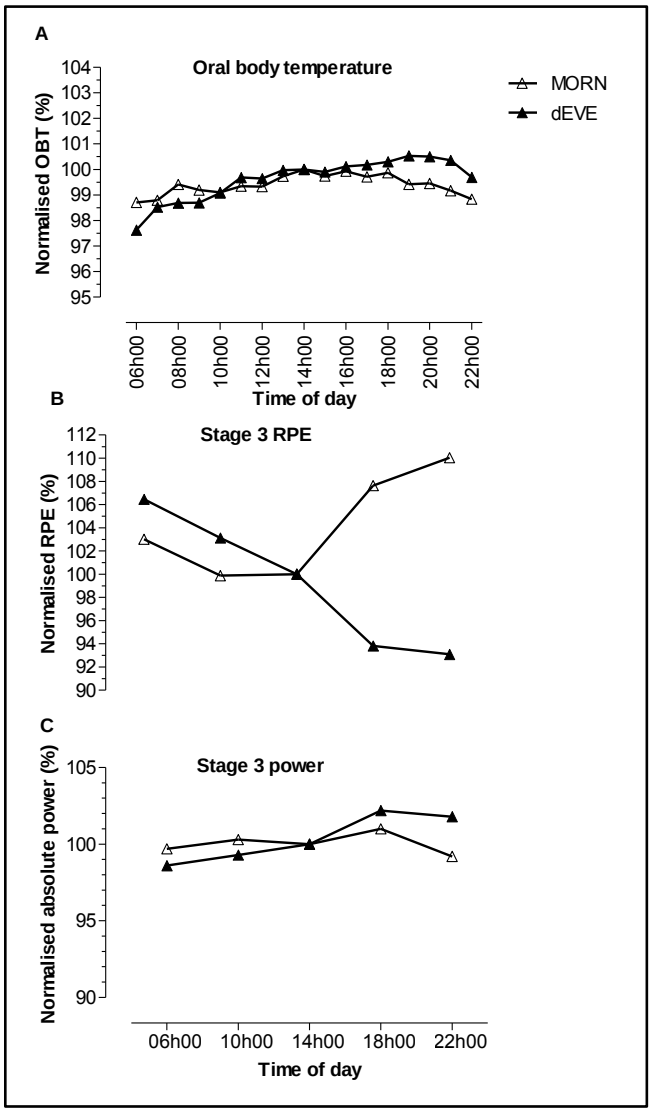

Figure 19: Time-of-day effects on (A) normalised oral body temperature, (B) normalised stage 3 rating of perceived exertion (RPE), and (C) normalised absolute power during stage 3 of the Lamberts and Lambert Sub-maximal Cycle Test in the MORN (n=20) and dEVE (n=4) groups. MORN: morning-type group, dEVE: designated evening-type group, OBT: oral body temperature and RPE: rating of perceived exertion. Note: error bars are not shown on these graphs as we were interested in only showing the trends.

3.2.6.4 Time-of-day effect on speed

There was no significant time-of-day effect for speed during any of the stages of the LSCT test in the MORN and dEVE cyclist groups (Figure 20A, B and C).

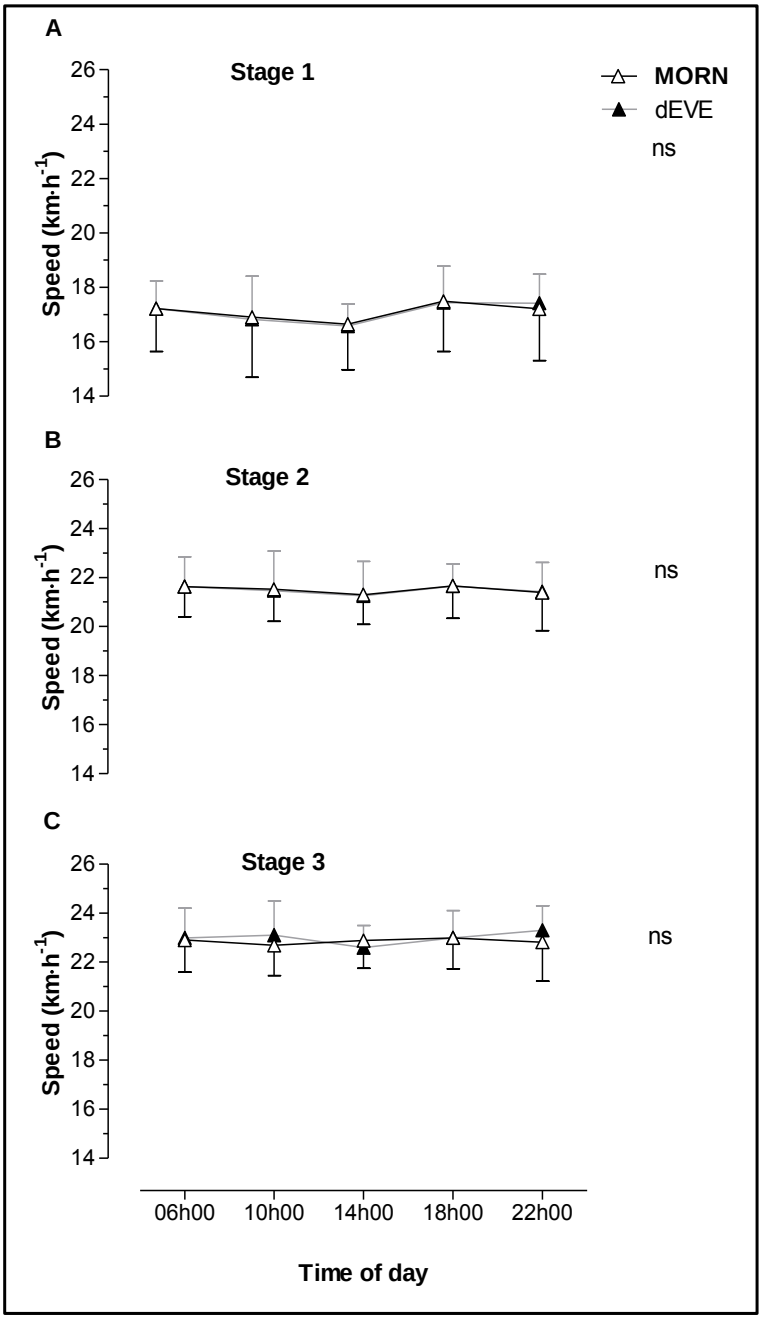

Figure 20: Time-of-day effect on speed during stages 1 (A), 2 (B) and 3 (C) of the Lamberts and Lambert Sub-maximal Cycle Test in the MORN (n=20) and dEVE (n=4) groups. MORN: morning-type group, dEVE: designated evening-type group. ns: not significant, represents the time-of-day effect as determined by a Friedman ANOVA test.

3.2.6.5 Time-of-day effect on cadence

There was no time-of-day effect observed for cadence in the MORN and dEVE cyclist groups at any of the stages of the LSCT (Figure 21A, B and C).

