Circadian rhythm, activity level, training habits and sports performance: the molecular and subjective components

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DECLARATION

I, Kim Jenna Stephenson, hereby declare that the work on which this dissertation/thesis is based 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.

Signature: ……

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TERMS AND DEFINITIONS

The following list contains useful terms and definitions for concepts presented in this thesis:

- **Caucasian** - White individuals of European descent.

- **Chronotype** – Individual differences in the preferred times of waking, activity and rest (sleep).

- **Circadian rhythmicity** - Is a property of a biological process, driven by a circadian clock, which displays an oscillation of approximately 24-hours even in the absence of external time cues.

- **Conditioning** - A process of behaviour modification by which an individual comes to associate a desired behaviour with a previously unrelated stimulus.

- **Diurnal variation** – Fluctuations that occur throughout the day.

- **Endogenous rhythm** – Is a rhythm that occurs within an organism, repeats with an intrinsic period of approximately 24-hours and is said to be "free-running" when in the absence of time cues.

- **Eveningness** – evening preference.

- **Evening-type** – Individuals who are more alert at night and prefer to sleep late in the morning. Also known as "owls".

- **Exogenous component** – This component re-sets the circadian clock in order to ensure that it is entrained to external time or environmental time. The exogenous component can therefore be light, environmental temperature, food or feeding, and social factors, all of which are known as zeitgebers.

- **Genotype** - The combination of alleles located on homologous chromosomes that determines a specific characteristic or trait.

- **Habitual training** – The time-of-day during which one routinely and consistently trains.

- **Maximal performance** – The highest possible exercise capacity. Measured variables include maximum volume of oxygen inspired (VO_2max), heart rate maximum (HRmax) and testing includes maximum voluntary contractions (MVC) and time-trials, amongst others.

- **Morningness** – morning-preference.

- **Morning-type** – Individuals who are able to get up easily in the early morning and are more alert in the morning than in the evening. Also known as a "lark".

- **Neither-type** - Most individuals tend to fall in the middle portion of the chronotype spectrum ranging from morning-type and evening and have no particular preference for mornings or evenings.

- ***PERIOD3*** **(*PER3*)** – This gene is a member of the PERIOD family of genes and is expressed in a circadian pattern. Genes in this family encode components of the circadian rhythms of locomotor activity, metabolism, and behaviour. The specific function of this gene is not yet known.

- **Physical activity** - Is any bodily activity that enhances or maintains physical fitness and overall health and wellness**.**

- **Polymorphism** – A discontinuous genetic variation that results in different forms or types of individuals among the members of a single species.

- **Submaximal performance** – Exercise performance that is less than the maximum of which an individual is capable. Submaximal testing includes an exercise test that is halted at a pre-determined point that is less than the maximal exercise capability of the individual, usually at a particular percentage of the maximal heart rate or after a set time interval. For example, when pace is controlled during exercise testing.

- **Variable number tandem repeat (VNTR)** - A location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and often show variations in length between individuals.

ABBREVIATIONS

ANOVA	Analysis of variance
ASPS	Advanced sleep phase syndrome
BALM	Basic Languages Morningness scale
BC	Breath count
BMAL1	Brain and Muscle Aryl Hydrocarbon receptor nuclear translocator (ARNT)-like gene
BMAL1	Brain and Muscle Aryl Hydrocarbon receptor nuclear translocator (ARNT)-like protein
BMI	Body mass index
bp	Base pairs
BPD	Bipolar disorder
BSA	Bovine serum albumin
BT	Body temperature
CBT	Core body temperature
CG	Control group
CKIε	Casein Kinase I ε
CLOCK	Circadian Locomotor Output Cycles Kaput
CRY	Cryptochrome protein
CRY1	*Cryptochrome1* gene
CRY2	*Cryptochrome2* gene

CSM	Composite scale of Morningness
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
DSPS	Delayed sleep phase syndrome
DTS	Diurnal Type scale
EDTA	Ethylenediaminetetraacetic acid
EMG	Electromyography
ESSM	Exercise Science and Sports Medicine Research Unit
ET	Evening-type
EtBr	Ethidium bromide
ETG	Evening training group
FASPS	Familial advanced phase sleep syndrome
FRP	Free-running period
H-Ö	Horne-Östberg Morningness-Eveningness Personality questionnaire
HR	Heart rate
HWE	Hardy-Weinberg equilibrium
LOWM	Male low physical activity group
MAPK	Mitogen-activated protein kinase
MCTQ	Munich ChronoType questionnaire
MEG	Morning and evening training group
mRNA	Messenger-RNA

MT	Morning-type
MTG	Morning training group
MVC	Maximum voluntary contraction
NT	Neither-type
PAS	PER-ARNT-SIM domain
PB	Personal best
PCR	Polymerase chain reaction
PER	PERIOD protein
PER1	*PERIOD1* gene
PER2	*PERIOD2* gene
PER3	*PERIOD3* gene
***PER3*^{4}**	shorter allele of *PER3*
***PER3*^{44}**	4-allele of *PER3*
***PER3*^{45}**	4- and 5- alleles of *PER3*
***PER3*^{5}**	longer allele of *PER3*
***PER3*^{55}**	5-allele of *PER3*
POMS	Profile of Mood states questionnaire
PS	Early/Late Preferences scale
REV-ERBα	Orphan nuclear receptor
RHT	Retino-hypothalamic tract
RNA	Ribonucleic Acid

RPE	Rating of perceived exertion
RUNF FULL	Female marathon and ultra-marathon runner group
RUNF HALF	Female half-marathon runner group
RUNF	Female runner group
RUNM FULL	Male marathon and ultra-marathon runner group
RUNM HALF	Male half-marathon runner group
RUNM	Male runner group
SA	South African
SC	Stroke count
SCN	Suprachiasmatic nuclei
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
Train AM	Habitual morning training
Train PM	Habitual evening training
TT	Time-trial
UV	Ultra-violet
VNTR	Variable number tandem repeat
VO_2max	Maximum volume of oxygen inspired

w/v	mass/volume
WBC	White blood cells
v	Volume
y	years

ABSTRACT

BACKGROUND

Circadian rhythmicity, which is driven by a circadian clock, is a property of a biological process that displays an oscillation of approximately 24-hours even in the absence of external time cues. Individual differences in the preferred times of waking, activity and rest (sleep) are known as chronotype or diurnal preference; which arise due to differences in circadian rhythmicity due to the fact that rhythms are not exactly 24-hours. Various polymorphisms of certain genes involved in circadian rhythm generation have been associated with extreme chronotype. Of interest to this study is the *PER3* gene as it has a variable number tandem repeat (VNTR) polymorphism in the coding region, which is repeated either four of five times, encoding proteins of different lengths.

An association between chronotype and the *PER3* VNTR polymorphism has been demonstrated: the longer *PER3*^{5} allele has been associated with morning preference and the shorter *PER3*^{4} allele with evening preference. This association has been observed in various populations around the world as well as in a South African population of individual sports athletes. However, the literature regarding physical activity status and the effects of gender are areas less well defined.

Sports performance has been shown to be superior in the early evening, coinciding with the peak in core body temperature. The majority of the literature regarding sports performance has focussed on various components of sports performance such as strength and flexibility. In addition, a handful of studies have reported that chronotype and training time-of-day appear to contribute to intra-daily variations in performance. However, the extent to which chronotype and training time-of-day influence performance at different times of the day is largely unknown. Although one study has reported *PER3* VNTR genotype and chronotype distributions within an active South African population, there was no performance analysis. Therefore it would be interesting to explore the influence or effect of both *PER3* VNTR genotype and chronotype on diurnal variation in sports performance. The latter exploration, together with training habits are all logical factors to consider when analysing performance at different times of the day and could be imperative in explaining intra-daily variations in performance.

OBJECTIVE

Therefore, the focus of this thesis was two-fold. Firstly, to examine the distribution of chronotype and *PER3* VNTR genotype within populations of low physical activity males, male runners and female runners (Study 1). Secondly, to assess diurnal variation in swimming performance in South African masters swimmers (Study 2). More specifically, the aims were:

(i) To describe and compare the distribution of chronotype and *PER3* VNTR genotype in South African Caucasian males of low physical activity status versus runners (physical activity level comparison).

(ii) To describe and compare the distribution of chronotype and *PER3* VNTR genotype in South African Caucasian male and female runners (gender comparison).

(iii) To compare swimming performance and other variables at 06h30 and 18h30 in well-trained South African Caucasian male and female masters swimmers , with reference to chronotype, *PER3* VNTR genotype and habitual training time-of-day.

METHODS

Study 1

One hundred and one Caucasian male runners (RUNM) (mean age ± SD: 34.4 ± 8.1 y) were compared to 104 Caucasian male low-activity (LOWM) individuals (mean age ± SD: 33.3 ± 8.4 y). The RUNM group were also compared to 102 Caucasian female runners (RUNF) (mean age ± SD: 34.2 ± 7.8 y). This study examined the distribution of chronotype using the Morningness-Eveningness Personality questionnaire, designed by Horne and Östberg (1976), in populations of South African participants. Additionally, *PER3* VNTR genotype was determined in these populations using polymerase chain reaction (PCR) and *Nco*1 restriction digestion, according to Ebisawa *et al.* (2001) and Archer *et al.* (2003). The association between chronotype and *PER3* VNTR genotype was also assessed in these populations.

Study 2

Diurnal variation in 200 m swimming time-trial performance, rating of perceived exertion (RPE), mood state and stroke count (SC) and breath count (BC) were assessed in 26 trained masters

swimmers (mean age ± SD: 32.6 ± 5.7 y: 18 males and 8 females), with respect to chronotype, *PER3* VNTR genotype and habitual training time-of-day. RPE, mood state and SC and BC were also assessed at 06h30 and 18h30.

RESULTS AND DISCUSSION

Study 1

The LOWM group contained fewer morning-type (MT) individuals compared to the RUNM group (RUNM: 61%; LOWM: 41%, p=0.006). The prevalence of the *PER3*^{4} allele was greater in the LOWM group (RUNM: 30%; LOWM; 48%, p<0.001). The RUNM group contained fewer morning-types (MTs) compared to the RUNF group (RUNM: 61%; RUNF: 83%, p=0<0.001). More RUNM were genotyped as *PER3*^{45} (57%) compared to the RUNF (40%, p=0.025), and a greater number of RUNF were homozygous for the *PER3*^{4} allele (p<0.05). Race discipline also had an influence on *PER3* VNTR genotype distribution with the marathon and ultra-marathon runners (RUNM FULL) having a higher *PER3*^{45} genotype frequency compared to the half-marathon runners (RUNM HALF) (RUNM FULL: 74%; RUNM HALF: 48%, p=0.003).

Runners are more likely to be MTs than low-activity individuals, and the morning-associated *PER3*^{5} allele was also more prevalent in runners. These findings are in line with the findings reported by Kunorozva *et al.* (2012). Study 1 also found the expected difference in diurnal preference between male and female runners, with the females being more morning-oriented. However, unlike previous studies, this study found that there was a gender difference in the genotype distribution of *PER3*, with the *PER3*^{4} allele being more common in females than males. *PER3* VNTR genotype appears to contribute to the race discipline or level of physical activity that individuals choose, especially in males.

Study 2

When all the swimmers were analysed together, there was no difference in 200 m swimming time-trial performance at 06h30 and 18h30 (p=0.590). However, with regard to chronotype and training time-of-day; 67% of the MT group and 71% of the habitual morning training (Train AM) group swam faster at 06h30 (p=0.032 and p=0.012 respectively). Additionally, when the swimmers were grouped according to a combination of chronotype and habitual training time-of-day there was a diurnal variation in performance (p=0.041). RPE was only different after the warm-up with regard to training

time-of-day (p=0.020). Mood states of total mood disturbance (TMD) (p=0.022), anger (p=0.008), depression (p=0.004) and tension (p=0.024) were greater at 06h30 for the group of masters swimmers as a whole. With regard to chronotype, the MT swimmers had a lower fatigue (p=0.012) and a higher vigour at 06h30 (p=0.010) compared to the neither-type (NT) swimmers. Habitual training time-of-day had an influence on vigour; vigour was higher at 06h30 for the swimmers that trained in the morning (Train AM) and higher at 18h30 for the swimmers who trained in the evening (Train PM) (p=0.007). Stroke count (SC) was greater 06h30 for the Train AM group and greater at 18h30 for the Train PM group (p=0.012).

The observed diurnal variation in performance with regard to habitual training time-of-day was only evident for the males; more specifically, the male swimmers showed a diurnal variation regarding habitual training (p=0.029). Similarly, when the male and female swimmers were analysed separately, only the female swimmers displayed a diurnal variation in RPE after the warm-up, with regard to habitual training time-of-day (p=0.022). The male swimmers displayed a difference in fatigue, with regard to chronotype, specifically, only the male swimmers showed a diurnal variation in fatigue (p=0.041) regarding chronotype group. Additionally, although the group as a whole showed a diurnal variation in vigour with regard to habitual training time-of-day, this variation was only significant for the male swimmers (p=0.007). Lastly, only the male swimmers (p=0.048) contributed to the observed diurnal variation in SC when the swimmers were grouped according to habitual training time-of-day group. The gender differences were most likely due to the small sample size of the female swimmers in proportion to the male swimmers and their relative chronotype and *PER3* VNTR genotype distributions.

It was evident that the initial lack of a diurnal variation in performance could to be attributed to the chronotype and habitual training time-of-day distributions within this group of masters swimmers. More specifically, morning-time trial performance was better for the majority of the morning-type individuals and the majority of the habitual morning training group. The observed diurnal variation in RPE with regard to habitual training time-of-day, after the warm-up, indicates that temporal specificity may contribute to diurnal variation in performance. Similarly, SC also displayed an intra-daily variation that was dependent on temporal specificity. This group of masters swimmers had a greater mood disturbance at 18h30, compared to 06h30. Additionally, it is apparent that mood state at different times of the day was also affected by both chronotype and habitual training time-of-day. Thus it appears that inter-individual differences in circadian rhythmicity should be accounted for as they may be useful in explaining peak performance of athletes at different times of the day. Inter-individual differences in circadian rhythmicity, such as chronotype, *PER3* VNTR genotype and

behaviour (training habits), need to be accounted for when analysing performance of individuals at different times of the day.

CONCLUSION

In conclusion, the data from this thesis showed that physical activity level is associated with chronotype and *PER3* VNTR genotype distribution. A higher physical activity level is coupled with a greater preference for mornings and a higher prevalence of the $PER3^{55}$ genotype. Females in this study carried a high frequency of the $PER3^{4}$ allele despite being skewed towards a greater "morningness" indicating that there must be other factors that contribute to morning-preference in females, such as the effects of hormones during certain developmental stages.

Diurnal variation in 200 m swimming performance as well as RPE, mood state and stroke count appear to be strongly related to and affected by chronotype and habitual training time-of-day. Additionally, that fact that diurnal variation with regard to chronotype, *PER3* VNTR genotype and habitual training time-of-day was different in males and females, reiterates that sex/gender needs to be taken into consideration when assessing performance. The findings of this thesis modify previous thinking, in which sports performance was concluded to be greater in the late afternoon/ early evening. This could be pertinent for coaches and athletes when planning training programs and scheduling competitive events.

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION AND SCOPE OF THE THESIS

The endogenous circadian clock is a highly organised and complex system that is responsible for driving the near-24-hour daily rhythms exhibited in physiology and behaviour in most living organisms (Takahashi *et al.,* 2008). This system is tightly controlled both internally, at a neural and molecular level by clock genes, and externally by time-cues, otherwise known as zeitgebers, such as light, temperature and social cues (Cermakian and Boivin, 2003; Takahashi *et al.,* 2008).

Polymorphisms in clock genes have been associated with human behavioural phenotypic traits such as depression (Barclay *et al.,* 2011), mood and seasonal affective disorders (Nievergelt *et al.,* 2006; Lamont *et al.,* 2007; Benedetti *et al.,* 2008), sleep disorders (Archer *et al.,* 2003; Lamont *et al.,* 2007), addiction (Abarca *et al.,* 2002; Zou *et al.,* 2008; Falcón and McClung, 2009) and diurnal preference or chronotype (Pereira *et al.,* 2005; Jones *et al.,* 2007). Specifically, chronotype has been associated with a single nucleotide polymorphism (SNP) in the *CLOCK* gene (T3111C; Katzenberg *et al.,* 1998; Mishima *et al.,* 2005) and a variable number tandem repeat (VNTR) in the *PERIOD3* gene (*PER3*) (Ebisawa *et al.,* 2001; Archer *et al.,* 2003). However, some studies have reported no associations regarding the *PER3* VNTR and chronotype (Osland *et al.,* 2011; Voinescu and Coogan, 2012) or the CLOCK T3111C SNP (Robilliard *et al.,* 2002; Iwase *et al.,* 2002).

Research to date has focussed largely on various populations and “mixed” ethnic groups from around the world (Nadkarni *et al.,* 2005; Ciarleglio *et al.,* 2008), of a large age-range (Archer *et al.,* 2003; Pereira *et al.,* 2005). Additionally, some studies have used students (Barbosa *et al.,* 2010; Kang *et al.,* 2011; Barclay *et al.,* 2011; Osland *et al.,* 2011) and others older participants (Ellis *et al.,* 2009; Viola *et al.,* 2011; Voinescu and Coogan, 2012). Lastly, populations of mixed gender (Archer *et al.,* 2003; Barbosa *et al.,* 2010; Kang *et al.,* 2011; Barclay *et al.,* 2011; Osland *et al.,* 2011; Voinescu and Coogan, 2012) and varying self-report questionnaires, to assess chronotype, have been used (Archer *et al.,* 2003; Jones *et al.,* 2007; Kang *et al.,* 2011; Barclay *et al.,* 2011; Osland *et al.,* 2011; Voinescu and Coogan, 2012). Thus, all of these factors could account for the conflicting results and make comparisons between studies difficult.