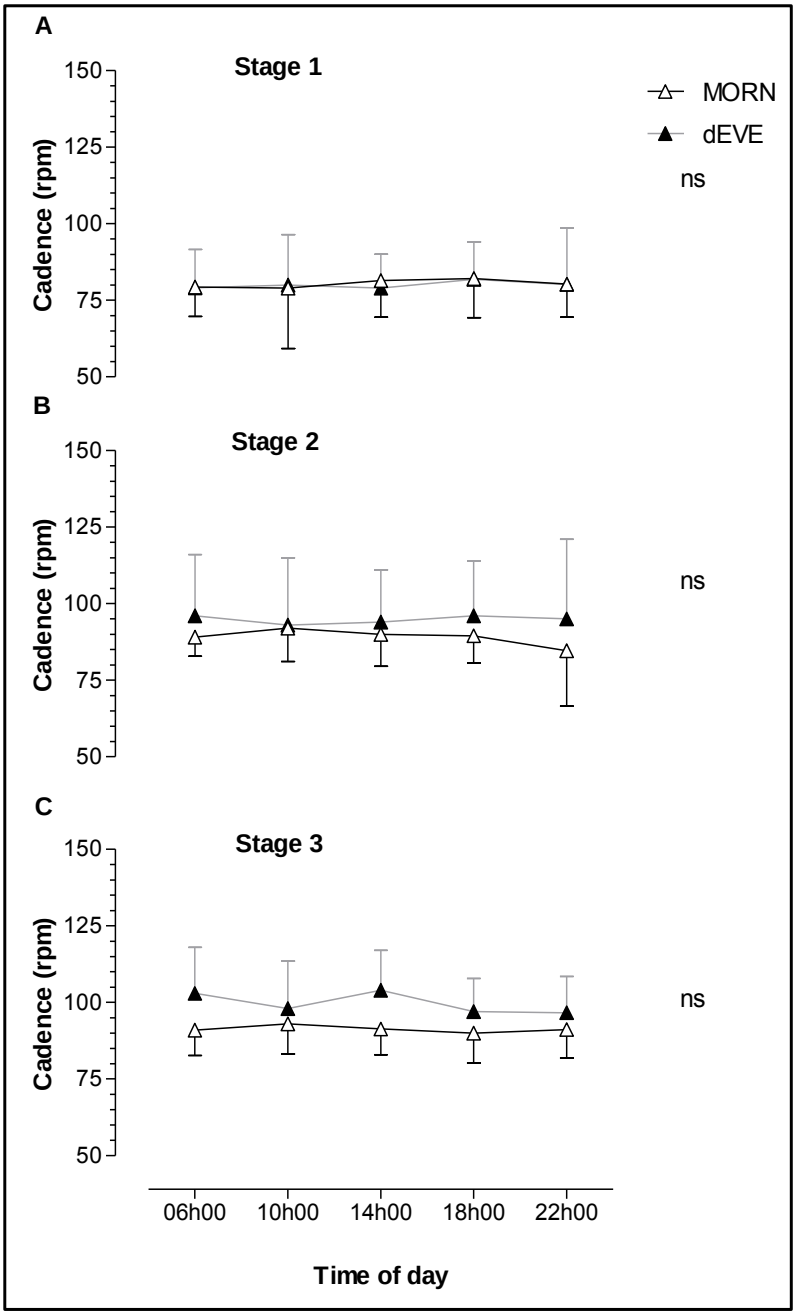

Figure 21: Time-of-day effect on cadence during stages 1 (A), 2 (B) and 3 (C) of the Lamberts and Lambert Sub-maximal Cycle Test in the MORN (n=20) and dEVE (n=4) groups. MORN: morning-type group, dEVE: designated evening-type group. ns: not significant, represents the time-of-day effect as determined by a Friedman ANOVA test.

3.2.6.6 Predicted peak power output, 40 km time trial time and VO_2max

Peak power output (PPO), 40 km time trial (TT) time and maximal oxygen uptake (VO_2max) were predicted based on the cyclists' power output generated during stage 3 of the LSCT and used as proxies for performance. There were no significant time-of-day effects for any of these predicted variables (Figure 22A, B and C).

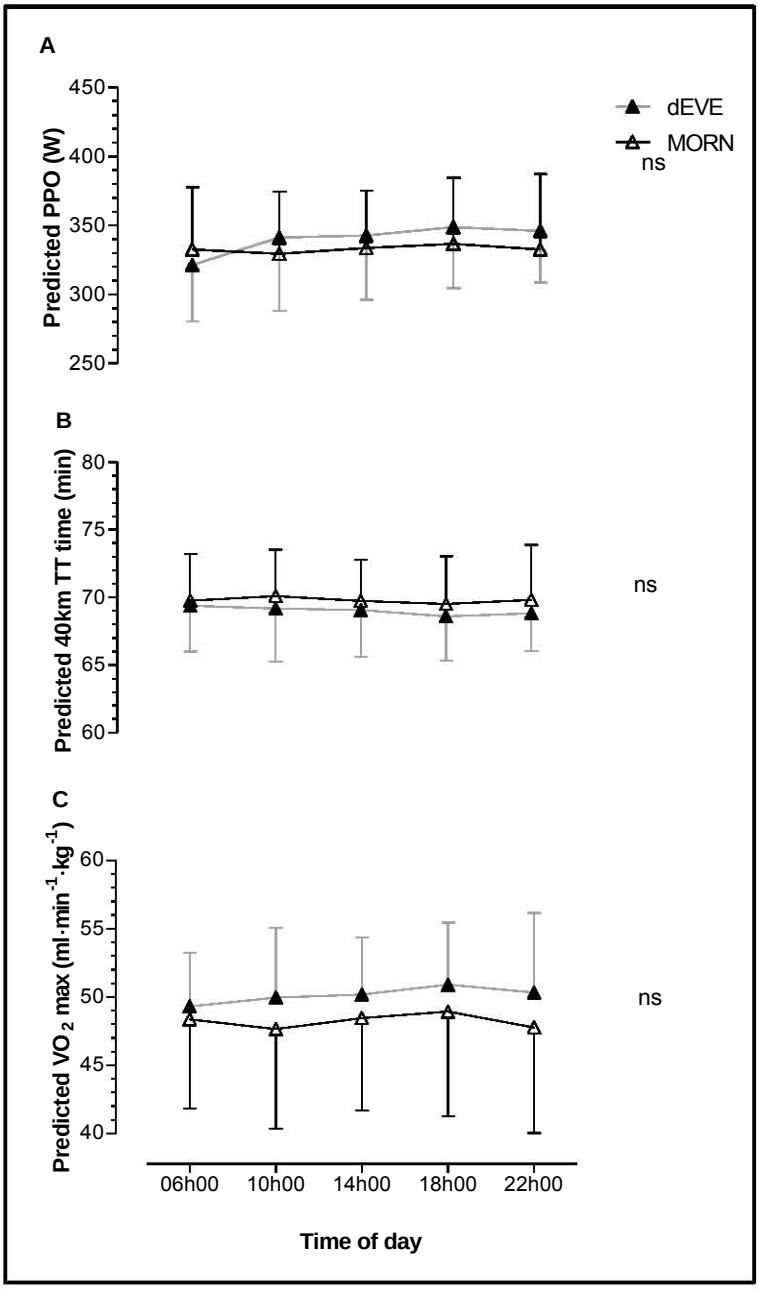

Figure 22: Time-of-day effects on predicted PPO (A), 40 km TT time (B), and VO_2max (C) based on absolute power produced during stage 3 of the Lamberts and Lambert Sub-maximal Cycle Test in the MORN (n=20) and dEVE (n=4) cyclist groups. MORN: morning-type group, dEVE: designated evening-type group, PPO: peak power output, TT: time trial, and VO_2max: maximal oxygen uptake. ns: not significant, represents time-of-day effect as determined by a Friedman ANOVA test.

3.2.6.7 Heart recovery rate (HRR) post exercise

HRR was assessed by measuring the decrease in HR for 90 seconds following the end of the LSCT test (from 15:30-16:30 min). Therefore, each cyclist's HRR was the average of the five readings for each of the five testing sessions. Mean HRR was similar in the MORN (42.6±9.2 bpm) and dEVE (42.8±8.6 bpm, p=0.824) groups and there was no time-of-day effect for HRR for the two groups as determined using a Friedman ANOVA test (Figure 23)

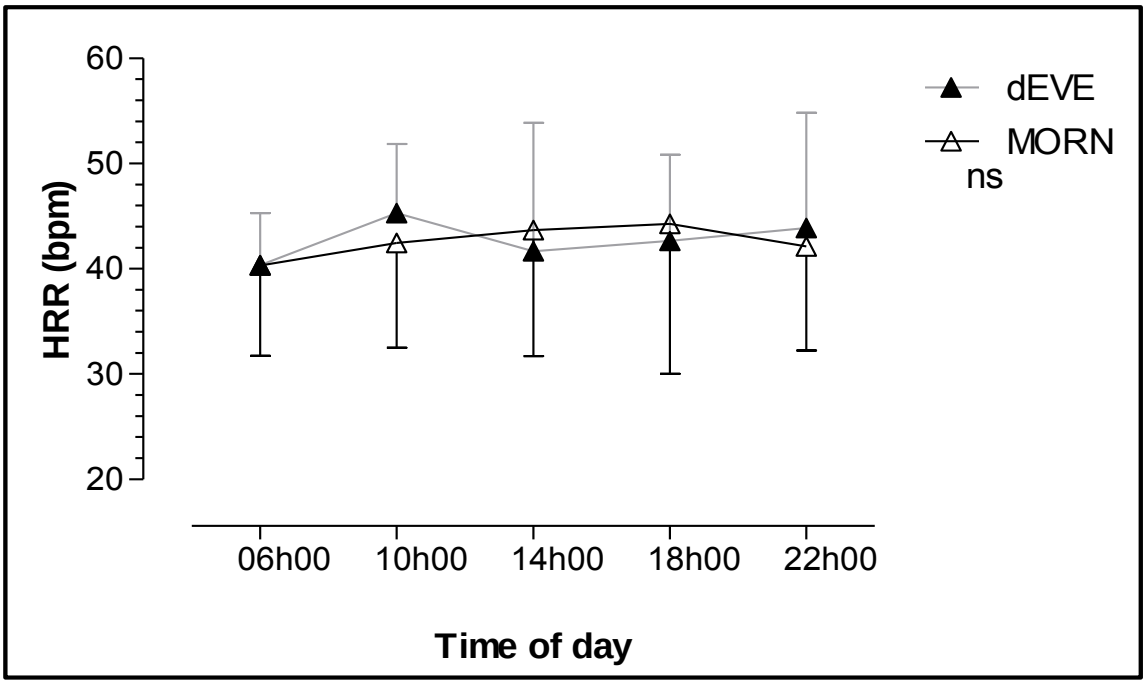

Figure 23: Time-of-day effect on heart recovery rate (HRR) following the Lamberts and Lambert Sub-maximal Cycling Test in the MORN (n=20) and dEVE (n=4) cyclist groups. MORN: morning-type group, dEVE: designated evening-type group, bpm: beats·min^{-1}, HRR: heart recovery rate, ns: not significant, represents the time-of-day effect as determined by a Friedman ANOVA test.

CHAPTER 4
DISCUSSION

This is the first study, to the best of my knowledge, to investigate the association between genotype and diurnal preference in well-trained, recreational athletes. The initial finding of this study was that the diurnal preference of trained populations of Caucasian male cyclists and Ironman triathletes was significantly different compared to a control group of active, but non-competitive Caucasian males (Figure 11). Specifically, the athletic groups contained more morning-types compared to the control group. In addition, morning-type was the dominant diurnal phenotype (between two thirds and three quarters) in the athletic populations whereas very few athletes where characterised as evening-types (less than 10%). While the control group had a similar proportion of evening-types compared to the athletic groups, just less than half of the control group were morning-types with the dominant category being neither-type. However, all three groups showed skewed distributions with more morning-type and fewer evening-type individuals, which is different to most other populations world-wide (Zavada et al., 2005, Tonetti et al., 2010, Chelminski et al., 1997).

For example, many studies have found that neither-types are the predominant diurnal phenotype, but that evening-types are more common than the morning-types. Studies conducted by Zavada et al. (2005) showed a 33:55:14 ratio of evening- to neither- to morning-types in a Dutch Caucasian population. Furthermore, Tonetti et al. (2010) and Chelminski et al. (1997) showed a 24:65:11 and 30:62:8 ratio of evening- to neither- to morning-types in Spanish and European American Caucasian populations, respectively. In contrast to these studies, Paine et al. (2006) found similar proportions to the control group, but not to the athlete groups in this study, showing a 6:45:49 ratio of evening- to neither- to morning-types in a New Zealand Caucasian population. Therefore, the diurnal preferences of both athletic and active, but non-competitive South African Caucasian males are quite different to other populations around the world.

One reason for the differences may be due to societal/cultural differences between South Africa and other countries. For example, the average school start time in Southern Africa is 07h45 vs. school start time in many parts of Europe and the United States of America being 09h00. Furthermore, in Brazil, where most of the population tends to be evening-types,

people are observed to start their personal activities in the evening (after work) and thus are more active and alert late at night, around 23h00 (Hidalgo et al., 2002). The same may be true for many European populations. Therefore, perhaps individuals are conditioned to being more morning-type or evening-type in nature by the society in which they live. Since the HÖ-MEQ scoring is based on answers where precise times are stated, societal practices may lead to a distortion of scores in different countries (Smith et al., 2002, Drennan et al., 1991). Another reason for the finding may be geographical differences between countries. Specifically, it is the difference in latitude which may be important, since this influences the photoperiod experienced in a given country during a given season. For instance, in northern European Russia (southern Komi Republic), the photoperiod is longer in summer (16 h light: 8 h dark) (Borisenkov et al., 2010) compared to an average of 13 h light: 11 h dark in South Africa; and at 22h00 individuals may still be active, while in South Africa at that time-of-day many individuals could already be asleep.