Most recently, Kunorozva *et al.* (2012) confirmed the association of chronotype and the *PER3* VNTR in a South African sporting population, and is the only study to have done so in a population selected

on the basis of their activity. Interestingly and in line with the findings of the latter study, it appears that participation in certain sports and even a greater physical activity level is constituted by various chronotypes, specifically morning- and neither-types (Rossi *et al.,* 1983; Lastella *et al.,* 2010; Kunorozva *et al.,* 2012). However, what does this mean for the association between chronotype and *PER3* VNTR genotype when comparing populations of varying activity level? The effect of gender on the association is also an area largely unknown, as most studies have used combined population samples of male and female participants. Lastly, there is limited data available regarding the extent to which chronotype, *PER3* VNTR genotype, as well as the behaviour of athletes (such as the time-of-day at which they choose to train) might influence sports performance at different times of the day.

Therefore the first aim of this thesis is to describe the distribution of chronotype and *PER3* VNTR genotype, as well as their association in a South African population of Caucasian "low-activity" (inactive population) males to that of "high-activity" (running population) males. The second aim is to assess if there are any sex-related differences in the distribution of chronotype, *PER3* VNTR genotype and the association between chronotype and *PER3* VNTR genotype in populations of Caucasian male and female runners. The third aim is to describe diurnal variation in swimming time-trial performance and other variables when chronotype, PER3 VNTR genotype and habitual training time-of-day are taken into account.

In preparation for the exploration and further discussion of the experimental chapters of this thesis, the remainder of Chapter 1 will provide a brief review of the circadian rhythmicity (Section 1.2), circadian rhythm and chronotype (Section 1.3), circadian rhythm genes and chronotype (Section 1.4), the *PER3* VNTR polymorphism (Section 1.5) and circadian rhythm and sport and exercise (1.6). There is considerable research regarding circadian rhythm and various genes. However, this review will primarily focus on the literature associated with the *PER3* gene. The subsequent experimental chapters will answer various research questions in order to achieve the first and second (Chapter 2) and third (Chapter 3) aims of this thesis.

1.2 CIRCADIAN RHYTHM

Almost all physiological, biochemical and behaviour variables, in most living organisms, are circadian rhythmic (Ko and Takahashi, 2006), that is they have an approximate 24-hour cycle. Circadian rhythms have periods of "about a day" (circadian is derived from two Latin words: *circa* = about and *dies* = a day) and are synchronised to the external, planetary 24-hour rhythm by light and temperature changes (Roenneberg *et al.,* 2007). This rhythmicity enables temporal organisation of physiological processes as well as the adaptation of the organism to the rhythmic environment, which includes the day-night cycle, seasons and latitude (Roenneberg *et al.,* 2007).

These rhythms are influenced by both endogenous and exogenous factors (Figure 1) (Dunlap, 1999). The endogenous component regulates rhythmicity at a molecular and neural level and is referred to as the "circadian clock or oscillator" (Dunlap, 1999). It controls physiology at many levels, from gene expression to complex behaviours, such as sleep and performance (Ko and Takahashi, 2006). This endogenous component is a rhythm that repeats with an intrinsic period slightly longer than 24-hours, when "free-running" or free of external time cues, in humans (Czeisler *et al.,* 1999; Roenneberg *et al.,* 2007) and slightly shorter than 24-hours in mice (Wager-Smith and Kay, 2000). A fundamental feature of circadian oscillations is that they will persist when isolated from environmental time cues (Rivkees, 2003). On the other hand, the exogenous component is affected by light, environmental temperature and social factors (Rivkees, 2003; Roenneberg *et al.,* 2007), which are known as zeitgebers. Therefore, the clock can be reset by these factors, which is of importance as it ensures that the circadian clock is entrained to 24-hour cycles (Sack *et al.,* 2007) and does not merely "free-run". The effect of these environmental time cues (zeitgebers) on the circadian system depends on the timing of their occurrence relative to the endogenous circadian cycle (Sack *et al.,* 2007). Mammals have evolved a set of anatomically discrete cell populations that function as a system to provide this temporal organisation (Sack *et al.,* 2007). These structures are commonly referred to as a circadian system (Figure 1) (Cermakian and Boivin, 2003).

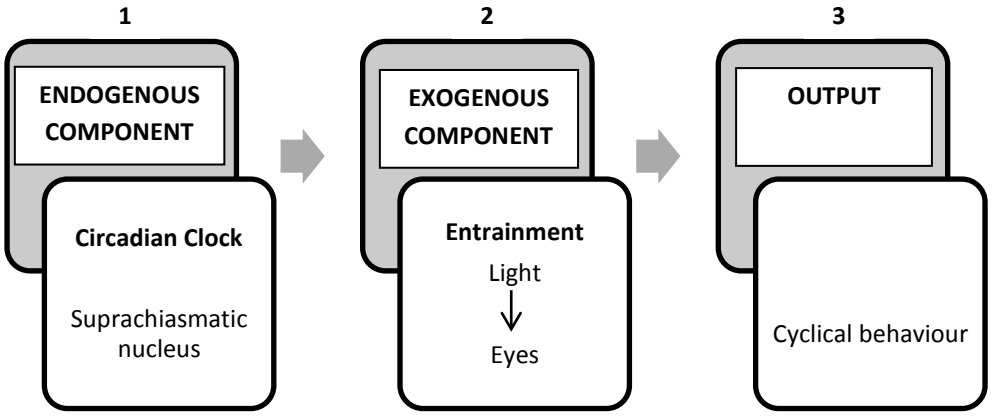

Figure 1: Schematic diagram of the circadian system. A circadian system can be modelled as having three components: 1) a clock responsible for the generation of the daily rhythm, 2) input pathways by which the environment and other components of the nervous system provide information to the clock, and 3) output pathways by which the clock provides temporal information to a wide range of physiological and behavioural control centres.

Thus, the combined interaction of the endogenous and exogenous components is essential in maintaining entrained circadian rhythmicity (Figure 1). The endogenous and exogenous components, respectively, will be discussed in sections 1.2.1 and 1.2.2 below.

1.2.1 Endogenous component

The circadian clock functions via neural control and cellular circadian oscillators (reviewed in Cermakian and Boivin, 2003).

1.2.1.1 Neural control and the suprachiasmatic nuclei (SCN)

Neural control of mammalian circadian systems is co-ordinated by three main components, namely; the SCN, photoreceptors and the pineal gland. The photoreceptors are responsible for providing input to the SCN and the pineal gland contributes to the output from the SCN.

In mammals the main circadian clock is located in the SCN of the anterior hypothalamus (Klein *et al.,* 1999; Moore *et al.,* 2002) (Figure 2), and is able to function autonomously without the need of any external time cues (Klein *et al.,* 1999; Moore *et al.,* 2002). Research on mice and hamsters has

consistently shown that ablation of the SCN not only prevents the expression of the endogenously driven daily rhythms of locomotor activity, feeding, drinking and body temperature (Ralph *et al.,* 1990; Meyer-Bernstein *et al.,* 1999; Filipski *et al.,* 2002; Takahashi *et al.,* 2008), but also eliminates other circadian oscillations such as melatonin, growth hormone (GH), adrenocorticotropic hormone (ACTH) and cortisol secretion (Meyer-Bernstein *et al.,* 1999). Moreover, the transplantation of the SCN into animals previously made arrhythmic by SCN lesions, reinstates behavioural circadian rhythmicity (Filipski *et al.,* 2002; Rivkees, 2003). Interestingly, the restored rhythms exhibits the period of the donor genotype regardless of the direction of the transplant or genotype of the host (Ralph *et al.,* 1990); thus, demonstrating the function of the pacemaker control of the SCN as the "master clock" in mammalian circadian systems (Takahashi *et al.,* 2008).

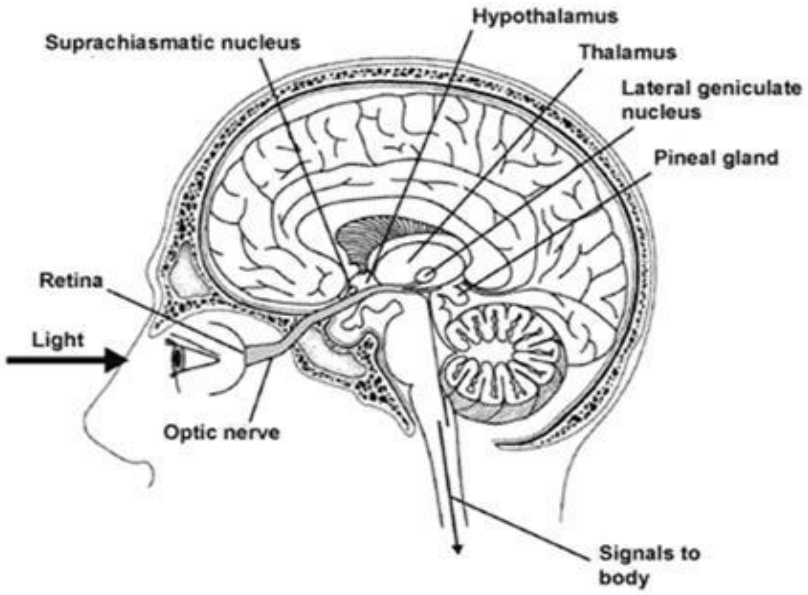

Figure 2: Diagram illustrating the location of the SCN within the brain. (Taken from: http://www.healthchoices-for-life.com/images/suprachiasmatic.jpg)

The circadian clock in the SCN receives photic input from the retina which results in the induction of various genes (Takahashi *et al.,* 2008). The photoreceptive cells that are involved in the entrainment of the SCN clock are different from those involved in vision (Ko and Takahashi, 2006) and form a subset of retinal ganglion cells (Albrecht *et al.,* 1997; Berson *et al.,* 2002). These ganglion cells express the retinal photopigment melanopsin, which integrates photic information for entrainment

within the retina and projects it to the core region of the SCN, via the retino-hypothalamic tract (RHT) (Berson *et al.,* 2002; Takahashi *et al.,* 2008).

Both neuronal and humoral signals originating from the SCN can regulate output pathways, which control various physiological functions (Takahashi *et al.,* 2008). The SCN neurons control rhythms such as body temperature and activity as well as hormone levels through nervous projections to other nuclei of the hypothalamus and other brain regions (Takahashi *et al.,* 2008). The paraventricular nucleus also enables the SCN to send signals to the periphery through the autonomic nervous system (Buijs and Kalsbeek, 2001). The circadian synthesis and secretion of melatonin by the pineal gland are direct outputs of the circadian oscillators within the SCN (Cassone, 1990; Lewy *et al.,* 1999; Cajochen *et al.,* 2003). Thus, the endogenous circadian rhythm of melatonin is generated in the SCN and entrained primarily by the light-dark cycle, acting via the RHT (Arendt, 1998). The melatonin rhythm is coupled tightly to the core temperature rhythm (Figure 3) and the release of melatonin is closely synchronized to the sleep-wake cycle, peaking in the dark phase (Arendt, 1998; Lewy *et al.,* 1999; Cajochen *et al.,* 2003).

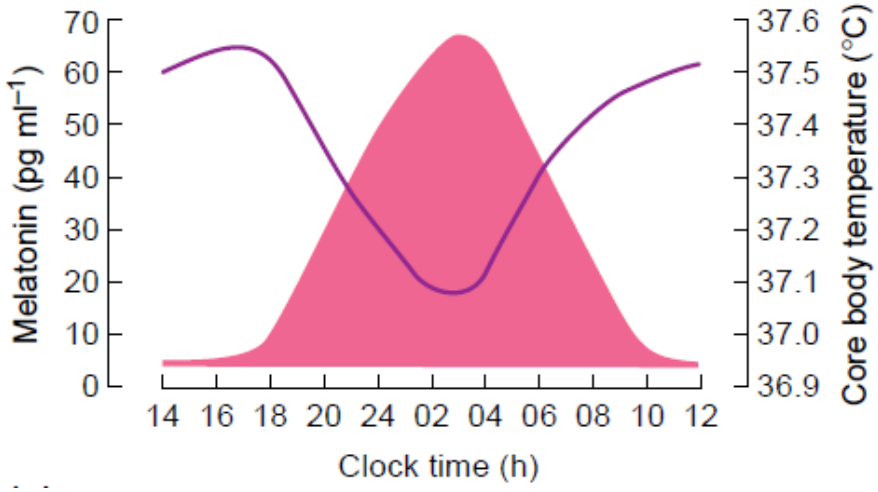

Figure 3: The melatonin and core body temperature rhythms (Taken from: Arendt, 1998).

Key: ■ = melatonin secretion profile; ▬ = core body temperature rhythm profile (Arendt, 1998).

Melatonin contributes to various physiological functions such as sleep propensity, control of the sleep-wake rhythm, circadian rhythm regulation, blood pressure regulation and immune function, amongst others (Cajochen *et al.,* 2003; Pandi-Peurnal *et al.,* 2006; Zeitzer *et al.,* 2007). Therefore, the pineal gland functions as a neuroendocrine transducer and the primary role of melatonin is to convey information regarding light-dark cycles in order to ensure the organization of seasonal and circadian rhythms (Arendt, 1998; Beckett and Roden, 2009) and to provide information to all cells, tissues and organs of the body about the time-of-day (Beckett and Roden, 2009).

1.2.1.2 Molecular components

The literature explaining the molecular basis and functioning of the mammalian circadian clock has been generated primarily from studies conducted on mice (Roenneberg and Merrow, 2003). Evidence suggests that the mechanism driving the clock is intracellular (Dunlap, 1999; Ko and Takahashi, 2006), and depends on the controlled and combined co-expression of specific clock genes (Takahashi *et al.,* 2008), which are a group of genes that constitute the generation and regulation of circadian rhythms (Hidalgo *et al.,* 2009). Humans appear to have a similar set of clock genes to that of rodents (Dunlap, 1999; von Schantz and Archer, 2003). The molecular clock machinery works through a core of interlocking positive and negative transcription-translation feedback loops of transcription and translation of these clock genes and clock proteins respectively, in order to produce a series of rhythmic processes (von Schantz and Archer, 2003), which will be discussed further below.

In the last several years a large number of clock genes have been discovered in various models of different organisms (von Schantz and Archer, 2003). In humans there are three groups of clock genes that are expressed with a near 24-hour rhythm, namely; the three *PERIOD* genes (designated *PER1*, *PER2* and *PER3*), the two *Cryptochrome* (*CRY1* and *CRY2*) genes and the orphan nuclear receptor *REV-ERB*α gene (Gekakis *et al.,* 1998; Jin *et al.,* 1999; Ueda *et al.,* 2002). The *Circadian Locomotor Output Cycles Kaput* (*CLOCK*) and *Brain and Muscle Aryl Hydrocarbon receptor nuclear translocator (ARNT)-like* (*BMAL1*) genes are two essential components of the circadian clock (Rudic *et al.,* 2004). CLOCK and BMAL1 proteins function to control the positive feedback loop, in order to activate the transcription of the aforementioned genes (Sangoram *et al.,* 1998; Bjarnason *et al.,* 2001; Harms *et al.,* 2004). The PER and CRY proteins function to control the negative feedback loop.

The proteins that compose the feedback loops control the period of the oscillations by the phosphorylation, degradation and nuclear translocation of proteins (Sanada *et al.,* 2000; Vielhaber *et al.,* 2001; Eide *et al.,* 2002). CLOCK and BMAL1 form heterodimers and bind to E-box elements of *PER1, PER2, PER3, CRY1, CRY2 REV-ERB*α, resulting in their activation and transcription (Figure 4) (Gekakis *et al.,* 1998; Bjarnason *et al.,* 2001; reviewed in Cermakian and Boivin, 2003). The PER proteins contain PER-ARNT-SIM (PAS) domains, through which they interact with each other as well as with other proteins (Shearman *et al.,* 2000; von Schantz, 2008). The protein products of the PER and CRY genes oscillate over the 24-hour cycle by inhibiting their own promoters (Shearman *et al.,* 2000; reviewed in Cermakian and Boivin, 2003) by negatively regulating the CLOCK: BMAL1 transcription of their own genes (Sangoram *et al.,* 1998), and thus operate in a negative feedback loop (Figure 4). *REV-ERB*α is a direct target of the CLOCK: BMAL1 transcription activator complex and represses *BMAL1* transcription (Sangoram *et al.,* 1998; Bjarnason *et al.,* 2001), as increased *BMAL1* would lead to increased heterodimerisation of CLOCK and BMAL1. During the latter part of the day and the early part of the night, the undisturbed activation of the PER and CRY promoters results in the accumulation of PER and CRY messenger-RNA (mRNA) in the SCN (Takahashi *et al.,* 2008). These mRNA transcripts are then exported into the cytoplasm and translated into the proteins PER1, PER2, PER3 and CRY1 and CRY2, with the highest concentrations peaking in the middle of the night (von Schantz and Archer, 2003). Sufficient quantities of these proteins enable the formation of trimeric complexes between PER and CRY and an additional clock component, casein kinase I ε (CKIε) (Sanada *et al.,* 2000; Vielhaber *et al.,* 2001).

This trimeric complex then enters the SCN, where it binds to the CLOCK: BMAL1 (positive component) complex and inhibits the transcription of PER and CRY mRNA (von Schantz, 2008). The PER proteins also enhance the transcription of *BMAL1*, which promotes the heterodimerisation of both CLOCK and BMAL1, thus restarting the cycle (Figure 4). Therefore it is the regulated degradation of the proteins which enables the cycle to restart (Reppert, 2000); a process that occurs during the day (Reppert, 2000). The entire cycle takes about 24-hours to complete (reviewed in Takahashi *et al.,* 2008).