Another explanation may be that our study looked at recreationally competitive male athletes, while many others have just taken cross-sections of the population, not taking into account sport or activity status. Lastly, a plausible explanation for the high number of morning-types in the athletic populations could be that repeated early morning training/competition may habituate individuals to become morning-types. There were very few evening-type individuals (less than 10%) in the athletic populations, suggesting that cycling and triathlons are individual sports that may attract individuals preferring mornings. Furthermore, it could be possible that even though evening-type individuals might take part in these types of sports, they do not maintain their engagement to become competitive in them due to the training and competition scheduling times. Morning-type individuals may find it easier to adapt and perform at the early times demanded by these disciplines. Thus, the diurnal phenotype of an individual may influence sport selection.

This is consistent with a previously published Australian study within a group of 23 Caucasian triathletes where no evening-type individuals were found (Lastella et al., 2010). These authors proposed that this was because evening-type athletes may not choose to take part in sports that require early morning training. Naturally, evening-type individuals have later bed times, thus they may find it difficult to wake-up early in the morning to train/compete even when they sleep early. Since an athlete's ability to maintain exercise intensity in physical sporting activities has been shown to adapt to his/her training time (Hill

et al., 1989, Cappaert, 1999), it would be beneficial for evening-type individuals to train in the morning, because individual sport races (i.e. cycling, running and triathlons) are usually scheduled at this time-of-day in South Africa. The reason for this may be that ambient temperatures are generally lower in the morning, and cooler temperatures are considered safer and allow for faster race times (Waterhouse et al., 2005). Expected summer temperature highs which are attained in the early afternoon can be in the region of 38^{o}C. Low ambient temperatures help maintain homeostasis in many physiological variables, while hot environments result in slower race times (Galloway and Maughan, 1997, Noakes, 2000). High ambient temperatures of the afternoon, in combination with metabolic heat produced through exercise, can modify the CBT of an individual (Dewasmes et al., 2003), possibly leading to a critically high CBT that may adversely affect performance. In high ambient temperatures the balance between heat loss and heat generation becomes more critical as there is less capacity to lose heat through sweating or radiation. The body naturally reduces metabolic heat production by forcing the individual to slow down and to prevent the irreversible damage of fundamental cellular processes.

The second finding of this study was that the athletic populations contained more individuals who were homozygous for the longer variant of the gene (*PER3*$^{5/5}$) compared to the control group, and the frequency of the 5-repeat allele was higher (60%) compared to the control group's (40%). In contrast, the control group had a high frequency (60%) of individuals who were homozygous for the shorter variant of the gene (*PER3*$^{4/4}$), which is in line with the results in other published studies (Pereira et al., 2005, Archer et al., 2003, Ciarleglio et al., 2008, Nadkarni et al., 2005). For example, studies conducted by Ciarleglio et al. (2008) demonstrated a high frequency of the 4-repeat allele in the European American (66%), African American (59%) and Ghanaian African (64%) populations. It is possible that the result of this study is different to others, due to random selection rather than a selection factor for the *PER3* allele in recreationally, well-trained athletes compared to the control group in the South African Caucasian male population. This is particularly interesting considering that genetic studies conducted by Jenkins (2005) indicated that even though the 5-repeat allele is common to all primates, it is the least frequent as it is the latest to emerge from the *PER3* coding region.

The third finding of this study was that there was a positive correlation between diurnal phenotype and genotype. Specifically, the *PER3* VNTR polymorphism genotype in this study showed a strong correlation with the HÖ-MEQ score (Figure 13). That is, individuals genotyped as $PER3^{5/5}$ were more likely to be categorised as moderate or definite morning-types, and those genotyped as $PER3^{4/4}$ were more likely to be scored as moderate or definite evening-types. This is in line with previous studies that indicated that diurnal preference appears to have a genetic component: the *PER3* VNTR polymorphic region has been associated with diurnal preference (Archer et al., 2003) and DSPS (Ebisawa et al., 2001). If it were the case that the training regime was driving a morning-type phenotype, we might expect that the *PER3* VNTR genotype would not necessarily match the phenotype in these athletic populations. This finding supports the link between diurnal preference and the *PER3* VNTR polymorphic region reported by Archer et al. (2003).

This suggests that the *PER3* gene is a good predictor of diurnal preference in humans and may be used alongside the HÖ-MEQ to determine an individual's diurnal phenotype. The realisation of a robust link between the *PER3* VNTR polymorphism and diurnal preference in humans prompts exploration into how genotype differences may contribute to diurnal phenotypic differences observed. One hypothesis may involve differential PER3 phosphorylation. The amino acid residues in the VNTR polymorphic region greatly influence residue recognition by kinases (Ebisawa et al., 2001, Archer et al., 2010) and each of the 4- and 5-repeat alleles has multiple potential casein kinase 1ε phosphorylation motifs. Phosphorylation by casein kinase 1ε is enhanced by the phosphorylation of the lead serine or threonine in the recognition motif. There are fewer amino acid residues in $PER3^4$ protein available as phosphorylation substrates due to a missing repeat. Thus, the $PER3^4$ protein may be phosphorylated to a lesser extent, resulting in a longer circadian period of abundance. Conversely, the $PER3^5$ protein is turned over at a faster rate allowing the cycle to start again, resulting in a shorter circadian period of its abundance.

We are not sure how *PER3* affects the overall circadian period. In this study, in order to correctly identify the 4-repeat vs. 5-repeat alleles, I exploited the 3110 (T→C) nucleotide substitution that is exclusive to the 4-repeat allele. This substitution results in an amino acid change from methionine to threonine, hence the (M1037T) polymorphism. I was also able to distinguish between the 4-repeat alleles that would encode 1037T or 1037M. It is possible that the threonine in $PER3^{4T}$ may provide an additional phosphorylation site over $PER3^{4M}$,

resulting in a slightly faster turnover. Furthermore, it may be true that the PER3^{4T} may have the same functional mechanism as the PER3^{5}, the analysis was beyond the scope of this thesis, but we observed that many individuals who had higher scores on the HÖ-MEQ and heterozygous for the *PER3* VNTR polymorphism carried the *PER3*^{4T} (56.4%) compared to PER3^{4M} (43.6%) in all groups studied.

In order to determine if a particular diurnal genotype-phenotype categorisation might have a performance advantage over another, morning- and evening-type cyclists who carried the *PER3* allele associated with morningness and eveningness, respectively, were invited to participate in a series of performance tests at different times of the day. This study was unique because we assessed both the subjective (i.e. using the HÖ-MEQ) and genetic aspects of diurnal phenotype and performance whereas other studies have only looked at the subjective component (using HÖ-MEQ) of diurnal phenotype and performance. The body clock produces oscillatory performance patterns that are linked to the physiological changes associated with the time-of-day, such as body temperature (Weinert and Waterhouse, 2007, Kerkhof and Van Dongen, 1996, Wright et al., 2002). Specifically, physical performance has been shown to peak when CBT is near its circadian peak (Waterhouse et al., 2005, Baxter and Reilly, 1983).

There were differences in the peak OBT phases between the MORN and dEVE groups: specifically, the peak in OBT of the MORN group occurred earlier compared to that of the dEVE group. This is consistent with previous studies that have demonstrated an earlier core or oral body temperature phase in the morning-type individuals compared to that of the evening-type individuals (Vidacek et al., 1988, Kerkhof and Lancel, 1991, Pati and Gupta, 1994, Horne and Östberg, 1976). This finding is indicative of the influence of the endogenous clock in regulating body temperature in morning- and evening-types during the 24 h day (Baehr et al., 2000), suggesting that the physiology of these two groups was different. This further justifies the use of individuals who were subjectively neither-type, yet had been genotyped as *PER3*$^{4/4}$ corresponding to an evening preference in the dEVE cyclist group. This is mainly so, since based on the genotype these individuals were similar to those categorised as evening-types using the HÖ-MEQ (Figure 15). During the LSCT, HR was fixed and the cyclists were free to change gears or to vary cadence and power output in order to maintain a HR of either 60%, 80% or 90% of HR_{max} for stages 1, 2 and 3 of the test, respectively. The main finding was that the MORN group reported lower RPE scores in the morning and higher

scores in the evening for stages 2 and 3, suggesting that cycling at higher intensities (i.e. 80 and 90% of HR_{max}) was easier in the morning and felt more difficult in the later part of the day. The reverse was true for the dEVE group. The use of perceived exertion offers an excellent method to study and understand human behaviour during sport and exercise. In addition, perceived exertion is thought to be the single best indicator of the sensation of physical strain and has been shown to complement physiological measures (MacKinnon, 1999, Glass and Chvala, 2001, Crewe et al., 2008). Furthermore, perceived exertion is reliable at cycling workloads of about 130 bpm and 170 bpm (Borg and Linderholm, 1970, Faulkner and Eston, 2007) corresponding to stages 2 and 3 of the LSCT in this study. RPE may vary depending on modalities, intensity, environmental conditions and duration of exercise (Crewe et al., 2008, Borg, 1973).

Performance studies using graded exercise testing, time trials, as well as training in rehabilitations have shown a linear relationship between HR and RPE (Gutmann and Meyer, 1981, Albertus et al., 2005, Chang et al., 2008, Green et al., 2007). Furthermore, some studies have shown a linear relationship between RPE and VO_2max with increasing exercise in a time to exhaustion test and during a sub-maximal cycling test (Al-Rahamneh and Eston, 2011, Buckley et al., 2004, Faulkner and Eston, 2007, Borg, 1998). Very few studies have been conducted to determine if RPE varies with time-of-day in morning- and evening-type individuals (Hill et al., 1989, Reilly and Baxter, 1983, Reilly and Brooks, 1990). The fact that we have shown a difference in RPE at different times of the day when HR was fixed suggests that RPE and HR can be dissociated. This finding offers additional support for the concept of a motivational component affecting performance at different times of the day (Reilly and Baxter, 1983). Thus, individuals may perform better at the time-of-day when they feel at their best, suggesting that the MORN group may have been motivated (intrinsic) to perform in the morning compared to the dEVE cyclist group.

Normalised RPE for the MORN group was lower in the morning compared to that of the dEVE group, which appeared to improve with time-of-day following the peak in OBT (Figure 19). Furthermore, the normalised power output for both groups appeared to follow the peak in OBT and appeared to peak at 18h00 in the later part of the day. This may be because muscle strength and flexibility which help determine power output among other things, may have been at their peak in the later part of the day (Reilly et al., 2007, Coldwells et al., 1994). The next finding was that in contrast to RPE, the relative and absolute power output, speed and

cadence of both the MORN and dEVE groups did not differ with time-of-day when cycling at a fixed HR. Furthermore, contrary to expectations, relative and absolute power output, speed and cadence did not seem to differ between the MORN and dEVE groups with time-of-day at any stage of the LSCT. It is remarkable that the MORN and dEVE group's absolute and relative power output, speed and cadence graphs are almost superimposable. This result suggests that at a fixed intensity of exercise, absolute power output, speed and cadence were more strongly associated with HR than with any time-of-day variation. No time-of-day effects on predicted 40 km TT time, PPO and VO_2max were observed. This seems intuitive since the variables are predicted from power output produced during stage 3 of the LSCT, which was shown not to vary in a time-of-day manner.

There is no doubt that a direct measure of performance (such as a time trial) would have been the best way to detect time-of-day performance differences between the morning-type and evening-type cyclists. However, such a protocol would have been open to serious performance confounders, such as training status, life stress, illness, as well as participant drop-out. Therefore, the LSCT was chosen since it allowed testing to take place within a short timespan (two weeks), thereby helping to eliminate confounding effects on performance, while simultaneously allowing for the prediction of a 40 km TT performance. Inter-individual differences in these predicted variables and the larger variability in the measured power output at stage 3 of the LSCT may be responsible for the inability to detect differences in these variables between the MORN and dEVE groups at the 5% significance level. In addition, performance variables have been shown to exhibit a higher intra-individual variability (Cappaert, 1999) than physiological variables, since performance depends largely on an individual's will power, for example, motivation.

The result of VO_2max is particularly interesting because very few studies have shown a significant difference using trained athletes. VO_2max is the highest rate of oxygen consumption obtainable during exercise (Brisswalter et al., 2007, Xu and Rhodes, 1999, Al-Rahamneh et al., 2011). Diurnal variation in VO_2max response to light- or moderate-intensity levels of exercise has not been consistently reported (Martin et al., 2001, Reilly and Brooks, 1990). There are a few studies that suggest that VO_2max peaks in the late afternoon when CBT is near its peak (Hill et al., 1988, Giacomoni et al., 1999). Numerous other studies have failed to show a significant difference in VO_2max between trained athletes (Stephenson et al., 1982, Hill et al., 1989, Burgoon et al., 1992, Powers et al., 1985). To date, significant changes

in VO_2max have mostly been observed in cycling studies examining the effects of prior exercise (Brisswalter et al., 2007, Torii et al., 1992) and only a few in trained athletes (Hill et al., 1988, Hoogeveen, 2000). Similarly, many studies have failed to show a clear time-of-day difference in PPO using trained cyclists, time trials, time to exhaustion and sub-maximal cycling tests (Reilly and Down, 1992, Bessot et al., 2011, Bessot et al., 2006, Balmer et al., 2000).