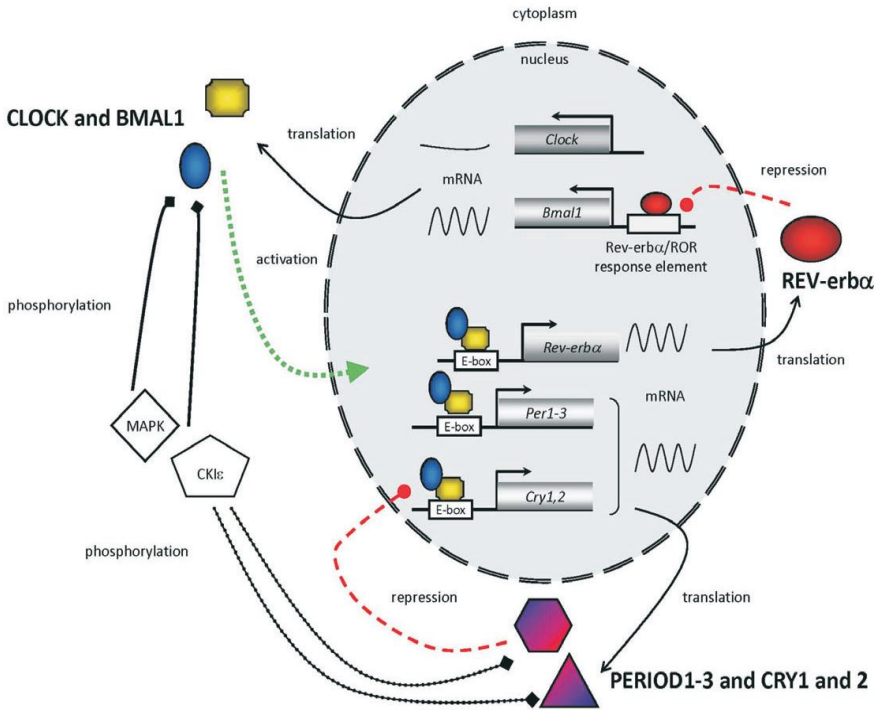

Figure 4: Model of the clock gene transcriptional-translational feedback loops in mammals. The CLOCK and BMAL1 proteins heterodimerise and function to bind to E-box elements, which activates the transcription of *PER1-3, CRY1, CRY2* and *REV-ERBα*. This process encodes the negative elements of the loop. The PER and CRY proteins then heterodimerise which negatively regulates their own transcription as well as the transcription of *REV-ERBα* (Taken from: Beckett and Roden, 2009).

The PER1 and PER2 proteins are central components of the clock, whereas, the PER3 protein does not appear to be essential for rhythm generation (Bae *et al.,* 2001). CKIε and mitogen-activated protein kinase (MAPK) function to phosphorylate the clock proteins (Sanada *et al.,* 2000; reviewed in Cermakian and Boivin, 2003). Phosphorylation regulates their activity, subcellular organisation and degradation (Reppert, 2000; reviewed in Takahashi *et al.,* 2008). The model proposes that the positive and negative loops are coupled to each other and function together in order to produce a self-sustained cycle (Dunlap, 1999).

1.2.2 Exogenous components

Animal experimentation and human studies have concluded that free-running periods (FRPs) are distributed around a species-specific mean (Roenneberg *et al.,* 2007). Thus, individuals assume a specific temporal relationship to a zeitgeber. For example, the time difference between dawn and

wake-up, the core body temperature minimum or the melatonin onset (Roenneberg *et al.,* 2007). This relationship between the external and internal time is called phase entrainment (von Schantz and Archer, 2003; Roenneberg *et al.,* 2007), which is an active process and ensures that the biological clock is stably synchronised to its zeitgebers (Roenneberg *et al.,* 2007).

When deprived of all exogenous zeitgebers, there is inter-individual variation in the periods of circadian clocks (Roenneberg and Merrow, 2003; Roenneberg *et al.,* 2007). Since there are various mechanisms of entrainment, circadian clocks with different FRPs will synchronise with different relationships to the 24-hour light-dark cycle (Roenneberg and Merrow, 2003; Figure 5A). These differences in the phase of entrainment that the cycle is in, depend on both the FRPs and the strength of the zeitgeber signal (Figure 5B and C) (Roenneberg and Merrow, 2003). The circadian clock responds differently to zeitgeber stimuli (Roenneberg *et al.,* 2007). For example, from late at night to the early morning, light advances the clock, whereas from the afternoon to the evening, light delays the response. Thus, the response is dependent on the phase of the circadian clock when the zeitgeber is applied (Roenneberg *et al.,* 2007; Takahashi *et al.,* 2008).

The primary environmental synchroniser (zeitgeber) is the daily light-dark cycle which is also responsible for the daily rhythmicity of all other environmental signals (Roenneberg *et al.,* 2007; Takahashi *et al.,* 2008). Light is detected exclusively by the eyes in mammals, via the RHT (Roenneberg *et al.,* 2007; Takahashi *et al.,* 2008). Thus, each day as the photic time cues are processed via retinal input pathways they synchronise the circadian pacemaker to the 24-hour day (Takahashi *et al.,* 2008). See Figure 5.

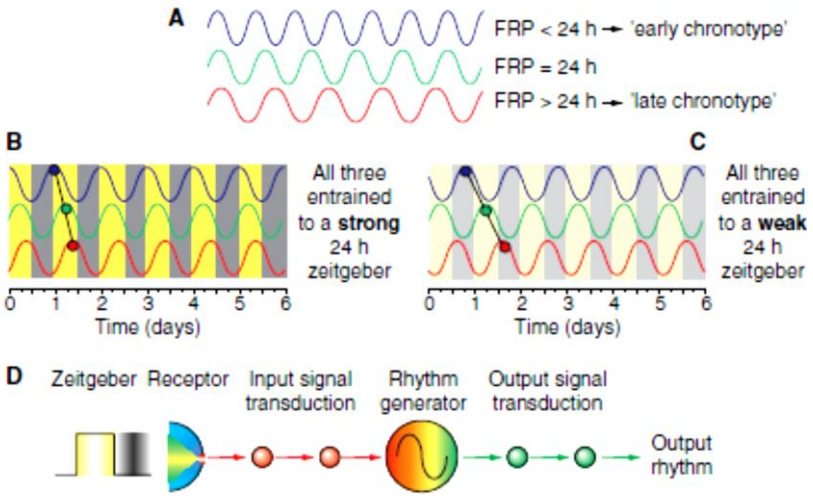

Figure 5: The active process of entrainment synchronises the biological clock to the 24-hour day. (A) Different chronotypes (subjective preference for mornings or evenings) have varying free running periods (FRPs). "Early chronotypes" (morning-types) have a shorter FRP compared to "late chronotypes" (evening-types). (B and C) The strength of the zeitgeber also has an influence on the FRP. (D) The circadian system is described as a pathway from the input that receives the zeitgeber signals to the output which leads to observable rhythms, with the mechanism that generates the circadian rhythm in the centre (SCN). Variations in the phase of entrainment may be due to differences that occur at any stage of this pathway (Taken (and edited slightly) from: Roenneberg and Merrow, 2003).

The circadian system is a highly organised structure that is controlled both endogenously and exogenously, in order to synchronise daily rhythmicity to the 24-hour day.

1.2.3 Measuring circadian rhythmicity

There are various measures available in order to assess human circadian rhythm, of which the most common include biological phase markers and self-report questionnaires (Hofstra and de Weerd, 2008). The most common biological markers used to measure phase and period of circadian rhythm are melatonin (plasma and saliva), core body temperature (CBT) and cortisol production (Dijk *et al.,* 1991; Dijk *et al.,* 1999; Hofstra and de Weerd, 2008). Plasma melatonin peaks in the middle of the dark/sleep episode, approximately two hours before the nadir of the CBT rhythm (Figure 3) and

approximately 4–6 hours before the peak of the cortisol rhythm (Arendt, 1998; Dijk *et al.,* 2012). However, a detailed review regarding the biological phase markers of circadian rhythmicity is beyond the scope of this thesis.

Over the past 30 years, various self-report questionnaires have been designed in order to collect information from individual's regarding the timing of daily activities and sleep (Horne and Östberg, 1976; Smith *et al.,* 1989; Brown*,* 1993). In contrast to biological markers, self-report questionnaires are a subjective tool used to gauge and individual's chronotype (preference for mornings or evenings).

Horne and Östberg (1976) created the Horne-Östberg Morningness-Eveningness Personality questionnaire (H-Ö questionnaire) which was the first English language questionnaire developed. The H-Ö questionnaire consists of 19 questions which address each participant's preferred rising and bed times and preferred times of physical and mental performances. Low scores (16-41) indicate "eveningness" and high scores (59-86) indicate "morningness", which will be discussed further in Chapter 2. The H-Ö questionnaire has been standardised in various countries (Posey and Ford, 1981; Mecacci and Zani, 1983; Benedito-Silva *et al.,* 1990).

Smith *et al.* (1989) created the Composite scale of Morningness (CSM) which is a 12-item Likert-type scale using nine questions from the H-Ö questionnaire (Horne and Östberg, 1976) and four questions from the Diurnal Type scale (DTS). The DTS is a seven item scale also based on questions regarding preferred awake and sleep times (Torsvall and Åkerstedt, 1980). The CSM was developed by Smith *et al.* (1989) in order to improve the analysis of psychometric properties and since then subsequent studies have concluded that the CSM is a reliable and valuable measurement tool (Brown*,* 1993; Bohle *et al.,* 2001; Natale and Alzani, 2001). The CSM has also been translated into other languages (Caci *et al.,* 1999; Natale and Alzani, 2001). Additionally, the reduced H-Ö questionnaire developed by Adan and Almirall (1991) together with the CSM were assessed by Randler (2009), who reported that both questionnaires were valid instruments to assess circadian preference.

The Basic Languages Morningness (BALM) scale developed by Brown (1993) is a simplified version of the CSM that was developed in order to assess a population of children (12-13 y) (Brown*,* 1993). Pornpitakpan (2000) concluded that the BALM scale was as equally reliable as the CSM and that it is a useful tool when analysing populations of a lower education level or with a lower reading ability (Brown*,* 1993; Pornpitakpan, 2000).

The Early/Late Preferences Scale (PS) was then created by Smith *et al.* (2002) in order to improve the psychometric problems associated with the CSM. The PS scale consists of 12 items and contains no references to specific times of the day (Smith *et al.,* 2002). The PS and CSM were both concluded to be equally valuable and reliable with regard to determining reliable differences between morning and evening oriented individuals (Smith *et al.,* 2002; Zickar *et al.,* 2002).

The Munich ChronoType questionnaire (MCTQ) was designed to collect information about the actual timing of daily sleep and activity (Zavada *et al.,* 2005). Zavada *et al.* (2005) analysed the reliability of the H-Ö questionnaire and the MCTQ questionnaire in a Dutch population of 2481 participants, concluding that both questionnaires provided good predictions with regard to chronotype.

Nevertheless, despite the fact that various self-report questionnaires have been developed and validated, the H-Ö questionnaire remains the most widely used and cited self-reported measure of chronotype (Tankova *et al.,* 1994; Ellis *et al.,* 2009). For the remainder of this thesis, unless stated, the H-Ö questionnaire has been used by the respective authors to determine chronotype.

1.3 CIRCADIAN RHYTHM AND CHRONOTYPE

Inter-individual differences in circadian rhythmicity have been attributed to factors such as age, gender, social and lifestyle factors, amongst others (Vink *et al.,* 2001). However, individual preference for either mornings or evenings (chronotype) is one of the main factors contributing to these inter-individual differences (Vink *et al.,* 2001; Ellis *et al.,* 2009). More specifically, humans display large variation with regard to organising their behaviour within the 24-hour day, the most obvious being the preferred timing of waking, sleep and activities (Roenneberg *et al.,* 2007).

Chronotype can be considered as a continuum between two extremes. On one extreme is the morning-type person (MT) who is able to get up easily in the early morning and is more alert in the morning than in the evening (Vink *et al.,* 2001; Cavallera and Giudici, 2008). On the other hand, evening-type (ET) persons are more alert at night and prefer to sleep until later in the morning (Vink *et al.,* 2001; Cavallera and Giudici, 2008). However, many individuals tend to lie near the middle of the spectrum ranging from morning- and evening-type extremes, and are described as neither-types (NT) (Vink *et al.,* 2001; Cavallera and Giudici, 2008). Sleep and wake times, and thus essentially chronotype, tend to display a near-normal distribution within a given population (Adan and Natale, 2002; Roenneberg *et al.,* 2007; Osland *et al.,* 2011). For example, Osland *et al.* 2011 observed a near-normal chronotype distribution in 432 male and female Norwegian university students (Figure 6).

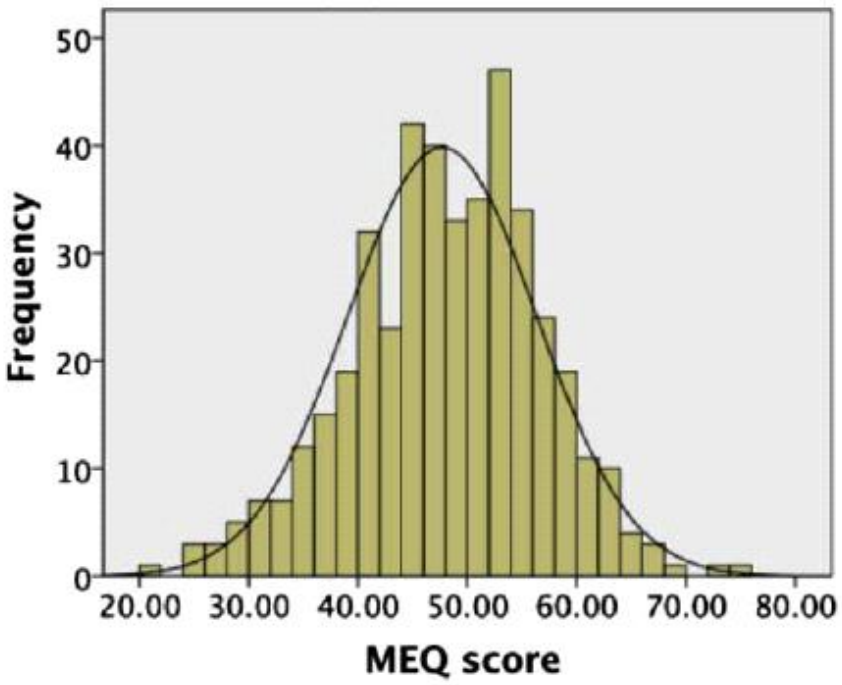

Figure 6: Normal distribution of the HÖ-MEQ scores for a sample of Norwegian students (n = 432). The majority of individuals scored as intermediates (42-58) or neither-types with few individuals scoring as extreme evening- (16-30) and extreme morning-types (70-86). (Taken from: Osland *et al.,* 2011).

Additionally, research has shown that there are physiological differences between morning- and evening-type individuals, as well as behaviour and social differences. The endogenous differences between morning- and evening-type individuals have been validated by measurements of various physiological markers under controlled constant routine protocols (Kerkhof and van Dongen, 1996; Waterhouse *et al.,* 2001). For example, Horne and Östberg (1976) reported that the body temperature profile of morning-type individuals peaked 68 minutes earlier than that of evening-type individuals. Similarly, subjective alertness and melatonin rhythms have all been shown to peak earlier in morning-type individuals (Kerkhof and van Dongen, 1996; Baehr *et al.,* 2000; Waterhouse *et al.,* 2001). Duffy *et al.* (2001) confirmed that shorter circadian periods (earlier phase relative to the entraining day) resulted in an earlier increase in body temperature which is associated with "morningness". Similarly, Lack *et al.* (2009) observed that the temperature difference between morning- and evening-type individuals was in the order of two to three hours. In conclusion, biological, social and environmental factors all contribute to an individual's preference for mornings or evenings. The relationship between chronotype and various variables will be discussed below.

1.3.1 Chronotype in different populations around the world

There are considerable differences in chronotype distribution when comparing different populations from around the world. For example; Taillard *et al.* (2004) reported that 62.1% of French workers (44-58 years) were morning- and 2.2% evening-type individuals. Prior to the latter study, Taillard *et al.* had analysed a group of French participants (17-81 y) and reported that 40.2% were morning- (mean age ± SD: 48.0 ± 12.0 y) and 10.8% were evening-types (mean age ± SD: 35.0 ± 11.0 y) (Taillard *et al.,* 1999). Thus, it appears that the larger the age-range analysed, the larger the "spread" in chronotype distribution. Similarly, Paine *et al.* (2006) showed that, in a general population of New Zealand participants, 49.8% and 5.6% were morning- and evening-types respectively. Most recently, Kunorozva *et al.* (2012) reported that a population of South African individual athletes and an active control group were significantly skewed towards a greater "morningness". On the other hand, Hidalgo *et al.* (2009) reported that the distribution of morning- (35.5%) and evening-types (35.5%) were the same in a Brazilian population (mean age ± SD: 34.4 ± 16.4 y). BaHamman *et al. (*2011) reported that the majority of 759 Saudi's were neither-type (54.9%) individuals, while only 18.9% were morning- and 26.9% evening-types. The latter findings were in line with those of Zavada *et al.* (2005) who showed that the chronotype distribution in Dutch students was normally distributed. In contrast, Borisenkov (2010) reported that human chronotypes in the North are significantly skewed towards "eveningness".

Chronotype distribution has also been shown to differ with regard to latitudinal distribution or geographic location. For example, Benedito-Silva *et al.* (1998) compared chronotype distribution, in 260 adults (21 - 40 y), from five different geographic locations, in Brazil (Benedito-Silva *et al.,* 1998). The authors concluded that geographic location, in terms of the city of residence, had a significantly greater effect on chronotype than did the sole effect of latitude; with the participants from the Northern parts of Brazil having a greater "morningness" compared to the Southern parts (Benedito-Silva *et al.,* 1998). The authors discuss that the findings could be attributed to the fact that geographic location, rather than latitude, is related to social and eating habits, the type of population and lifestyle (Benedito-Silva *et al.,* 1998) of the people studied; all of which can influence an individual's chronotype.

Smith *et al.* (2002) then analysed chronotype in male and female university students from six different countries: United States of America (USA) (n=579; 171 males and 408 females; 19.0 y); England (n=183; 72 males and 111 females; 20.3 y); The Netherlands (n=283; 77 males and 206 females; 21.1 y); Colombia (n=303; 106 males and 197 females; 23.6 y); Spain (n=159; 34 males and

125 females; 19.9 y); and India (n=300; 187 males and 113 females; 21.8 y). The less temperate countries in terms of climate (USA, England and the Netherlands) had a lower "morningness" (using the CSM) compared to the temperate countries (Colombia, Spain and India) (Smith *et al.,* 2002). However, it was also noted that data was collected at different times of the day; mainly in the mornings in the temperate climate countries and at varying times of the day for the less temperate countries (Smith *et al.,* 2002). This could have potentially influenced the results, with more "morning-type" students from the temperate countries participating during the morning data collection. Similarly, Adan and Natale (2002) observed that Italian students had a greater "morningness" compared to Spanish students. There is only a small latitudinal difference between Italy and Spain, thus the differences may not be attributed to latitude alone.