HRR is a measure of the speed at which HR returns to resting state after cessation of moderate-to-heavy intensity exercise (Shelter, 2001) and is generally used as a measure of fitness and/or training status. HRR is faster in trained compared to untrained athletes after exercising at similar exercise intensities (Short and Sedlock, 1997, Lamberts et al., 2009b). The increase in HR during exercise is mainly due to the withdrawal of the parasympathetic nervous tone and an increase in the sympathetic tone. Therefore, HRR at the end of exercise is thought to be a function of the reactivation of the parasympathetic nervous system, since the sympathetic system continues into the early phases of recovery (Kannankeril et al., 2004). An abnormal autonomic nervous system response in terms of HRR post exercise is a good indicator of athletes who are fatigued and those at risk of cardiac diseases (Lamberts et al., 2009b, Lamberts et al., 2004, Hedelin et al., 2000). In this study we used HRR for two reasons. The first was to determine whether there was a time-of-day effect on recovery from a standardized bout of exercise in the MORN and dEVE cyclist groups, and the second was to monitor the training status of the cyclists throughout the trial.

No time-of-day differences were observed for HRR in either group, suggesting that regulation of the autonomic nervous system may not be dependent on time-of-day. The fact that HRR was similar between the two groups throughout the study suggests that training status of the cyclists did not change sufficiently to compromise or improve performance over the two weeks of observation. In addition to the physiological measurements, we also measured the attention of cyclists as a proxy for cognitive function before the start of each LSCT. Attention was investigated by asking participants to complete the computer-based ANT test before the start of each testing session. The data from the ANT test showed no differences in attention between the two groups at different times of the day. Furthermore, no time-of-day differences were observed within individual groups. Attention is the cognitive process responsible for selecting relevant stimuli, responses, memory and thoughts, while obstructing irrelevant information (Raz et al., 2004, Frascina, 2004, Fan et al., 2002) and varies with time-of-day,

seasons, and emotional states (Rueda et al., 2005, Gasper and Clore, 2002, Wright et al., 2002). The ANT has been used to test for time-of-day differences in attention in other studies (Reilly et al., 1997b, Blatter et al., 2006, Blatter and Cajochen, 2007, Wright et al., 2002). We expected to see a time-of-day effect on attention and one reason for not observing such differences may be that this test was not sensitive enough to detect these subtle changes, given the large inter-individual variation as evidenced by the large standard deviations. Alternatively, this result suggests that time-of-day did not differentially influence attention in the MORN and dEVE groups. Finally, given the very small changes in performance that we wished to detect, it was important to control for possible confounders on performance. Two such confounders include sleep deprivation and caffeine ingestion.

Sleep deprivation is thought to lead to an increase in homeostatic sleep drive, which subsequently impairs various aspects of cognitive and physical performance (Daniels et al., 1998, Hobson, 2005, Reilly and Walsh, 1981). While there is conflicting data regarding caffeine (Smith, 2002, Lieberman, 1992, Loke, 1988, Green and Suls, 1996), some studies have shown that it may improve performance (Bell et al., 2001, Marshall, 2010, Walsh et al., 2010, Rogers and Dinges, 2005). Therefore, the cyclists were encouraged to have at least six hours of sleep prior to each testing session and to abstain from taking caffeine within three hours prior testing. Controlling for these possible confounders, as achieved through the use of a diary kept for the 24 h period prior to testing, therefore increased the likelihood that any time-of-day differences observed between these groups could reasonably have been attributed to the effects of the body clock or diurnal preference.

4.1 Limitations

The HÖ-MEQ scoring is based on answers where precise times are stated, and thus it does not allow for flexibility: individuals are constrained in the answers they can give. This may account for the differences in proportions of evening-type individuals found in this study compared to others. The finding for the dEVE group has to be treated with caution because of the very small sample size. Furthermore, since the dEVE group included three neither-types, it is possible that these individuals may have influenced both cognitive and physical performance as they may not have had a particular time-of-day preference. Another possible limitation could be that we used a sub-maximal test as a proxy for a time trial, when an actual time trial could have been the best to measure direct changes in performance.

4.2 Practical applications and future work

Awareness of an athlete's diurnal preference could possibly enhance the competitive edge of the athlete in races, especially where training times can be matched to competition times. Furthermore, knowledge of an athlete's diurnal preference might allow him/her to schedule physically demanding tasks at his/her preferred training time-of-day (i.e. time-of-day where low RPE scores were observed) and leave the least demanding tasks to any part of the day or when he/she can. Understanding the body clock in particular, may prove helpful today when there is increased connectivity and inter-dependence between global societies, resulting in activity schedules that may not be in line with one's circadian rhythms. Since the dEVE cyclist group used in performance studies included three neither-types as part of the group, in future it will be ideal to have moderate or definite evening-type individuals, homozygous ($PER3^{4/4}$) for the 4-repeat allele or heterozygous ($PER3^{4/5}$) for the *PER3* VNTR polymorphism, in order to accurately compare the performance of morning- and evening-type cyclists. The *PER3* VNTR polymorphism varies between ethnic populations (Paine et al., 2006, Frascina, 2004, Ciarleglio et al., 2008, Barbosa et al., 2010, Nadkarni et al., 2005), thus further studies would be invaluable in describing the distribution of the polymorphism in other ethnic groups within the South African population. Therefore, in future we intend to extend the study to the black and coloured (mixed race) South African populations.

4.3 Summary and Conclusions

This study shows that a South African Caucasian male population of trained athletes participating in individual sports contains more individuals with a preference for mornings than a control group of active, but non-competitive individuals. Furthermore, 60% of the athletic populations had the 5-repeat allele of the *PER3* gene compared to 40% of the control population. Both the genotype and phenotype distributions in all populations measured in this study were different to other populations around the world, suggesting that the level of athletic activity may be related to diurnal preference, but also perhaps the cultural/societal differences between South Africa and other countries. A strong correlation was found between phenotype and genotype for both morning- and evening-type individuals extending the work of Archer et al. (2003). The MORN and dEVE cyclist groups were distinct with respect to their circadian rhythms as evidenced by their 24 h CBT phases. In addition, they perceived the same relative intensity of exercise to be different at different times of the day. It is possible that since most of the cycle races are scheduled in the morning (i.e. a time-of-day

when morning-types may perceive effort to be easier) in South Africa, perhaps morning-type cyclists persist with or are drawn to the sport because it feels easier for them to perform at this time-of-day. Furthermore, we speculate that the over-representation of morning-types in the athletic populations studied here is not because they are able to perform better than evening-types, but rather only because people select sports practiced at times of day that match their diurnal phenotype. A future study based on both phenotype and genotype would need to compare the performance of morning-type and evening-type individuals in the field (i.e. in real race situation). Most of the results obtained here were expected, however in future it will be ideal if an independent study could examine performance in a different type of athletic population such as rugby. This study suggests that, appreciation of the relationship between diurnal preference and preferred time-of-day of training/practice may lead to a better understanding of inter-individual differences in athletic performance in particular sports.