Randler and Diaz-Morales (2007) used the CSM to assess chronotype in 1086 German and Spanish students. The German students (n=540; 108 males and 432 females; mean age ± SD: 22.22 ± 2.9 y) had a greater "morningness" compared to the Spanish students (n=546; 107 males and 439 females; mean age ± SD: 21.60 ± 2.6 y) (Randler and Diaz-Morales, 2007). The authors reason that the greater "morningness" seen in the German students could be due to an earlier exposure to daylight in the morning, climate and lifestyle differences. For example Spain is known for its afternoon siesta period (Randler and Diaz-Morales 2007). Randler (2008) analysed chronotype, using the CSM, in 2621 German adolescents (mean age ± SD: 12.61 ± 1.53 y to 22.68 ± 1.38 y). The participants attended German schools around the world (n=1911) or in Germany (n=710). Six schools were located in temperate regions, five in sub-tropical regions and six in tropical regions. Interestingly, the authors observed that German adolescents living in tropical regions had a greater "morningness", whereas those living in sub-tropical regions had a greater "eveningness" (Randler, 2008). The students living in temperate regions displayed a similar chronotype distribution to the students living in tropical regions (Randler, 2008). This was unexpected as Smith *et al.* (2002) mentioned that people living in hotter climates had a greater "morningness" which would have resulted in the tropics having the greatest "morningness" followed by the sub-tropics regions and then the temperate regions. Randler (2008) also analysed the relationship between longitude and chronotype and concluded that the relationship is related to sunrise times which are earlier in the east within a given time zone.

The contrasting findings discussed above may be due to the fact that most studies have analysed populations constituted by adolescents and/ or students (Smith *et al.,* 2002; Randler, 2007; Randler, 2008). The relationship between chronotype and age will be discussed in section 1.3.3. Additionally most studies have used different chronotype self-report questionnaires; which makes comparisons between studies difficult. Moreover, the various studies have collected data in different seasons. For

example, Benedito-Silva *et al.* (1998) collected data between October and December and Rander (2008) between June and October. Seasonality is known to affect chronotype (Portaluppi *et al.,* 2008, 2010) and thus this makes accurate comparisons between studies difficult. Nevertheless, it is evident that trans-cultural comparisons are important as there are differences in chronotype when comparing different populations living all around the world as well as in populations living on the same continent. The presented literature highlights the fact that chronotype appears to be influenced by biological factors, environmental factors (climate), geographic location as well as social (lifestyle) and cultural differences. Thus, in the future, concise research regarding cross-cultural and lifestyle differences amongst different countries and the corresponding chronotype profiles is required.

1.3.2 Chronotype and gender

For years the available literature regarding the relationship between gender and chronotype was considered inconsistent and controversial (as reviewed in Kerkhof, 1985 and Tankova *et al.,* 1994). The majority of studies, which mainly focussed on workers and students, reported no significant differences between gender and chronotype (reviewed in Tankova *et al.,* 1994). For example, Posey and Ford (1981) conducted a study on 259 students (62 males and 187 females) and concluded no significant gender differences between sex and chronotype. Similarly, studies conducted by Mecacci *et al.* (1986), Ishihara *et al.* (1988), Wilson (1990), Adan and Almirall (1991) and Neubauer (1992), all observed no significant gender related differences in chronotype.

However, the majority of the above-mentioned studies all have two shortcomings in common 1) a relatively small sample size and 2) a large age range of participants. For example, the participant group analysed by Ishihara *et al.* (1988) consisted of 346 workers and students (183 males and 163 females), with the age of participants ranging from 19-64 years. Thus, the combination of the relatively small sample sizes studied and the large variation in age between participants, within and between studies, could have resulted in non-significant findings. Chronotype is affected by age (which will be discussed below), thus using large age ranges within a population group could be problematic. Another factor which makes comparisons between studies difficult is the variation in self-report questionnaires that were employed in the various studies.

In recent years, although the findings are still somewhat conflicting, the relationship has been understood better through the use of larger sample sizes (reviewed in Cavallera and Giudici, 2008).

Thus, most of these studies have concluded that females tend to have a greater "morningness" (Cavallera and Giudici, 2008). Chelminski *et al.* (1997) analysed the chronotype of 1617 participants (576 males and 1041 females; 18-53 y) and Natale and Danesi (2002) 1319 university students (614 males and 705 females; mean age ± SD: 23.6 ± 3.7 y). Both studies concluded that females were more morning-oriented in comparison to their male counterparts. Adan and Natale (2002) then conducted a study on 2135 participants (mean age ± SD: 22.2 ± 2.9 y) of Italian (n=1256) and Spanish (n=879) origin, and again confirmed that there was a greater "morningness" in females. In contrast, Steele *et al.* (1997) observed that males were more morning-oriented in a sample of American Emergency Medical residents (n=2047; mean age: 29 y). However, in this particular study, a modified version of the H-Ö questionnaire was administered, three-quarters of the population sample were males and it could be argued that the participants were not "normal" due to the nature of their shift-work, associated with their job.

Brazilian females were reported to have a greater "morningness", when comparing individuals from Sao Paulo (n=464; 120 males and 344 females; mean age ± SD: 22.1 ± 4.3 y) and Curitila (n=585; 280 males and 305 females; mean age ± SD: 20.5 ± 3.5 y) (Louzada *et al.,* 2004). Contrastingly, Paine *et al.* (2006) showed that chronotype was independent of ethnicity, gender and socio-economic status in a New Zealand population of 1060 males and 1466 females (30-49 years). The authors did note a possible response bias due to the limited English literacy capabilities of the participants (Paine *et al.,* 2006).

Randler (2007) then used meta-analytical tools to analyse 52 studies regarding the chronotype-gender relationship. Of the 52 studies, 11 used CSM, 29 the H-Ö questionnaire, one the BALM scale, eight the morningness-eveningness (M-E) questionnaire (Carskadon *et al.,* 1993) and three the Torsvall scale (Torsvall and Åkerstedt, 1980). The authors reported a significant gender-related difference, with girls and woman having scores skewed towards the higher scores and thus having a greater "morningness". Randler (2007) suggested that with larger sample sizes, gender differences were detected as the standard deviations of the mean age were smaller. Recently, Barclay *et al.* (2011) reported that females had a significantly higher H-Ö questionnaire score and thus a greater "morningness" compared to males in a population sample of 947 participants (362 males and 585 females; mean age ± SD: 20.3 ± 1.8 y). Randler (2007) pointed out that there has been and still continues to be a large inconsistency with regards to the self-report questionnaires that are administered in different studies.

In conclusion, although the majority of the studies have used population samples consisting of students (few studies have used older participants and children under 13), overall it appears that females tend to have a greater "morningness".

1.3.3 Chronotype and age

There is a large body of evidence available regarding the association between age and chronotype; however a detailed review of the literature is beyond the scope of this thesis. It has been reported that there is a developmental predisposition towards "eveningness" in the adolescent population (Carskadon, 2005) and thereafter, daily preferences and activities begin reversing and the shift towards "morningness" occurs (Dijk and Archer, 2010). For example, Kim *et al.* (2002) analysed chronotype in 989 children (8-16 y) and concluded that children tend to shift towards a preference for evenings around the age of about 13 years. Various other studies have replicated these findings in children (Takeuchi *et al.,* 2002; Roenneberg *et al.,* 2004; Mateo *et al.,* 2012). With regard to adults, a positive correlation between H-Ö questionnaire score, as well as other various self-report questionnaires, and age has consistently been reported (Mecacci *et al.,* 1986; Adan and Almirall, 1991; Chelminski *et al.,* 1997; Thoman, 1999; Diaz-Morales and Sanchez-Lopez, 2004; Talliard *et al.,* 2004; Paine *et al.,* 2006; Caci *et al.,* 2009).

Generally, from the age of 13, there is a trend for superior evening preference which continues throughout adolescence, after which there is a shift towards greater morning preference, around the age of 20 (Roenneberg *et al.,* 2007). Kerkhof (1985) mentioned that there is an age-related reduction of the rhythm amplitude which could be driving the increased preference for mornings with age. Therefore, it has been postulated that with age the phase relationship with the sleep-wake cycle changes, thus with an increase in age the sleep onset and offset occur earlier, leading to a shift towards "morningness" (Vink *et al.,* 2001; Ellis *et al.,* 2009). This shift in chronotype has also been hypothesised to occur due to changes in work, lifestyle and or domestic relationships (Adan and Natale, 2002; Jones *et al.,* 2007).

1.3.4 Chronotype, physical activity and sport

Cavallera *et al.* (2011) assessed chronotype (reduced H-Ö questionnaire) in 61 students (33 males, 26 females, 2 un-specified; 19-31 y). Interestingly, there was a negative correlation between the hours spent participating in sport and activity per week and H-Ö score. Thus, the morning-type

individuals spent more time playing sport (Cavallera *et al.,* 2011). Similarly, a study conducted on 678 children (mean age ± SD: 13.8 ± 2.0 y) determined that the morning-type individuals, as determined by the CSM, spent more time on reading and physical activity while the evening-type individuals had a greater screen time, in terms of time spent watching television and playing on a computer (Kauderer and Randler, 2012).

Most of the research regarding chronotype and physical activity level has been conducted on athletes. For example, Rossi *et al.* (1983) compared chronotype and performance of male golfers (n=34) and water-polo players (n=23), who ranged from 22-28 years of age. All participants had seven years of experience regarding their respective sports. The authors reported that there were no differences in chronotype between low-performing and high-performing individuals when comparing the two sports. The high-performing golfers, however, had a greater morning preference compared to the high-performing water-polo players (Rossi *et al.,* 1983). Similarly, Zani *et al.* (1984) assessed chronotype in 87 athletes (gender not specified) who participated at the top National levels in their respective sports. There was a decrease in H-Ö questionnaire score from golf (n=10; mean age ± SD: 27.6 ± 2.6 y) to shooting (n=10; mean age ± SD: 35.8 ± 9.0 y) to volley-ball (n=12; mean age ± SD: 26.0 ± 2.2 y) to basketball (n=15; mean age ± SD: 25.9 ± 3.9 y) and to water-polo (n=8; mean age ± SD: 25.8 ± 4.7 y) (Zani *et al.,* 1984). Since, the above-mentioned sports all take place at different times of the day, these studies provided the first indication that chronotype might be influenced by the time-of-day that the respective sports matches/ competitions or training sessions occurred.

More recently, Lastella *et al.* (2010) analysed chronotype in 23 elite triathletes (16 males; mean age ± SD: 20.9 ± 2.1 and 7 females; mean age ± SD: 20.8 ± 3.6 y) and found that 11 scored as morning-types and 12 as neither-types. The authors state that a possible explanation for no evening-type individuals could be due to the fact evening-types do not select sports which require repetitive early morning training and that an athlete's preference for mornings or evenings could determine an individual being a sub-elite versus an elite triathlete. Kunorozva *et al.* (2012) conducted a chronotype-genotype analysis on individual sports athletes and a control group of active gym members. The individual sport athletes had higher H-Ö questionnaire scores and were more likely to be morning-types. For example, 72% of the cyclists, 67% of the runners and 59% of the Ironman triathletes were morning-types, while only 41% of the controls (active, but non-competitive individuals) were morning-types (Kunorozva *et al.,* 2012).

The studies discussed above all focussed on athletes; recreational, sub-elite and elite, the majority being morning-type individuals. However, are the athletes accustomed to morning training/ morning behaviour or is this really their actual preference? Since, cycling, running and triathlon disciplines all involve early morning training and racing, this could be another possible explanation as to why there are more morning-types associated these sports. Perhaps morning-type individuals persist with sports that have an early morning race time.

1.4 CIRCADIAN RHYTHM GENES AND CHRONOTYPE

Given the fundamental role of the circadian system in the timing of different physiological processes and in synchronising them with the daily environmental changes, it is probable that a dysfunction of the clock may have an impact on health (Cermakian and Boivin, 2003; Lamont *et al.,* 2007; Beckett and Roden, 2009). In man and other mammals mutations in a number of the core clock genes have been shown to alter circadian parameters (Barbosa *et al.,* 2010). This could ultimately result in phenotypic differences between individuals on one end of the spectrum or physiological problems and psychological disorders such as familial advanced phase sleep syndrome (FASPS), delayed sleep phase syndrome (DSPS), metabolic syndrome, bipolar disorder (BPD), substance abuse and depression amongst others (Dagan, 2002; Archer *et al.,* 2003; Zhu *et al.,* 2005; Nievergelt *et al.,* 2006; Artioli *et al.,* 2007; Sack *et al.,* 2007 Benedetti *et al.,* 2008), on the other end.

Katzenberg *et al.* (1998) were the first authors to report the association between a clock gene polymorphism and circadian rhythm phenotype in humans. These authors concluded that in a single nucleotide polymorphism (SNP) in the 3'-untranslated region of CLOCK (T3111C), the minor allele (C) was associated with "eveningness", in a Caucasian American population (Katzenberg *et al.,* 1998). Similarly, Mishima *et al.* (2005) confirmed these findings in a Japanese population. In contrast, these findings were not replicated in British (Robilliard *et al.,* 2002), Japanese (Iwase *et al.,* 2002), mixed European (Johansson *et al.,* 2003) and Brazilian (Pedrazzoli *et al.,* 2007) populations. The controversial findings may be due to ethnic differences or that only one polymorphism was studied; thus the differences could possibly arise from linkage to another polymorphism.

Polymorphisms in other clock genes have also been associated with chronotype. For example, polymorphisms in the *PER1* and *PER2* genes have been associated with chronotype in a British population (Carpen *et al.,* 2005; Carpen *et al.,* 2006). Carpen *et al.* (2005) reported that in a polymorphism of the *PER2* gene (C111G), the minor G allele was associated with morning preference. Similarly, Carpen *et al.* (2006) concluded that the C2434 allele within the *PER1* gene is associated with "morningness". In addition, Toh *et al.* (2001) reported that there was an association between a polymorphism within the *PER2* gene with a familial case of Advanced Sleep Phase Syndrome (ASPS). However, in a Japanese population, with FASPS, the latter findings were not confirmed by Satoh *et al.* (2003).

Ebisawa *et al.* (2001) were the first authors to analyse the human *PER3* gene as a possible candidate for rhythm disorder susceptibility. It was suggested that structural polymorphisms in the *PER3* gene were potentially implicated in the pathogenesis of DSPS in a Japanese population of 48 participants

(Ebisawa *et al.,* 2001). The *PER3* gene is unique in that it has a VNTR in which a 54-nucleotide coding-region segment is repeated either four or five times (Dijk and Archer, 2010) (Figure 7). Archer *et al.* (2003) then reported an association between the *PER3* VNTR polymorphism and chronotype. Of the 484 British participants recruited (16 - 27 y), genotype was analysed in 7% of the participants with the highest, 7% with the lowest and 7% with intermediate H-Ö scores. The authors concluded that the frequency of the 5-repeat allele was significantly higher in the extreme morning-type individuals compared to the evening-type individuals (Archer *et al.,* 2003).

Pereira *et al.* (2005) also established that there was an association between the shorter allele and "eveningness" in a Brazilian population of mixed ethnicity (mean age ± SD: 25.9 ± 7.1 y), which is in line with the findings of the latter study. Jones *et al.* (2007) then analysed the association between the *PER3* VNTR polymorphism and chronotype in the 5% extreme chronotypes from a British population. They found that the 4-repeat allele was significantly associated with evening-types and the 5-repeat allele with morning-types. Interestingly, the strength of the association decreased with age (Jones *et al.,* 2007). The authors concluded that perhaps diurnal preference is more strongly influenced by genotype and less by exogenous factors in young individuals; whereas, in older individuals chronotype is not as closely linked with genotype, but rather influenced to a greater extent by social factors such as family time and work schedules (Jones *et al.,* 2007). Additionally, Ellis *et al.* (2009) confirmed this association by conducting a study that assessed the association of specific H-Ö questionnaire items and *PER3* VNTR genotype in a British population (mean age ± SD: 40.2 ± 14.4 y). More recently, Kunorozva *et al.* (2012) established that there was an association between *PER3* VNTR genotype and chronotype, in both a South African population of male Caucasian athletes and a group of active but non-competitive males.

In contrast, other studies have reported no *PER3* VNTR genotype-chronotype association (Viola *et al.,* 2007; Goel *et al.,* 2009; Osland *et al.,* 2011). For example, Goel *et al.* (2009) analysed chronotype, using the CSM in 14 *PER3*^{55}, 63 *PER3*^{45} and 52 *PER3*^{44} individuals. The authors reported no association between *PER3* VNTR genotype and chronotype in these North American participants. Viola *et al.* (2011) conducted a study on older participants, of whom 13 were homozygous for the *PER3*^{5} allele and 13 the *PER3*^{4} allele. They observed no association between *PER3* VNTR genotype and chronotype. It is important to note that the mean age (± SD) of the participants was 62.23 ± 1.01 y for the *PER3*^{4} group and 62.38 ± 1.39 y for the *PER3*^{5} group. Based on the findings of Jones *et al.* (2007), a possible reason for the lack of an association reported by Viola *et al.* (2011) could be attributed to the age of population sample studied. Kang *et al.* (2011) assessed *PER3* VNTR genotype and chronotype, using the CSM, in a Korean population (mean age: 22.9 y). The authors observed

that although the *PER3*^{55} participants had a tendency towards higher CSM scores, no significant differences between genotype and CSM score were observed. Voinescu and Coogan (2012) analysed the *PER3* VNTR polymorphism in 72 older Romanian participants, with self-reported sleep problems. Interestingly, 43% of the participants were homozygous for the longer (*PER3*^{5}) allele; however there was no genotype-chronotype association. With the exception of one study (Viola *et al.,* 2011) the participants in the latter study were older in comparison to other studies that have assessed the association *PER3* VNTR polymorphism and chronotype. Osland *et al.* (2011) also observed no association between *PER3* VNTR genotype and chronotype, as determined by the CSM and H-Ö questionnaires in Norwegian students (mean age ± SD: 22.0 ± 6.0 y). The authors note that a possible reason for finding no association in their study could be due to the fact that previous studies have used "sub-sets" of participants that obtained the highest and lowest chronotype questionnaire scores and thus focussed on "extreme" chronotypes. Similarly, another study conducted on young (mean age ± SD: 20.30 ± 1.77 y) British participants reported no association between *PER3* VNTR genotype and chronotype (Barclay *et al.,* 2011).

The discrepancies surrounding the association between *PER3* VNTR genotype and chronotype could be attributed to a few factors. For example; populations of different ethnicity have been studied; there is a large variation in the age of participants that have been analysed, both within and between studies; the sample sizes employed by varying studies differ greatly; and chronotype has been assessed by different questionnaires. Nevertheless, to date, it appears that the *PER3* VNTR polymorphism displays the most robust association with chronotype so far, compared to other polymorphisms. The *PER3* VNTR polymorphism will be discussed further below (section 1.5).

1.5 *PER3* VNTR POLYMORPHISM

The *PER3* gene is a member of the Period family of genes, however its specific role within the circadian system is not fully understood (Ebisawa *et al.,* 2001). It has been proposed that the *PER3* gene may function in tracking day length throughout the course of the year, rather than having a direct effect on the maintenance of the circadian intrinsic period (Pereira *et al.,* 2005).

Figure 7: **A schematic diagram showing the *PER3* length polymorphism located on exon 18 of chromosome 1.** Grey boxes indicate the flanking regions. White boxes indicate the 54 base pair (bp) repeats which are repeated either four (*PER3*^{4}) or five (*PER3*^{5}) times. The four-repeat allele derives from the loss of the third repeat.

The *PER3* VNTR polymorphism is not situated within any identified functional domain; however it is near to the putative binding site for Casein Kinase I (Archer *et al.,* 2003; Nadkarni *et al.,* 2005). This polymorphism is absent in non-primate mammals such as mice, rats and dogs (Jenkins *et al*., 2005; Nadkarni *et al.,* 2005; Dijk and Archer, 2010). Moreover, the repeated motifs in the protein contain several probable phosphorylation sites, thus indicating that this polymorphism could affect post-translational modification and stability of the protein as well as having an effect on tertiary structure (Dijk and Archer, 2010). Of additional interest is that the *PER3* gene has been reported as the one *PER* paralogue which is not essential for maintaining circadian rhythmicity (Bae *et al.,* 2001; Pereira *et al.,* 2005).

1.5.1 *PER3* VNTR polymorphic frequencies around the world

Over the past few years, there has been an increase in the body of evidence produced with regard to the *PER3* gene, in both healthy and “unhealthy” populations. However, there are large variations in

the distribution of the 4- and 5-repeat allele frequencies when comparing populations worldwide. For example, some authors have reported no differences in the distribution of *PER3* VNTR genotypic frequencies when comparing different ethnic groups (Goel *et al.,* 2009); whereas others have reported significant ethnic differences (Barbosa *et al.,* 2010).

1.5.1.1 Healthy populations

A Continental trend?

When assessing the European populations from various studies, the 4-repeat allele frequency tends to range from 67% to 69% (Archer *et al.,* 2003; Nadkarni *et al.,* 2005; Osland *et al.,* 2011). For example, the Norwegian population assessed by Nadkarni *et al.* (2005) had a 4-repeat allele frequency of 66%. Similarly, Norwegian students had a reported 4-repeat allele frequency of 67% (Osland *et al.,* 2011). Similar findings were reported for British populations (Archer *et al.,* 2003; Nadkarni *et al.,* 2005), a Finnish population sample (Nadkarni *et al.,* 2005), a Ukrainian population (Nadkarni *et al.,* 2005) as well as populations of Anatolians and Tyrolians (Nadkarni *et al.,* 2005).

African populations seem to have a greater variation in 4- and 5-repeat allele frequencies, compared to European populations. For example, the 4-repeat allele frequencies range from 48% to 75% in African populations (Nadkarni *et al.,* 2005; Ciarleglio *et al.,* 2008). Recently, Kunorozva *et al.* (2012) reported the 4- and 5-repeat allele frequencies in sporting individuals. Cyclists (n=125; 37.2 ± 7.1 y), runners (n=113; mean age ± SD: 35.6 ± 7.1 y) and Ironman triathletes (n=287; mean age ± SD: 36.8 ± 6.5 y) all had very low 4-repeat allele frequencies, 39%, 42% and 44% respectively (Kunorozva *et al.,* 2012). This is in reverse to what was seen in the European groups. Interestingly, the control group (n=88; mean age ± SD: 32.9 ± 8.1 y), which consisted of active but non-competitive individuals (i.e. exercised in a gym approximately twice per week) had a 4-repeat allele frequency of 62% (Kunorozva *et al.,* 2012), which is similar to the European groups. In the latter study, the 4-repeat allele frequency of the active control group was similar to South African Bantu (65%), Nigerian (66%) and Malawian (65%) populations (Nadkarni *et al.,* 2005)

When observing the findings of Asian populations, the range in 4-repeat allele frequency distribution is more widespread in comparison with European and African population samples (Nadkarni *et al.,* 2005; Barbosa *et al.,* 2010). For example, the 4-repeat allele frequencies were lower in Indian (58%) and Yemeni (48%) populations compared to those of Afghan (66%), Iranian (70%), Chinese (82%) and Mongolian (89%) populations (Nadkarni *et al.,* 2005). Similarly, the 4-repeat allele frequency in a Han Chinese population (82%) (Ciarleglio *et al.,* 2008), was similar to the Japanese population (81%)

analysed by Ebisawa *et al.* (2001). Lastly, Papau New Guineans appear to have a much higher frequency of the 5-repeat allele (81%) in comparison to all other populations (Nadkarni *et al.,* 2005; Ciarleglio *et al.,* 2008).

The considerable variation in the 4- and 5-repeat allele frequency distributions when comparing various global populations is most likely due to the fact that most studies have used relatively small sample sizes, of both males and females and although the various studies analysed specific populations groups, the exact origin of all of the participants is largely unknown.

1.5.2 *PER3* VNTR genotype and gender

With the exception of Jones *et al.* (2007) very few studies have analysed the direct relationship between *PER3* VNTR genotype and gender, in large cohorts of male and female participants. Jones *et al.* (2007) reported that gender did not have an influence on *PER3* VNTR genotype distribution in a population of 707 (44%) males and 883 (56%) females. A number of smaller studies have replicated this finding in both healthy and diseased populations. For example, Ellis *et al.* (2009) observed no gender effect on *PER3* VNTR genotype, in a British population of 113 male and 127 female participants. Similarly, Goel *et al.* (2009) also found no sex difference in *PER3* VNTR genotype in 63 males and 66 female American participants. Similarly, Benedetti *et al.* (2008) also reported no significant relationship between *PER3* VNTR genotype and gender, in an Italian population of Bipolar disorder patients (36% males and 64% females). Additionally, in a Romanian population sample of 40 males and 32 females with self-reported sleep problems 25% of the males and 16% of the females were genotyped as *PER3*^{44} and 43% of the males and 44% of the females as *PER3*^{55} (Voinescu and Coogan, 2012). Moreover, Chellappa *et al.* (2012) reported the *PER3* VNTR frequency distribution in a population of 50 young males and females (20-31 y). Although the authors did not include the female participants in any statistical analyses it was apparent that of the 21 *PER3*^{44} individuals, almost 60% (n=13) were female and of the 19 *PER3*^{45} individuals, 74% (n=14) were female. On the other hand, only one female was genotyped as *PER3*^{55} (10%). The latter study, albeit a small sample, shows a bias towards the *PER3*^{44} and *PER3*^{45} genotype in females.

As discussed above, *PER3* has been shown to be associated with chronotype and since females are more “morning-oriented” (section 1.3.2) (Tankova *et al.,* 1994; Randler, 2007; Cavallera and Giudici, 2008) it is appropriate to examine *PER3* and gender.

1.5.3 *PER3* VNTR genotype and sleep

There is a large body of evidence available regarding the association between the *PER3* VNTR genotype and sleep; however a detailed review of the literature is beyond the scope of this thesis. Nevertheless, the *PER3* VNTR polymorphism has been associated with sleep homeostasis (Viola *et al.,* 2007). More specifically, the *PER3* VNTR polymorphism contributes to differences in sleep structure between genotypes (Viola *et al.,* 2007). For example, slow-wave sleep (SWS) and electroencephalogram (EEG) slow-wave activity in non-rapid eye movement (non-REM) sleep were increased in $PER3^{55}$ compared to $PER3^{44}$ participants (Viola *et al.,* 2007). Additionally, with regard to sleep loss, $PER3^{55}$ participants displayed a greater decline in cognitive performance (Viola *et al.,* 2007). Similarly, Dijk and Archer (2010) concluded that $PER3^{55}$ participants had a higher sleep pressure. One could thus postulate that this could potentially result in earlier bed times, earlier wake times and poorer performances as they day increases for $PER3^{55}$ participants. Homozygosity for the $PER3^{5}$ allele has also been associated with sleep timing and duration, with $PER3^{55}$ individuals reporting earlier awake and bed-time times (Lázar *et al.,* 2012).

1.6 CIRCADIAN RHYTHM AND SPORT AND EXERCISE

Peak athletic performance varies at different times of the day, of which the possible causes include environmental factors, the sleep-wake cycle and the internal "body clock" (Reilly and Waterhouse, 2009). The existence of circadian rhythms in human physical performance has potential implications for athletes in both training and competition (Reilly and Bambaeichi, 2010). However, current evidence for the role of the body clock, with regard to physical activity, is controversial.

The definition of sport or exercise "performance" in an athletic context is a nebulous term, as sport events typically comprise a combination of components such as psychomotor performance, motor skills and the physiologic responses to exercise (reviewed in Atkinson and Reilly, 1996 and Drust *et al.,* 2005), all of which may vary in a circadian-like manner and have varying effects on overall "performance". The literature that has focussed on circadian variation and sports performance has often isolated separate components of performance in the laboratory and described the circadian characteristics of each, thereby relating the ecological validity of the data to the real event (Drust *et al.,* 2005), with the trend being that most measured variables tend to peak in the late afternoon and early evening (around 16h00 to 20h00) (Atkinson and Reilly, 1996; Drust *et al.,* 2005).

Additionally, world records, specifically in track and field athletics and swimming, are customarily broken in the late afternoon and/ or early evening (Atkinson and Reilly, 1996), coincidentally at the time-of-day at which body temperature is highest. Such observations have resulted in a considerable body of indirect evidence advocating that sports performance capability in the field is greatest in the evenings and close to the time-of-day that body temperature nears its peak value (Atkinson and Reilly, 1996; Drust *et al.,* 2005). However, this must surely in part reflect the fact that these events are usually scheduled for evening meetings, presumably either to meet television schedules or to avoid excessively hot environmental conditions, thus skewing the number of opportunities for world record attempts to occur at other times of the day.

1.6.1 Evidence for diurnal variation in exercise and sport

The experimental manipulation of the time-of-day is not feasible in competitive circumstances or situations (Reilly and Waterhouse, 2009). Thus the research for circadian rhythms relevant to exercise has largely focussed on three main aspects of performance: 1) the components of performance, 2) task performance and 3) event performance, which will be discussed further below.

1.6.1.1 Diurnal variation and components of performance

While individual components of sport and exercise are not direct indicators of "real" athletic performance (Drust *et al.,* 2005) they may be useful from a mechanisms perspective when studying circadian rhythms and response to exercise. Over the past 30 years a large body of evidence has focussed on components of performance and circadian rhythmicity (Rossi *et al.,* 1983; Winget *et al.,* 1985; Hill *et al.,* 1988). Some of the data from various studies is presented in Table I, however a detailed review of the studies observing the components of performance that have displayed circadian variation is beyond the scope of this thesis.

From Table I it is evident that there are large variations in performance at different times of the day. Possible reasons for the discrepancies in the literature include the varying age-range of participants used in each of the studies, the sample sizes analysed in different studies, the training status of the participants is largely unknown, participants have been tested at different times of the day and varying protocols and methodologies have been employed to assess the same variables. Nevertheless, the general trend is that measures of the components of performance tend to peak in the late afternoon and early evening (Table I), the same time that body temperature is said to be at its maximum (Atkinson and Reilly, 1996).

Table I: Studies that have analysed various components of performance

Components of performance	Measure/ Variable	Time of peak effect	Reference
Psychomotor performance, motor skills and psychological variables	Simple reaction time	15h00-18h00	Colquhoun, 1972
	Hand/eye co-ordination	14h30	Winget *et al.,* 1985; Cappaert, 1999
	Information processing	14h00-21h00	Winget *et al.,* 1985; Drust *et al.,* 2005
	Logical reasoning & arithmetic	14h00 & 14h21	Winget *et al.,* 1985; Drust *et al.,* 2005
	Short term memory	08h00-13h34	Winget *et al.,* 1985
	Mood	14h00-16h00	Atkinson and Reilly, 1996; Cappaert, 1999
	Arousal	06h00-14h00	Winget *et al.,* 1985
Joint flexibility and stiffness	Flexion, extension, rotation	12h00-24h00	Winget *et al.,* 1985; Gifford, 1987
	Whole body flexion	17h00-18h00	Atkinson *et al.,* 1993; Edwards and Atkinson, 1998
	Stiffness	Lowest levels in early evening	Wright *et al.,* 1969
Strength measures	Grip strength	14h00-19h00	Atkinson *et al.,* 1993; Jasper *et al.,* 2009
	Elbow flexion strength	17h44	Winget *et al.,* 1985; Gauthier *et al.,* 1997
	Back strength	17h00-19h00	Atkinson *et al.,* 1993;
	Leg strength	18h00-19h30	Atkinson *et al.,* 1993; Drust *et al.,* 2005
	Concentric and eccentric strength	18h00-19h00	Wyse *et al.,* 1994; Souissi *et al.,* 2002
Anaerobic exercise variables	Peak power output (Wingate test, stair-run test, broad jump test etc.)	17h00-18h00	Hill and Smith, 1991; Hill *et al.,* 1988; Reilly and Down, (1992) Melhim, 1993; Chtourou *et al.,* 2012 c
	Maximal muscle power	17h00-19h00	Racinais *et al.,* 2005
	Power output (Biokinetic swim bench)	16h13	Reilly and Marshall, 1991
Metabolic and aerobic exercise variables	Minute ventilation	16h42	Winget *et al.,* 1985; Cappaert, 1999; Brisswalter *et al.,* 2007
	Heart rate	12h00-18h00	Winget *et al.,* 1985; Sugawara *et al.,* 2001
	Oxygen consumption (VO_2)	17h25-1800	Winget *et al.,* 1985; Hill *et al.,* 1988
	VO_{2max}	15h30-18h30	Hill *et al.,* 1988; Drust *et al.,* (2005)

1.6.1.2 Diurnal variation and task performance

Tasks related to specific skills have also been shown to display diurnal variations (Reilly and Waterhouse, 2009). For example, Atkinson and Speirs (1998) concluded that time-of-day affects the performance of tennis serves in a way that suggests a nonlinear relationship between velocity and

accuracy, with first serves being faster at 18h00. Deschodt and Arsac (2004) observed that there was diurnal variation in swimming technical ability, in eleven swimmers (mean age ± SD: 19.0 ± 1.3 y; five males and six females) tested at 08h00, 13h00 and 18h00. Specifically, swimming technical ability changed as the velocity changed; with stroke length decreasing and stroke rate increasing from the morning to evening (Deschodt and Arsac, 2004). In addition, forward movement of the hand was increased by 16% and maximal depth of the hand by 6% from the morning to evening (Deschodt and Arsac, 2004). Sinnerton and Reilly (2002) also noted that stroke rate was higher in the evening (17h30) compared with the morning (06h30) in eight swimmers aged between 19 and 28 years, which is in line with the findings of the latter study.

Reilly *et al.* (2007) conducted a study on football players and concluded that the peak performance of juggling and dribbling speed occurred in the evening (20h00), around the same time that body temperature peaked. Similarly, Rahnama *et al.* (2009) also observed diurnal variation in tasks specifically related to soccer skills, namely dribbling, wall-volley and soccer chipping with performance being significantly greater between 19h00-21h00 compared to 07h00-09h00, in twelve players (mean age ± SD: 22.6 ± 3.0 y). Recently, Chtourou *et al.* (2012 c) concluded that variables pertinent to soccer performance, such as the Yo-yo test and repeated sprint ability, in twenty junior football players (mean age ± SD: 17.6 ± 0.6 y), all peaked at 17h00, as opposed to 07h00.

1.6.1.3 Diurnal variation and laboratory-based performance

While a wide range of "events" have been simulated in the laboratory, a detailed review is beyond the scope of this thesis, which will rather focus on swimming. Swimming performance in simulated time-trials or contests also displays a similarly timed diurnal rhythm to that of body temperature (Reilly and Waterhouse, 2009). For example, maximal swimming performance has been determined to peak in the late evening (Baxter and Reilly, 1983; Deschodt and Arscac, 2004; Kline *et al.,* 2007). More specifically, Baxter and Reilly (1983) observed that there was a linear improvement in time-trial performance throughout the day, when swimmers (four males and ten females; mean age: 14.7 y) were tested at 06h30, 09h00, 13h30, 17h00 and 22h00, in a counterbalanced study design. The authors reported an improvement of 3.5% and 2.5% from 06h30 to 22h00, in 100 m and 400 m time-trials respectively (Baxter and Reilly, 1983). Similarly, Deschodt and Arsac (2004) found a total improvement of 4% in 50 m swimming performance from morning to evening and Kline *et al.* (2007) reported that swimming performance was impaired from 02h00 to 08h00, compared to all other times of the day (11h00, 14h00, 17h00, 20h00 and 23h00), with the peak in performance being

recorded at 23h00 in twenty-five swimmers (12 males and 13 females; mean age ± SD: 20.7 ± 0.6 y). Additionally, Sinnerton and Reilly (2002) observed that 50 m swimming performance was 1.9% greater in the evening for 4×50 m freestyle sprints and 3.5% greater, also in the evening for a 400 m freestyle sprint. Conroy and O'Brien (1974) also reported that performance was significantly greater in the evening for various sporting populations. Superior evening performance was reported for 9 of the 16 swimming participants, all of the running participants (n=6) and all of the shot-put participants (n=3) (Conroy and O'Brien, 1974).