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APPENDICES

Appendix A1: Participant information sheet

PARTICIPANT INFORMATION SHEET

Dear Volunteer,

Thank you for agreeing to participate in the University of Cape Town's study entitled **Diurnal preference and Sports performance: A genetic and subjective view (Part 1)** to be conducted by researchers from the UCT/MRC Research Unit for Exercise Science and Sports Medicine within the Department of Human Biology and the Department of Molecular and Cell Biology.

Background

Human physiology, metabolism and behaviour are coordinated, controlled and influenced by circadian rhythms (i.e. 24 hour day/night cycles). Furthermore there appear to be genetically determined inter-individual differences in circadian rhythm. While it has been well established that elements of performance in athletes are affected by circadian rhythm, the extent to which these disruptions affect individuals who prefer mornings compared to those who prefer evenings has not been established. We hypothesise that well-trained and elite populations may contain different proportions of individuals with a preference for mornings or evenings compared to the general population.

Therefore, the **aims of this research** are:

1. To describe the distribution of individuals preferring either mornings or evenings in
    (a) a healthy, regularly active population of gym-goers,
    (b) well-trained cyclists,
    (c) Ironman triathletes and
    (d) well-trained runners
2. To genotype the same four populations for the variable number tandem repeat (VNTR) polymorphism within the *Per3* gene.

Procedures

You will be asked to complete a General Questionnaire (personal details, medication / supplement use, training history and cycling, triathlon or running racing history) and the Horne-Östberg Morningness / Eveningness personality questionnaire. The investigator will then measure your weight and height and take a buccal sample from the inside of your cheeks using a sterile swab. This is a non-invasive process. DNA will be extracted from your buccal sample to establish your genotype for the VNTR polymorphism within the *Per3* gene. This will enable us to determine whether, from a genetic perspective, you are more of an "Owl" or a "Lark" regarding your preference for a particular time of day. We anticipate that this study will lead to a better understanding of inter-individual athletic performance. Perhaps in the future, with studies investigating the role of circadian rhythm on training efficacy, this area of research could improve management of athletes' training and health based on our understanding of the role of the internal molecular clock and chronobiological principles.

Potential Risks

There are no risks related to donating a buccal sample for subsequent DNA analysis.

Benefits

You will receive feedback as to your preference for either mornings or evenings as established by the Horne-Östberg questionnaire, as well as the general results of the study once it has been completed in its entirety.

Ethical considerations
This study will be performed in accordance with the principles of the Declaration of Helsinki (October 2008, Seoul), ICH and South African Good Clinical Practice (GCP) guidelines, the laws of South Africa. The UCT Research Ethics Committee (please see contact information below) has approved this study.

Participants will not be included unless they have signed a consent form, after the investigator has provided substantial verbal and written explanation of the study, including any potential risk factors. You are invited to ask the investigator any questions you may have relating to the tests and the procedures throughout the study. Participation in the study is entirely voluntary and you have the right to withdraw from the study at any time without stating a reason. The investigator may also withdraw you from the study at any time.

Your DNA sample will only be used for the purposes explained to you, namely to determine your genotype for the VNTR polymorphism within the *Per3* gene, and will be destroyed on completion of the Circadian Rhythm and Sports Performance study. You may request that your blood and DNA sample be destroyed before the completion of the study.

Privacy, confidentiality and liability
All records and results generated from this study will be stored in a computer database in a secure facility, and in a manner that maintains your confidentiality. All participants will remain anonymous in any ensuing publication of the results of the study in a peer-reviewed scientific journal. Finally, the University of Cape Town has a no-fault insurance or public liability cover should some unforeseen event occur whilst you are participating in this study.
Please do not hesitate to contact us should you require any additional information. Our contact information is listed below.

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Appendix A2: Informed consent

INFORMED CONSENT

ALL PARTICIPANTS TO READ AND SIGN

I, the undersigned, have been fully informed about the University of Cape Town's study entitled **Diurnal Preference and Sports Performance: A Genetic and Subjective View (Part 1)** to be conducted by researchers from the UCT/MRC Research Unit for Exercise Science and Sports Medicine within the Department of Human Biology and the Department of Molecular and Cell Biology.

I agree to complete a questionnaire disclosing my personal details and information relating to my training and racing history. I understand that the following measurements / tests **may** be conducted on me during this study, as described in Participant Information document:

- Body composition assessment – height and weight measurements.
- Buccal samples will be obtained from the insides of my cheeks using sterile swabs. DNA will be extracted from these samples to establish my genotype for the VNTR polymorphism within the *Per3* gene.

I have been fully informed about the risks inherent in participation in this trial. I understand that my DNA sample will only be used for the purposes explained to me, namely to determine my genotype for the VNTR polymorphism within the *Per3* gene, and will be destroyed on completion of the Circadian rhythm and sports performance study.

I understand that all the information collected during the study will be treated confidentially, will only be used for scientific research purposes and that my name and personal particulars will not be released under any circumstances.

I have been informed that I will be free to withdraw from the study at any time if I so wish without explanation. I also understand that I may request that my samples are destroyed before the completion of the study. I will be free to ask any questions about the procedures and results of the study. I understand that I will receive, where applicable, feedback pertaining to my morning-evening personality type as well as general results of the study once the entire study has been completed.

I agree to participate in the study.

Participant's name: _____

Signature: _____

Investigator's name: _____

Signature: _____

Witness's name: _____

Signature: _____

Date: _____

Appendix A3: Horne-Östberg morningness-eveningness personality questionnaire

Horne-Östberg morningness-eveningness personality questionnaire

N A M E : ____

INSTRUCTIONS

a) Please read each question very carefully before answering.
b) Answer ALL twenty questions.
c) Answer questions in numerical order.
d) Each question should be answered independently of others. **DO NOT** go back and check your answers.
e) For some questions, you are required to respond by placing a cross alongside your answer. In such cases, select **ONE** answer only.
f) Please answer each question as honestly as possible. Both your answers and results will be kept in strict confidence.

QUESTION 1

Considering your own feelings about when you are "at your best", at what time would you get up if you were entirely free to plan your day?
Time: .....

QUESTION 2

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

QUESTION 3

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

a. Not at all dependent ..... ☐
b. Slightly dependent ..... ☐
c. Fairly dependent ..... ☐
d. Very dependent ..... ☐

QUESTION 4

Assuming adequate environmental conditions, how easy do you find getting up in the morning?

a. Not at all easy ..... ☐
b. Slightly easy ..... ☐
c. Fairly easy ..... ☐
d. Very easy ..... ☐

QUESTION 5

How alert do you feel during the first half hour after having woken in the morning?

a. Not at all alert ☐
b. Slightly alert ☐
c. Fairly alert ☐
d. Very alert ☐

QUESTION 6

How is your appetite during the first half hour after having woken in the morning?

a. Not at all good ☐
b. Slightly good ☐
c. Fairly good ☐
d. Very good ☐

QUESTION 7

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

a. Very tired ☐
b. Slightly tired ☐
c. Fairly refreshed ☐
d. Very refreshed ☐

QUESTION 8

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

a. Seldom or never later ☐
b. Less than one hour later ☐
c. 1-2 hours later ☐
d. More than 2 hours later ☐

QUESTION 9

You have decided to engage in some physical exercise. A friend suggests that you do this one hour twice a week and the best time for him/her is between 7.00-8.00 am. Bearing in mind nothing else but your own inclinations, how do you think you would perform?

a. Would be on good form ☐
b. Would be on reasonable form ☐
c. Would find it difficult ☐
d. Would find it very difficult ☐

QUESTION 10

At what time in the evening do you feel tired and in need of sleep?