Martin and Thompson (2000) analysed the physiological and kinematic responses to the same training set twice a day (06h30-08h00 and 16h30-20h00) on three separate days in seven, male collegiate swimmers (mean age ± SD: 22.0 ± 4.0 y). The testing protocol consisted of a 600 m warm-up followed by 10 x 100 m sub-maximal freestlye swims, twice a day. Swimming speed was controlled by a pacing device (Aquapacer©). The authors observed a diurnal variation in resting heart rate, oxygen consumption (VO_2), minute ventilation (VE) and volume of expired carbon dioxide (VCO_2) on two of the three days of testing (Martin and Thompson, 2000). During the submaximal swimming set, there was no reported diurnal variation in heart rate or stroke rate, but RPE was reported to be higher in the morning on two of the three days (Martin and Thompson, 2000). Mean swimming performance was faster in the evening on days one and two and faster in the morning on day three; but the difference was not significant (Martin and Thompson, 2000). Additionally, although it was mentioned above that there was no diurnal variation in stroke rate; stroke rate was higher in the morning on all three days, but the differences were not significantly different (Martin and Thompson, 2000). This is in contrast to the findings of Deschodt and Arsac (2004) and Sinnerton and Reilly (2002). Post exercise, there was a significant diurnal variation in oral temperature and blood glucose, on two of the three days. Thus, Martin and Thompson (2000) concluded that there was no diurnal effect on physiological and kinematic responses during a sub-maximal set. The findings of this study are of interest as they suggest that the exercise bout possibly had the same extent of disturbance at different times of the day, or that the swimmers were able to adapt to the training set equally in the mornings and evenings. Moreover, the authors did not analyse chronotype, thus there is the possibility that the group consisted of more morning- than evening-types, which could hypothetically blunt any time-of-day effects.

In conclusion, it is clear that there is evidence for a circadian variation in many measures of athletic performance. However, the peaks in performance are reported to occur from about 15h30 to 20h30, with amplitudes in peak performance ranging from 2% to 11% of the daily mean, indicating that there is large inter-individual variation (Kerkhof, 1985; Drust *et al.,* 2005; Reilly and Waterhouse,

2009) and that there is a large window of time during which athletes are able to achieve this maximal performance (Cappaert, 1999; Drust *et al.,* 2005; Reilly and Waterhouse, 2009). As such, inter-individual characteristics of athletes need to be considered. For example, training time-of-day and chronotype may potentially have an impact of performance at different times of the day, which will be discussed below.

1.6.2 The influence of time-of-day training on diurnal variation in sports performance

1.6.2.1 Strength training and anaerobic power output

Souissi *et al.* (2002) assessed the effect of training at the same time-of-day on diurnal variation in muscular performance. Fourteen males (mean age ± SD: 19.0 ± 1.2 y) were required to do baseline tests for peak torque and anaerobic power (Isokinetic test and Wingate test), in the morning and evening. Thereafter, the participants were randomly assigned to two training groups. Seven participants were assigned to the morning training group (MTG) (trained in the morning between 07h00 and 08h00) and seven the evening training group (ETG) (trained in the evenings between 18h00 and 19h00). Two weeks after completing the six week training protocol (habitual training in either the morning or evening), the participants underwent the same testing prior to the start of the training protocol. The results showed that before the six week training protocol began, peak torque and peak anaerobic power were greater in the evening, for all participants. However, after the six week training protocol there was no longer a difference between peak torque and peak anaerobic power between morning and evening testing for the MTG (Souissi *et al.,* 2002). Thus, it appears that habitual morning training abolished the diurnal variation in muscular performance.

Sedliak *et al.* (2007) assessed the diurnal patterns of testosterone, cortisol and maximal isometric strength of the knee extensors in 38 untrained male participants (20-45 years). All participants underwent a 10-week strength training period, training between 17h00 and 19h00. Thereafter, the participants were required to train for another 10 weeks in either the morning or evening. The MTG (n=20) trained between 07h00-09h00 and the ETG (n=18) between 17h00-21h00. The authors concluded that the diurnal pattern of isokinetic strength was blunted after time-of-day specific training, in both groups and that morning cortisol concentration was decreased, for the MTG only (Sedliak *et al.,* 2007).

Similarly, Sedliak *et al.* (2008) then analysed maximum strength and electromyography (EMG) in males (n=27) who had undergone the same 10 week preparatory strength training protocol as described in the previous study. Thereafter, fourteen participants were grouped to form the MTG (mean age ± SD: 32.0 ± 7.0 y), thirteen the ETG (mean age ± SD: 33.0 ± 7.0 y) and seven the control group (CG) (mean age ± SD: 34.0 ± 8.0 y), who did not train but performed all the strength and EMG testing. Prior to the time-of-day specific training protocol, peak torque was significantly higher at 17h00 compared to 07h00. This difference decreased after time-of-day specific training in the MTG but not in the ETG or CG (Sedliak *et al.,* 2008). In contrast, Blonc *et al.* (2010) conducted a 5-week training study analysing the effect of habitual training on maximal muscle power performance. The study was very similar in design to the aforementioned studies in that 16 students were randomly assigned to either a MTG (07h00-09h00) or an ETG (17h00-19h00), with testing taking place prior to and after the 5-week specific time-of-day training protocol. The authors concluded that after five weeks of habitual training, although there were improvements from baseline testing within the MTG and ETG, there were no reported time-of-day effects on performance in any of the variables (sprints, jumps and other exercises), in either training group (Blonc *et al.,* 2010).

More recently, Chtourou *et al.* (2012 a) analysed the effect of strength training at the same time-of-day on diurnal variations in muscular anaerobic performances. Thirty participants were assigned to three groups: 10 in the MTG (07h00-08h00), 10 in the ETG (17h00-18h00) and 10 in the CG. The two training groups underwent eight weeks of lower extremity training, three times a week, at their assigned time-of-day training times. Before the 8-week training protocol began, anaerobic performance was significantly greater in the evening, compared to the morning, with an acrophase between 17h00 and 18h00, for all groups. Follow-up testing after the 8-week intervention showed that this diurnal variation was reduced for the MTG while daily variations in muscle power and strength remained evident for the ETG and CG (Chtourou *et al.,* 2012 a). Similarly, Chtourou *et al.* (2012 b) then recruited 31 male participants (mean age ± SD: 23.1 ± 1.98 y) who underwent a 12-week lower-extremity resistance training protocol, followed by a 2-week taper period. Again, 10 participants were randomly assigned to the MTG, 11 to the ETG and 10 to the CG. The main finding being that training altered the diurnal variation of anaerobic performance, with the greatest improvements occurring at the time-of-day at which training had been habitually conducted (Chtourou *et al.,* 2012b). More specifically, baseline testing showed that performance during the Wingate test, vertical jump test and maximum voluntary contraction (MVC) was higher in the evening, for all participants. Then, after the 12-week protocol of habitual training, these diurnal differences were blunted for the MTG but persisted in the ETG and CG.

Thus, there is evidence to suggest that habitual training does have an impact on time-of-day performance in activities such as anaerobic power, peak torque, strength and that morning training may reduce diurnal variation. However, most studies have tested young, untrained participants and the sample sizes have been small.

1.6.2.2 Time-trial performance

Arnett (2001) analysed the effect of a morning and afternoon practice schedule on morning and afternoon swim performance. Ten competitive swimmers (six males and four females; mean age ± SD: 15.3 ± 0.95 y) were required to train in both the morning and evening, for four months. Prior to the four-month standardised training period, all swimmers performed two time-trials, one in the morning and one in the evening. On average, the swimmers were faster in the evening time-trial. Swimming (prior to and after the training period) performance was assessed by a 91.44 m freestyle sprint, which took place at the same time-of-day as the training sessions (06h15 and 17h00), with each swimmer completing one test session per day in a counterbalanced study design. After the training intervention, the swimmers' time-trial performance in the morning equalled that of the evening (Arnett, 2001). This reduction in diurnal variation could be pertinent for swimmers required to swim personal best times in morning heats, in order to qualify for evening finals.

Arnett (2002) then investigated the effects of variations in warm-up length on diurnal variation in body temperature and swim performance. Ten competitive swimmers, six male and four female (mean age ± SD: 15.0 ± 1.0 y) performed time-trials in the morning and evening. Prior to the morning time-trial, the participants performed a standardised warm-up of either a standard volume (2,011.68 m) or a warm-up of 200% of that volume. Prior to the evening time-trials, the warm-up volumes were either 33% or 100% of the standard warm-up volume (2,011.68 m). It was concluded that an increased warm-up time in the morning eliminated the diurnal variation in body temperature; however swimming performance was still superior in the evening (Arnett, 2002).

Recently, Martin *et al.* (2007) conducted a study to determine if the diurnal variation in swim performance was reduced by training twice a day, compared to training only once a day. Sixteen competitive swimmers were separated into two groups, determined by the squad with whom they trained with: 1) the morning and evening group (MEG; n=8; mean age ± SD: 15.2 ± 1.0 y) and 2) the evening-only group (EOG; n=8; mean age ± SD: 15.4 ± 1.4 y). Both squads trained five days per week. The MEG habitually trained, in both the morning (06h30-07h30) and the evening (16h00-18h00) and

the EOG swimmers in the evenings only (19h00-22h00). All of the MEG swimmers were neither-types and the EOG group moderate evening-types (MET) (Martin *et al.,* 2007). The testing protocol consisted of a 100 m freestyle morning and evening time-trial, performed in a random order with 48-hours separating each time-trial, which was then repeated within seven days. The morning time-trials took place at similar times for both groups (MEG: 06h30-07h30, EOG: 07h00-09h00), however the evening time-trials were dictated by the habitual training routines of the two squads and were not equally matched (MEG: 16h30-18h30, EOG: 20h00-22h00). This study design is flawed; not only is the chronotype of the two groups that are being compared, different, but so is the morning time-trial time. Thus, the study design was innately biased because the neither-types in the MEG group were racing earlier in the morning compared to the moderate evening-types in the EOG. This is further exacerbated by the evening time-trial schedule. Nevertheless, oral temperature was significantly lower during morning time-trials, for both the groups (Martin *et al.,* 2007). Maximal swimming performance was significantly slower during morning time-trials for the MEG (1.7% slower) and EOG groups (1.4% slower) (Martin *et al.,* 2007). Additionally, the MEG group had a higher stroke rate in the evening and the EOG group had similar stroke rates at both times of day (Martin *et al.,* 2007). The level of intra-daily variation for the 100 m maximal sprint was higher in the MEG group (2.5%) compared to the EOG (2.0%) (Martin *et al.,* 2007). This is interesting as one would expect that since the MEG group habitually trains twice a day, that they would be able to adapt better and "reduce" the amount of intra-daily variation. Martin *et al.* (2007) also acknowledge that although the two swimming squads are of a similar standard, their respective training programs were highly variable. This could possibly account for the differences observed in intra-daily variation. The authors discuss that the diurnal variation was evident in both groups and was thus irrespective of training in the mornings and evening or in the evening only (Martin *et al.,* 2007). These findings are in contrast to those of Arnett (2001). The differences may be related to training status, since the participants of the latter study were recruited after only four weeks of habitual afternoon/evening only training.

It is evident, from the research discussed above, that there is a large amount of variation with regards to the protocols employed, the age, gender and training status of the participants and the environmental time-of-day that testing took place. In addition, the majority of the studies involved relatively small sample sizes. Also, although chronotype was assessed in some of the studies above, none of the studies analysed the direct relationship between swimming performance and chronotype.

1.6.3 The influence of chronotype on diurnal variation in performance

There is conflicting evidence regarding the effect that chronotype has on performance at different times of the day. As discussed in Section 1.3.4, a significant difference in chronotype distribution has been observed when comparing different sports (Zani *et al.,* 1984; Lastella *et al.,* 2010). However, the extent to which chronotype influences performance is largely unknown.

Hill *et al.* (1988) assessed chronotype and performance in 32 college students (8 males, mean age ± SD: 26.0 ± 4.0 y and 24 females, mean age ± SD: 24.5 ± 5.0 y). Fourteen of the participants were classified as morning-types and 11 as evening-types. The participants were then required to perform two continuous maximal cycle ergometer tests in one day; one in the morning (06h00-08h30) and one in the evening (15h30-18h00). In order to ensure that an accurate VO_2max measurement was obtained, the participants also performed two discontinuous maximal tests on a different day, one in the morning and one in the evening. The results showed that although the group as a whole had a higher VO_2max in the evening, the morning-type participants showed no diurnal variation in VO_2max. This was the first study pioneering that chronotype may influence components of performance at different times of the day.

In contrast, Burgoon *et al.* (1992) analysed chronotype and running performance in 26 untrained males (mean age ± SD: 23.3 ± 4.4 y). The participants were required to perform two maximal treadmill tests; one between 07h30 and 08h30 and the other between 19h30 and 20h30, 48-hours apart. The authors observed that there were no significant differences in maximum exercise performance according to chronotype (Burgoon *et al.,* 1992). More specifically, significant differences were observed for the time-of-day and time to exhaustion, with higher values being obtained in the evening session. Additionally, there was no interaction between time-of-day and VO_2max nor were there any chronotype differences in VO_2max and time to exhaustion (Burgoon *et al.,* 1992).

Sugawara *et al.* (2001) then analysed diurnal variations in post-exercise parasympathetic nervous reactivation in different chronotypes. The authors reported that the evening-type participants (n=6; mean age ± SD: 24.0 ± 3.3 y) had a significantly slower heart rate recovery after the morning time-trial compared to the evening time-trial, whereas heart rate recovery was the same, after both time-trials, for the morning-type individuals (n=6; mean age ± SD: 24.0 ± 1.7 y) (Sugawara *et al.,* 2001). A limitation of this study was that the training status of the participants was not homogenous, as each group contained one athlete and the other participants were non-athletes.

Atkinson *et al.* (2005) assessed 16.1km cycling time-trial performance, at 07h30 and 17h30 in eight male cyclists (mean age ± SD: 24.9 ± 3.5 y). Based on CSM score, seven cyclists scored as "morning-type" and one as "evening-type". Although the cyclists were skewed towards "morningness", as determined by mean CSM score, mean cycling time was significantly slower in the morning compared to the evening. Brown *et al.* (2008) then conducted a study on 16 collegiate rowers (8 males and 8 females) who had a near-daily (habitual) morning training programme. The BALM and H-Ö questionnaires were administered to the participants. Interestingly, the morning-type individuals rowed significantly faster in the 2000m ergometer time-trial that was conducted in the morning. On the other hand, the evening-type and neither-type rowers (who also habitually trained in the morning) rowed faster in the evening time-trial (Brown *et al.,* 2008). This was the first clue that in addition to habitual training time-of-day, an individual's chronotype may be key to anticipating the extent to which peak performance time might change.

Lastly, Tamm *et al.* (2009) reported that the chronotype of 18 participants (19-54 y) was related to their ability to generate torque during a MVC at different times of the day. Specifically, torque production of the evening-type individuals (n=9) was 13% higher in the evening compared to the morning, whereas torque production was the same in the morning and evening, in the morning-type individuals (n=9) (Tamm *et al.,* 2009).

Thus, based on the research discussed above, it is evident that the magnitude to which chronotype has an influence on performance, at different times of the day, is somewhat unknown. Understanding the influence of chronotype on performance, at different times of the day, could be a useful tool for coaches and athletes, with regards to improving training and racing principles.

1.7 SUMMARY AND CONCLUSIONS OF THE LITERATURE REVIEW

Circadian rhythmicity is an organised system that is responsible for driving the near 24-hour rhythm exhibited in most living, eukaryotic and prokaryotic, organisms. This system is controlled internally, by clock genes, and externally by zeitgebers such as light, temperature and social cues. Due to individual differences in circadian rhythmicity and that fact that this rhythm is not exactly 24-hours, individual differences arise. For example, there are individual differences in the preferred times of activity and rest (known as chronotype or diurnal preference). Of interest to this thesis is that fact that the *PER3* gene, which is a clock gene involved in circadian rhythm generation, has been associated with chronotype. However, there are contrasting findings regarding the association between chronotype and *PER3* VNTR genotype. Additionally, females have been shown to be more morning-oriented, compared to males, and few studies have focussed on *PER3* VNTR distribution in large cohorts of female participants. Therefore, it is unknown if the association between chronotype and *PER3* VNTR genotype will hold when females are analysed separately. Therefore, there is a need to analyse chronotype and *PER3* VNTR genotype distribution, as well as the relationship between the two, in a large cohort of females.

Recently, a study showed that there was a difference between chronotype and *PER3* VNTR genotype distribution when comparing South African endurance athletes to active, but non-competitive, individuals. However, it is unknown whether these observations were due to the fact that the participants were male Caucasian South Africans or due to the activity level or physical activity status of the individuals studied. Therefore, future research is required in order to compare chronotype and *PER3* VNTR genotype in South African active and inactive individuals, in order to determine if physical activity status contributes to chronotype distribution.

Studies documenting exercise performance at different times of the day have consistently reported that performance tends to be superior in the early evening, around the same time-of-day that body temperature nears its peak value. However, inter-individual variation with regard to habitual training time-of-day and chronotype appear to have an influence on diurnal variation in performance. Additionally, the extent to which *PER3* VNTR genotype has an influence on individuals' during physical activity level, at different times of the day, is unknown. Therefore, exercise performance with regard to individual differences in circadian rhythmicity as well as training habits and habitual behaviour need to be accounted for when analysing exercise performance at different times of the day.