Time: .....

QUESTION 11

You wish to be at your peak for a test which you know is going to be mentally exhausting and last for two hours. You are entirely free to plan your day. When would you do this task?

a. 8.00 am – 10.00 am ☐
b. 11.00 am – 1.00 pm ☐
c. 3.00 pm – 5.00 pm ☐
d. 7.00 pm – 9.00 pm ☐

QUESTION 12

If you went to bed at 11.00 pm at what level of tiredness would you be at that time?

a. Not at all tired ☐
b. A little tired ☐
c. Fairly tired ☐
d. Very tired ☐

QUESTION 13

For some reason you have gone to bed several hours later than usual, but there is no need to get up at any particular time the next morning. Will you:

a. Wake up at the usual time and not go back to sleep ☐
b. Wake up at the usual time and doze ☐
c. Wake up at the usual time and go back to sleep ☐
d. Wake up later than usual ☐

QUESTION 14

One morning you have to remain awake between 4.00 am and 6.00 am in order to carry out a watch duty. You have no commitments the next day. Which ONE of the following alternatives suits you best?

a. Would NOT go to bed until 6.00 am ☐
b. Nap before 4.00 am and sleep after 6.00 am ☐
c. Sleep before 4.00 am and nap after 6.00 am ☐
d. Only sleep before 4.00 am and remain awake after 6.00 am ☐

QUESTION 15

You have to do 2 hours of hard physical work. If you were completely free to plan your day, and considering only your "feeling best" rhythm, which hours would you prefer to do it between:

a. 8.00 am – 10.00 am ☐
b. 11.00 am – 1.00 pm ☐
c. 3.00 pm – 5.00 pm ☐
d. 7.00 pm – 9.00 pm ☐

QUESTION 16

You have decided to engage in some physical exercise. A friend suggests that you do this between

10.00 pm and 11.00 pm twice a week. How do you think you would perform?

a. Would be on good form .....☐

b. Would be on reasonable form .....☐

c. Would find it difficult .....☐

d. Would find it very difficult .....☐

QUESTION 17

Suppose that you can choose your own work hours, but had to work FIVE hours in the day. Assume that your job is interesting and paid by results. Which FIVE CONSECUTIVE HOURS would you choose?

Hours…

QUESTION 18

At what time of day do you feel at your best?

Time: …..

QUESTION 19

One hears of "morning" and "evening" types. Which do you consider yourself to be?

a. Morning type .....☐

b. More morning than evening .....☐

c. More evening than morning .....☐

d. Evening type .....☐

QUESTION 20

At what time(s) of day do you regularly exercise?

Time: ……..

Appendix B1: Cyclits' scheduling options

DIURNAL PREFERENCE AND SPORTS PERFORMANCE: A MOLECULAR AND SUBJECTIVE VIEW

Cyclists' Scheduling Options

Visit	Option 1	Option 2	Option 3	Option 4	Option 5	Option 6
1	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
2	Thursday	Friday	Saturday	Sunday	Monday	Tuesday
3	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday
4	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
5	Wednesday	Thursday	Friday	Saturday	Sunday	Monday
6	Friday	Saturday	Sunday	Monday	Tuesday	Wednesday
Note	*No Sun*				*No Sat*	

Visit	Option 7	Option 8	Option 9
1	Sunday	Tuesday	Friday
2	Wednesday	Friday	Monday
3	Friday	Monday	Wednesday6
4	Sunday	Wednesday	Friday
5	Tuesday	Friday	Monday
6	Thursday	Monday	Wednesday
Note	*No Sat*	*No w/end*	*No w/end*

Option 8
Tuesday
Friday 10h00
Monday 4h00
Wednesday 1
Friday 22h00
Monday 06h00

Appendix B2: Training, diet, sleep and temperature dairy

TRAINING, DIET AND SLEEP DIARY

ALL participants to complete diary 24 hours prior to each exercise testing trial

Name: _____ Date: _____

Period time: _____ (e.g. 06h00 – 06h00) Test time: _____

Training

Exercise session type: _____

Time of day: _____

Duration: _____ (min)

Distance: _____ (km)

Intensity: _____ (use scale)

Exercise session type: _____

Time of day: _____

Duration: _____ (min)

Distance: _____ (km)

Intensity: _____ (use scale)

Sleep

What time did you go to sleep? _____ (AM / PM)

What time did you wake up? _____ (AM)

Body temperature (ºC)

05h00 _____	06h00 _____	07h00 _____
08h00 _____	09h00 _____	10h00 _____
11h00 _____	12h00 _____	13h00 _____
14h00 _____	15h00 _____	16h00 _____
17h00 _____	18h00 _____	19h00 _____
20h00 _____	21h00 _____	22h00 _____
23h00 _____	00h00 _____	01h00 _____
02h00 _____	03h00 _____	04h00 _____

Diet

Please log all items of food that you either ate or drank (including water) in this period, as well as any supplements or medication that you may have taken.

Time	Food/beverage type	Amount

Appendix B3: Perception of perceived effort during training

PERCEPTION OF EFFORT DURING TRAINING SCALE

Score	English	Afrikaans	Xhosa	Zulu
0	Rest	Rus	Ukhuphumula	Phumula
1	Really easy	Baie maklik	Ilula kakhulu	Kulula kakhulu
2	Easy	Maklik	Ilula	Kulula
3	Moderate	Matig	Phakathi	Kulula kahle
4	Sort of hard	Effens moeilik	Inobunzuma	Kululi-khuni nje!
5	Hard	Moeilik	Inzima	
6	HARD!	MOEILIK	INZIMA	Kulikhuni kabi
7	VERY HARD	BAIE MOEILIK	INZIMA KAKHULU	KULIKHUNI KAKHULU
8	The coach tried to kill us!	Die afrigter wou ons doodmaak	Umqeqeshi ubezama ukisibulala	Umqeqeshi ubezama ukisibulala
9	I feel like death warmed over!	Ek Voel asof ‘n trein my getrap het	Ndiva ngathi ukufa kunifikele	Ngizwa sengathi ngiyafa
10	Oh!	Ai!		Ngiyafa manje!

Use this scale to complete the 5-day dietary evaluation and the cycling and running training diary.