1.8 AIMS OF THIS THESIS

Each aim or focus of this thesis will be addressed in a separate chapter:

Therefore, the first aim of this thesis is to describe and compare the *PER3* VNTR genotype and chronotype distributions within (i) South African male populations of varying physical activity level and (ii) South African male and female runners (Chapter 2).

The second aim of this thesis is to analyse exercise performance at different times of the day and to assess if *PER3* VNTR genotype, chronotype and training habits, play a role or have an effect on diurnal variation in exercise performance (Chapter 3).

CHAPTER 2: CIRCADIAN RHYTHM, PHYSICAL ACTIVITY LEVEL AND GENDER

A DESCRIPTION OF CHRONOTYPE AND *PER3* VNTR GENOTYPE

STUDY 1

2.1 INTRODUCTION

2.1.1 Rationale

The relationship between physical activity and chronotype is an area that has been poorly explored, with fewer than ten studies focussing on the topic (Rossi *et al.,* 1983; Lastella *et al.,* 2010; Cavallera *et al.,* 2011; Kauderer and Randler, 2012). Nevertheless, two of the common findings are that evening-types are under-represented in the athletic groups studied so far (Lastella *et al.,* 2010; Kunorozva *et al.,* 2012), and that morning-type individuals tend to be more active than evening-type individuals (Cavallera *et al.,* 2011; Kauderer and Randler, 2012).

Kunorozva *et al.* (2012) reported that there was a significant difference between chronotype distribution and activity-type when comparing South African male Caucasian individual sport endurance athletes (cyclists, runners and Ironman triathletes) to an active control group, who did not compete in any races or events, but trained at least twice a week in a gym. The endurance athletes were more morning-oriented in comparison to the active controls (Kunorozva *et al.,* 2012). Furthermore, Kunorozva *et al.* (2012) were the first researchers to have analysed *PER3* VNTR genotypic and allelic frequency distributions within a sporting population and an active population, showing that the individual sport athletes had a higher frequency of the *PER3*^{5} allele compared to the active population.

Additionally, the data reported by Kunorozva *et al.* (2012) show that the chronotype and genotype distributions of athletic South African populations are different to those of other populations around the world (Archer *et al.,* 2003; Jones *et al.,* 2007; Osland *et al.,* 2011). However, it is not clear whether these findings were particular to Caucasian male South Africans or to the physical activity level of the individuals studied. Therefore, by comparing South African participants of low physical

activity versus high activity we will be able to determine if physical activity level is the driving force behind the findings of the Kunorozva *et al.* (2012) study or if Caucasian male South Africans in general display different chronotype and genotype distributions to those of other populations.

Another interesting point raised by Kunorozva's study was whether their findings would also hold for females. When one examines the literature regarding the relationship between gender and chronotype, it is apparent that females tend to be more morning-oriented than males (Randler, 2007; Cavallera and Giudici, 2008). On the other hand, the literature associated with the relationship between gender and *PER3* VNTR genotypic and allelic distribution is relatively scanty. With the exception of Jones *et al.* 2007, no studies have deliberately compared males to females with respect to the *PER3* VNTR genotype, in large population samples. Some studies have performed gender sub-analyses and showed that the *PER3* VNTR genotype distribution was not different when comparing males and females (Benedetti *et al.,* 2008; Ellis *et al.,* 2009; Goel *et al.,* 2009). On the other hand, other studies have reported male and female *PER3* VNTR genotypic and allelic frequencies, in small population samples, but again have not separated gender in the statistical analyses. For example, in a population sample of 40 males and 32 females, Voinescu and Coogan (2012) reported that 25% of the males and 16% of females were homozygous for the 4-repeat allele. In addition, Chellappa *et al.* (2012) reported that in a population of 50 participants, nine males and 13 females were genotyped as $PER3^{44}$, nine males and one female as $PER3^{55}$ and five males and 14 females as $PER3^{55}$. However, the latter findings should be interpreted with caution as extreme chronotypes were excluded from the study, which could potentially have an influence on genotype distribution. The sample size was also relatively small, which makes genetic comparisons between *PER3* VNTR genotype groups difficult. Jones *et al.* (2007) is the only study to have separately analysed a large cohort of male and female participants with respect to chronotype and the *PER3* gene. It was reported that the distribution of *PER3* allele frequencies of male and female participants did not differ to that of the combined sample and that gender did not have an effect on the relationship between chronotype and the *PER3* gene (Jones *et al.,* 2007).

Therefore, it is largely unknown if the association between chronotype and *PER3* VNTR genotype is still persistent when male and female participants are analysed separately. Thus, based on the findings by Kunorozva *et al.* (2012) and the fact that females have been shown to be more morning-oriented (as mentioned above) than males, one could question the role or influence of the *PER3* VNTR genotype on chronotype in women. Additionally, does the chronotype and genotype association observed in the latter study persist in female runners?

2.1.2 Aims

This study was designed to answer two major research questions. Firstly, are the chronotype and *PER3* VNTR genotype distributions and relationships similar in South African physically active compared to low physical activity individuals? Secondly, are the chronotype and *PER3* VNTR genotype distributions and relationships similar in South African male and female runners?

Therefore the first aim of this study was to determine the extent to which level of physical activity can account for differences in chronotype and *PER3* VNTR genotype in South African Caucasian male runners and male low physical activity individuals.

The objectives relating to this aim were:

1) To describe and compare the chronotype distributions of South African Caucasian male runners and low physical activity individuals.
2) To describe and compare the distribution of the VNTR polymorphism within the *PER3* gene in the same two populations.
3) To determine the level of association between chronotype and *PER3* VNTR genotype in the same two populations.

The second aim of this study was to determine if there are gender differences in chronotype and *PER3* VNTR genotype distribution when comparing South African Caucasian male runners to South African Caucasian female runners.

The objectives relating to this aim were:

1) To describe and compare the chronotype distributions of South African Caucasian male and female runners.
2) To describe and compare the distribution of the VNTR polymorphism within the *PER3* gene in the same two populations.
3) To determine the level of association between chronotype and *PER3* VNTR genotype in the same two populations.

2.1.3 Hypotheses

2.1.3.1 Research question 1

It was hypothesised that the South African Caucasian male runner group would contain a greater proportion of morning-type individuals and the low physical activity group a higher proportion of evening-type individuals, based on the H-Ö questionnaire. It was also hypothesised that the runner group would have a higher frequency of the *PER3*^{5} allele, compared to the low physical activity group. Lastly, it was hypothesised that there would be a relationship between chronotype and *PER3* VNTR genotype in both groups. More specifically, it was hypothesised that the *PER3*^{4} allele would be associated with lower H-Ö scores and a preference for evenings, and the *PER3*^{5} allele with higher H-Ö scores and a preference for mornings.

2.1.3.2 Research question 2

It was hypothesised that the South African Caucasian female runner group would contain a higher proportion of individuals being classified as definite morning-types (DMT) or moderate morning-types (MMT) in comparison to the male runner group, based on the H-Ö questionnaire. Secondly, it was hypothesised that there would be no gender related differences with regards to *PER3* VNTR genotype distribution. Lastly, it was hypothesised that there would be a relationship between chronotype and *PER3* VNTR genotype, in the male runner group. More specifically, it was hypothesised that the *PER3*^{4} allele would be associated with lower H-Ö scores and a preference for evenings, and the *PER3*^{5} allele with higher H-Ö scores and a preference for mornings.

2.2 MATERIALS AND METHODS

2.2.1 Participants

The participants constituted three groups, namely: the male low physical activity group (LOWM); the male runners group (RUNM) and the female runners group (RUNF). The health of the runners (both male and female) and low-activity male groups was self-reported, both verbally and in written form. Prior to participation all participants were questioned by the author regarding their current health state. Additionally, all participants completed Appendix 4 (page 151) which documented current medication and supplement use. Participants that reported illness or had been on medication within the last month were excluded from the study. Thus, all participants were apparently healthy, between the ages of 25 and 50 years, and were excluded from participation if they were currently taking amphetamines, such as pemoline, alerting drugs such as modafinil, soporific drugs or hypnotics (e.g. benzodiazepines and minor tranquilizers, diazepam, lorazolam, zopiclone) or melatonin or had taken these drugs, within the three months prior to the study. Selective serotonin reuptake inhibitors (SSRI's) may have an effect on chronotype, and although not listed as medication that would exclude a participant, none of the participants reported taking these or any other medications for depression/ anxiety.

The LOWM group comprised 104 Caucasian males, whom, since school, had not participated in any physical activity or exercise on two or more days per week. The participants were recruited at various shopping malls (V & A Waterfront, Cape Town; Bayside Mall, Table View, Cape Town), from various companies (Old Mutual Park, Pinelands, Cape Town; Moffatt Optical, Pietermaritzburg) and from staff and eligible students at the University of Cape Town. Recruitment took place throughout the day in order to ensure that sample collection was not biased towards any particular chronotype, at a certain time-of-day.

The RUNM and RUNF groups comprised 101 and 102 trained South African Caucasian male and female runners respectively. All runners were recruited from the 2011 Old Mutual Two-Oceans Ultra-marathon, the 2011 Pick 'n Pay Cape Times Knysna Forest Marathon and the 2011 Mr Price Winelands Marathon, as well as through advertising at running clubs in the Western Cape and Kwa-Zulu Natal, in 2011 and 2012. The runners were reasonably well trained, having trained at least three times per week for the three months prior to the study, and all currently participated in amateur running races, of at least 21km in distance.

2.2.2 Study design

All participants were informed as to what the purpose of the study was, the testing to be undertaken and the risks and benefits of the trial, both verbally and in writing (Appendix 1). On agreeing to take part in the study all participants signed a consent form (Appendix 2). All participants completed the Horne-Östberg Morningness-Eveningness Personality questionnaire (Appendix 3) to determine their chronotype as well as another questionnaire which documented personal details and physical activity history (Appendix 4). Additionally, the two running groups completed a questionnaire which documented running training and racing history (Appendix 5).

Wherever possible a 5 ml venous blood sample was obtained from each participant by venipuncture of a forearm vein and collected into an ethylenediaminetetraacetic acid **(**EDTA) vacutainer tube. Blood samples were stored at -20°C until subsequent deoxyribonucleic acid (DNA) extraction. Alternatively, buccal cell samples were obtained from participants, by scraping the inside of both cheeks using Epicentre Catch-All™ Sample Collection Swabs (Epicentre Biotechnologies, WI, USA). DNA was extracted from the blood and buccal samples and each individual was genotyped for the VNTR polymorphism within the *PER3* gene (Archer *et al*., 2003). Questionnaire administration, height and mass measurements as well as blood and/or buccal cell sample collection took place in the field (i.e. at the shopping malls or at the running race registration sites) and at the UCT/MRC Research Unit for Exercise Science and Sports Medicine (ESSM), Department of Human Biology, University of Cape Town. The DNA extraction took place at ESSM, while genotyping was performed at the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town. This study was approved by the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee (Ref: 412/2009 and 066/2009), and performed in accordance with the principles of the Declaration of Helsinki (October 2008, Seoul), the International Conference on Harmonization (ICH) and the South African Good Clinical Practice (GCP) guidelines. The protocol used in this study conformed to the international ethical standards for biological rhythm research described by Portaluppi *et al.* (2010).

2.2.3 Detailed testing procedures

2.2.3.1 Chronotype

The H-Ö questionnaire served as a measure of chronotype. As mentioned in the literature review, low scores (16-41) indicate eveningness and high scores (59-86) morningness. Based on the scores obtained, individuals are placed into one of five chronotype categories: definite evening-type (DET, 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) (Horne and Östberg, 1976).

2.2.3.2 Anthropometry

All participants' height (centimetres) and mass (kilograms) were measured by the same investigator and used to determine their body mass index ($kg \cdot m^{-2}$).

2.2.3.3 Laboratory analysis

DNA EXTRACTION FROM WHOLE BLOOD

Total genomic DNA was extracted from the blood samples using the procedure described by Lahiri and Nurnberger (1991), with slight modifications (Mokone *et al.*, 2006). Briefly, the blood samples were transferred to sterile 15 ml polypropylene tubes, to which 10 ml of TKM1 buffer (10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$ and 2 mM EDTA) containing 2.5% Nonidet P-40 were added to lyse the red blood cells. After incubating at room temperature for 10 min the white blood cells (WBC) were then be pelleted by centrifugation at 1200 X g at room temperature for 10 min and washed at least once with one volume of TKM1 buffer. The washed WBC pellets were resuspended in 800 µl of TKM2 buffer (10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 0.4 M $NaCl_2$ and 2 mM EDTA) containing 50 µl of 10% SDS and incubated for at least 60 min at 55°C to lyse the WBC. One hundred and fifty µl of 5 M $NaClO_4$ and 500 µl of chloroform were added to each sample, which was then be mixed thoroughly by vortexing for 15 - 20 s. The samples were transferred to 1.5 ml microfuge tubes and the protein was precipitated by centrifugation at 15 000 X g for 5 min at room temperature. Five hundred microlitres of the top aqueous phase was transferred to new microfuge tubes containing 1 ml of absolute ethanol, mixed and the DNA pelleted by centrifugation at 15 000 X

g for 2 min at room temperature. The precipitated DNA was air dried for at least 30 min and resuspended in 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Each tube was incubated at 65°C for 15 min before being stored at -20°C under secure conditions in the Biochemistry laboratory at ESSM, until subsequent polymerase chain reaction (PCR) analysis.

DNA EXTRACTION FROM BUCCAL SAMPLES

Total genomic DNA was extracted from the buccal samples, according to the method described in Aljanabi and Martinez (1997). This method was also used by Kunorozva *et al.* (2012). The DNA samples were then stored at -20°C under secure conditions in the Biochemistry laboratory at ESSM, for later genetic analysis.

PER3 VNTR GENOTYPING: PCR ANALYSES AND DIGESTION OF PCR PRODUCT

DNA samples were genotyped for the VNTR and the M1037T polymorphisms within the *PER3* gene. Exon 18 containing *PER3* and the flanking regions was amplified by PCR and digested with *Nco*I as described by Ebisawa *et al*. (2001) and Archer *et al*. (2003) and the M1037T polymorphism as described by Ebisawa *et al.* (2001).

The following primers were used to amplify the region in exon 18 containing the *PER3* VNTR polymorphism Forward: (*PER3*F) 5'-CAAAATTTTATGACACTACCAGAATGGCTCAC-3', Reverse: (*PER3*R) 5'-AACCTTGTACTTCCACATCAGTGCCTGG-3'. For the blood samples, the PCR was carried out in a total volume of 25 µl containing 80 - 100 ng of genomic DNA, 0.5 µl of 20 mM forward and reverse *PER3* primers, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 40 µM deoxy-ribonucleic acid triphosphates (dNTPs), 5 µl of 5X Gotaq flexi buffer and 0.125 µl of 0.5 U Gotaq DNA polymerase (Promega, Madison, WI, USA). The total volume was then made up to 25 µl, using distilled, autoclaved water. For the buccal samples, the PCR was carried out in a total volume of 25 µl containing 80 - 100 ng of genomic DNA, 0.5 µl of 20 mM forward and reverse *PER3* primers, 5 µl of 5X MyTaq reaction buffer and 0.2 µl of 5.0 U MyTaq Hot Start (Bioline, USA, Inc; supplied by Celtic Molecular diagnostics, South Africa) DNA polymerase. The total volume was then made up to 25 µl, using distilled, autoclaved water. Amplification was performed in the GeneAmp® PCR system 2700 (Applied Biosystems, Singapore). Initial denaturing was performed at 94°C for 5 min, followed by 35 cycles of a denaturing step of 45 s at 94°C, an annealing step of 45 s at 60°C, an elongation step of 1 min at 72°C and a final elongation

step of 5 min at 72°C. The *PER*3^4 amplified fragment was approximately 600 base pairs (bp) in length and the *PER*3^5 approximately 650 bp in length.

In addition to the *PER3* VNTR polymorphism, a second polymorphism (3110 (T→C), alternatively known as the M1037T polymorphism), present exclusively on the *PER3*^{4} allele, was also analysed. Genotyping the samples for this polymorphism, using 10 U *Nco*1, was done to accurately distinguish between the *PER3*^{4} and *PER3*^{5} alleles which are very similar in size. Briefly, the amplified DNA template contains the sequence CCA**T**GG. When the nucleotide T is present, the amino acid methionine is encoded. However, when a nucleotide substitution occurs (only ever on the *PER3*^{4} allele), the T is replaced with a C and the resultant amino acid is threonine. Therefore, this is a known functional polymorphism. *Nco*l recognises this sequence and cuts the DNA strand when the nucleotide T is present (Ebisawa *et al.,* 2001; Archer *et al.,* 2003).

The reaction mix used for this digestion contained 2 µl Tango buffer (Promega), 10 µl of DNA template from the PCR, 0.15 µl of bovine serum albumin (BSA, which aided digestion and stabilised the enzymes in the reaction), and 0.1 µl of the restriction enzyme *Nco*l. The PCR products were digested overnight at 37°C. Digestion products were analysed by electrophoresis on 2% agarose gels in 1X TAE buffer, run at 100 V for 40 minutes, and visualised under ultra-violet (UV) light using ethidium bromide (EtBr) staining.

Digestion with *Nco*l yielded fragments of 600, 420, 380 and 200 bp in size, depending on the VNTR and M1037T polymorphisms present. As a result, an individual was assigned one of six genotypes: homozygous 5M/5M, 4M/4M, 4T/4T or heterozygous 4M/5M, 4T/5M, 4M,4T (Table II). The numbers 4 and 5 represent the *PER3*^{4} and *PER3*^{5} repeats respectively of the VNTR polymorphism. The letters M and T represent the resultant methionine and threonine amino acid residues respectively from the 3110 (T→C) substitution or M1037T polymorphism.

Table II: **Expected fragment sizes for the *hPER3* VNTR and M1037T polymorphisms post restriction digest by *Nco*1.**

VNTR Genotype	M1037T Genotype	Band 1 (bp)	Band 2 (bp)	Band 3 (bp)	Band 4 (bp)
PER3^{55}	5M/5M	-	420	-	200
PER3^{45}	4M/5M	-	420	380	200
PER3^{45}	4T/5M	600	420	-	200
PER3^{44}	4T/4M	600	-	380	200
PER3^{44}	4M/4M	-	-	380	200
PER3^{44}	4T/4T	600	-	-	-

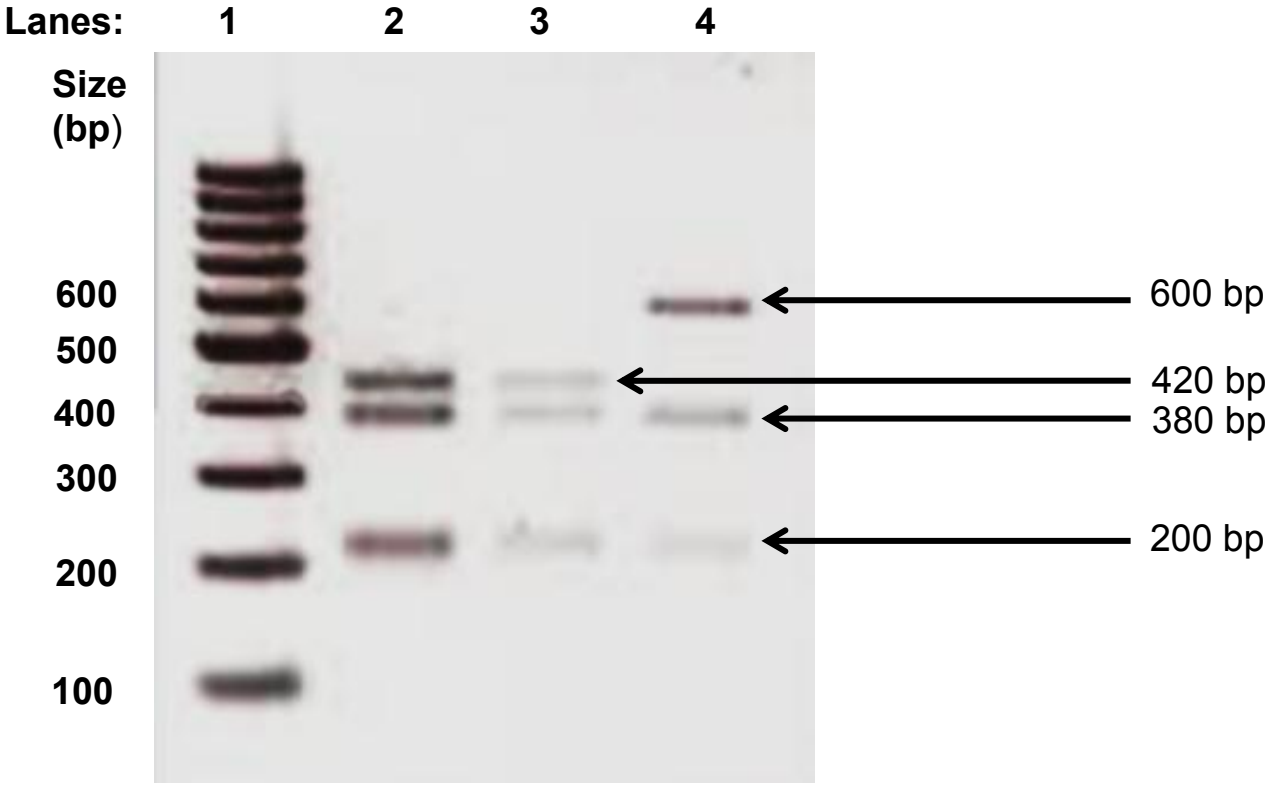

Figure 8: *Nco*1 restricted *PER3* amplicons from genomic DNA of Runners. Lane 1: 100 base pair DNA ladder (O' GeneRuler, Fermentas), Lane 2: individual genotyped as 4M/5M, Lane 3: individual genotyped as 4M/5M, Lane 4: individual genotyped as 4T/4M. Amplification products were resolved by electrophoresis on a 2% agarose gel and visualised under UV light using EtBr.

2.2.4 Statistical analyses

All data are expressed as the mean ± standard deviation. The Shapiro-Wilks test was used to determine if the data were normally distributed. Participant characteristics were compared using a one-way analysis of variance (ANOVA) for normally distributed data, and a Mann-Whitney U test for data that were not normally distributed. Post hoc analyses were performed used Scheffe's post hoc test. The internal consistency of the H-Ö questionnaire was estimated using Cronbach's α co-efficient. Correlations were performed using Pearson's product-moment correlation or a multinomial logistic regression. Hardy-Weinberg exact tests (GenePop, web version 4.2, http://genepop.curtin.edu.au) were used to test whether the proportions of 4- and 5-repeat alleles were in Hardy-Weinberg equilibrium. Pearson's chi-squared and Fisher's exact tests were used to compare the chronotype, genotype and allelic frequencies between the groups and the Freeman-Halton extension of the Fisher's exact test was used to compare the chronotype, genotype and allelic frequencies within the groups. Post-hoc analyses were conducted using a two-tailed Fisher's exact chi-squared test to compare the chronotype and *PER3* VNTR genotype frequencies within groups. This was done by grouping chronotype categories within groups (MT vs. NT and ET, NT vs. MT and ET and ET vs. MT and NT) and similarly, genotype within groups ($PER3^{44}$ vs. $PER3^{45}$ and $PER3^{55}$, $PER3^{45}$ vs. $PER3^{44}$ and $PER3^{55}$ and = $PER3^{55}$ vs. $PER3^{44}$ and $PER3^{45}$). Data were analysed using STATISTICA version 10 (Soft Inc., Tulsa Oklahoma, USA) and Stata version 10 (StataCorp LP, Texas, USA). Statistical significance was accepted when $p<0.05$.

2.3 RESULTS

A) PHYSICAL ACTIVITY LEVEL COMPARISON: CHRONOTYPE AND *PER3* VNTR GENOTYPE

2.3.1 Participant characteristics

The general characteristics of the male runner group (RUNM) and the male low physical activity group (LOWM) are presented in Table III. The RUNM group was significantly lighter ($p<0.001$) and had a lower body mass index (BMI) ($p<0.001$) compared to the LOWM group. The RUNM group exercised more days per week ($p<0.001$) compared to the LOWM group. The number of days of exercise per week was running-specific for the RUNM group.

Table III: General characteristics of the male runner (RUNM) and the male low physical activity (LOWM) groups

	RUNM	LOWM	p-value
Age (y)	34.4 ± 8.1 (101)	33.1 ± 8.2 (104)	0.107
Height (cm)	1.79 ± 0.06 (101)	1.81 ± 0.07 (104)	0.172
Body mass (kg)	77.92 ± 9.22 (101)	85.84 ± 12.05 (104)	p<0.001*
BMI ($kg \cdot m^{-2}$)	24.2 ± 2.6 (101)	26.3 ± 3.1 (104)	p<0.001*
Exercise ($day \cdot week^{-1}$)	4.05 ± 1.33 (99)	0.35 ± 0.69 (104)	p<0.001*

The data are presented as mean ± SD (n). The p-values represent significance as determined by a one-way ANOVA for normally distributed data and a Mann-Whitney U test for data that were not normally distributed. * indicates statistical significance. RUNM = male runner group; LOWM = male low physical activity group; BMI= body mass index.

2.3.2 Chronotype

All participants (205) completed the H-Ö questionnaire. Cronbach's α (George and Mallery, 2003) was used to determine the internal consistency of the H-Ö questionnaire in the RUNM and LOWM groups and was considered to be satisfactory ($\alpha=0.87$). The mean H-Ö questionnaire score for the RUNM group (mean ± SD: 61.6 ± 10.6) was significantly higher ($p<0.001$) than that of the LOWM group (mean ± SD: 54.5 ± 10.7). The H-Ö score data for the RUNM and LOWM groups ranged from 31 - 78 and 33 - 78, respectively. Although both groups displayed a similar range in H-Ö scores, the RUNM and LOWM groups were both skewed towards higher scores, as evident by the negative

"skewness" value which indicates that distribution was left-tailed, with few low scoring values. In particular, the RUNM group was significantly skewed towards the higher scores (skewness RUNM group: -0.46 ± 0.24, p<0.001; LOWM group: -0.10 ± 0.24, p<0.001).

The chronotype frequencies of the RUNM and LOWM groups are presented in Figure 9. For ease of comparison the MMT and DMT individuals were combined to form the MT group (RUNM: n=62, LOWM: n=43). The ET group was only made up of MET individuals, as no individuals were classified as DET (scored below 30) (RUNM: n=4; LOWM: n=13). From Figure 9 it can be seen that there was a significant difference in the chronotype distribution (p=0.006) between the RUNM and LOWM groups. Specifically, as determined by post-hoc analyses, there were significantly more MT individuals in the RUNM group compared to the LOWM group (p=0.005), and, there were fewer ET individuals in the RUNM group compared to the LOWM group (p=0.040).

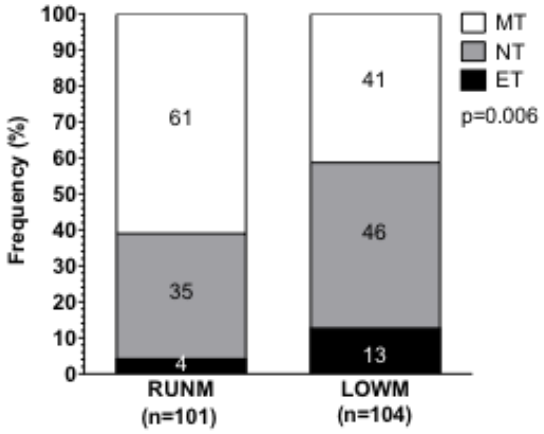

Figure 9: Chronotype frequencies of the RUNM and LOWM groups. RUNM = male runner group; LOWM = male low physical activity group; MT = morning-type individuals; NT = neither-type individuals; ET = evening-type individuals. The p-value represents the overall frequency distribution significance as determined using the Freeman-Halton extension of the Fisher's exact chi-squared test.

2.3.2.1 Chronotype and age

There was no correlation between chronotype and age for the LOWM group (r=0.07; p>0.05). This is an interesting finding as there was no difference in age between the RUNM and LOWM groups (p=0.107), thus one would expect the relationship between chronotype and age to be similar for the

two groups. Nevertheless, the lack of an association between chronotype and age within the LOWM group was most probably due to the fact that the distribution of morning-type and neither-type individuals was very similar which could have potentially masked any correlation with age.

2.3.3. *PER3* VNTR genotype

The *PER3* VNTR genotypic distribution of all participants (RUNM and LOWM) combined did not deviate significantly from Hardy-Weinberg equilibrium (HWE) (X^2=15.73, p=0.070). Similarly, the *PER3* VNTR genotype distribution of the RUNM group was in HWE (X^2=-0.177, p=0.100). However, the *PER3* VNTR genotype distribution of the LOWM group was not in HWE (X^2=-0.299, p=0.040). Of the 104 LOWM individuals, only 93 were genotyped successfully. This was due to very low yields of genomic DNA in eleven of the samples which were thus not able to be amplified.

The *PER3* VNTR genotype frequencies of the RUNM and LOWM groups are presented in Figure 10A. There was a significant difference in genotypic distribution between the RUNM and LOWM groups (p=0.000). Post-hoc analyses indicated that more LOWM were genotyped as $PER3^{44}$ compared to the RUNM group (p=0.008), and a greater number of RUNM were homozygous for the $PER3^{5}$ allele (p=0.001). Only one LOWM individual was homozygous for the $PER3^{5}$ allele.

The *PER3* VNTR allele frequencies of the RUNM and LOWM groups are presented in Figure 10B. Although the 4-repeat allele was predominant in both groups, the LOWM group had a significantly higher 4-repeat allele frequency compared to RUNM group, and the 5-repeat allele was more prevalent in the RUNM group (p=0.002).

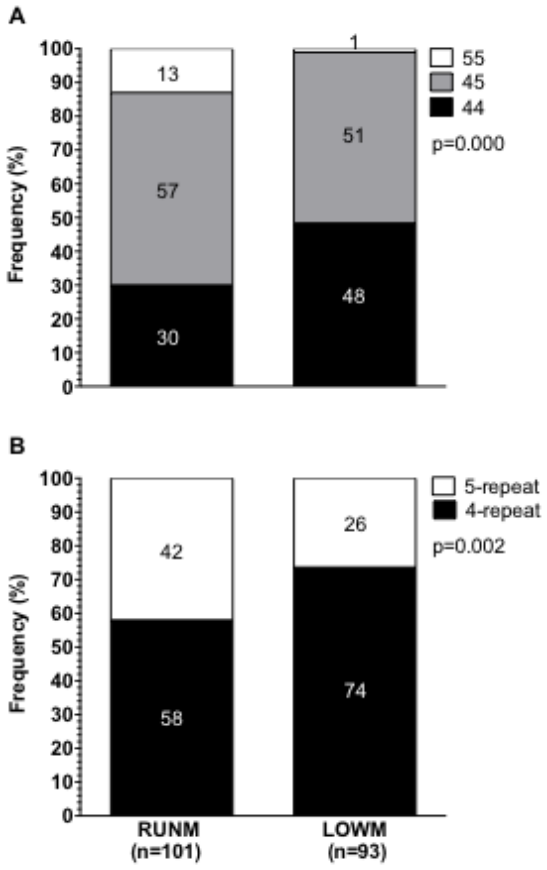

Figure 10: *PER3* VNTR genotype (A) and allele (B) frequencies of the RUNM and LOWM groups. RUNM = male runner group; LOWM = male low physical activity group; 55 = *PER3*^{55}; 45 = *PER3*^{45}; 44 = *PER3*^{44}; 4-repeat = *PER3*^{4} allele; 5-repeat = *PER3*^{5} allele. The p-values represent the overall frequency distribution significance as determined using the Freeman-Halton extension of the Fisher's exact chi-squared test for (A) and the Pearson's Chi-squared test for (B).

2.3.4 Relationship between chronotype and genotype

Figure 11 shows the H-Ö questionnaire scores for each RUNM and LOWM individual relative to his genotype. There was an association between H-Ö score and *PER3* VNTR genotype when all data were grouped, as determined by a multinomial logistic regression ($p<0.001$). When the RUNM and LOWM groups were analysed separately, there was no correlation for the RUNM group ($p=0.889$), however, there was a correlation for the LOWM group ($p<0.001$). Of the LOWM individuals genotyped as *PER3*^{44}, only two were classified as MMT individuals, with the remainder being NT (n=33) or MET (n=10) individuals. In contrast, 7 of the RUNM individuals genotyped as *PER3*^{44} were classified as DMT individuals and 10 as MMT, with only seven being NT and one being MET. No *PER3*^{55} LOWM or RUNM individuals were classified as MET.

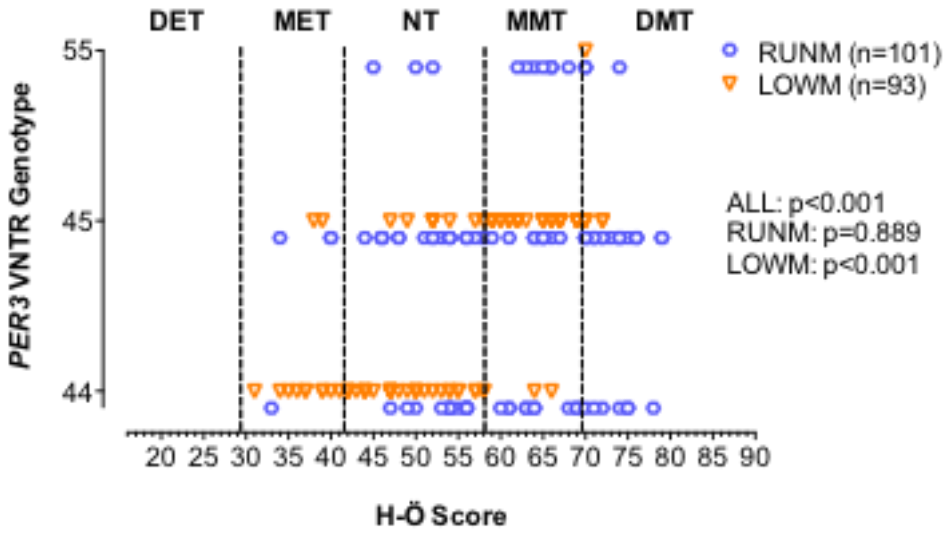

Figure 11: Relationship between the H-Ö questionnaire score and *PER3* VNTR genotype in the RUNM and LOWM groups. RUNM = male runner group; LOWM = male low physical activity group; DET = definite evening-type; MET = moderate evening-type; NT = neither-type; MMT = moderate morning-type; DMT = definite morning-type; VNTR = variable number tandem repeat polymorphism; 44 = *PER3*^{44}; 45 = *PER3*^{45}; 55 = *PER3*^{55}; H-Ö = Horne-Östberg. The vertical dotted lines delineate the chronotype categories. The p-values represent the correlation significance as determined by a multinomial logistic regression.

B) GENDER COMPARISON: CHRONOTYPE AND *PER3* VNTR GENOTYPE

2.3.5 Participant characteristics

The general characteristics of the male runner group (RUNM) and the female runner group (RUNF) are presented in Table IV. Not all participants gave all requested information on the questionnaire; as such the sample sizes for each variable are indicated in parentheses. The RUNM group was significantly taller ($p<0.001$), heavier ($p<0.001$) and had a higher BMI ($p<0.001$) compared to the RUNF group. The RUNM group had raced for more years ($p=0.014$) compared to their RUNF counterparts, and had faster current ($p<0.001$) and personal best (PB) 21km race times ($p<0.001$).

Table IV: General characteristics of the male (RUNM) and female (RUNF) runners.

	RUNM	RUNF	p-value
Age (y)	34.4 ± 8.1 (101)	34.2 ± 7.8 (102)	0.767
Height (m)	1.79 ± 0.06 (101)	1.67 ± 0.07 (102)	0.000*
Body mass (kg)	77.92 ± 9.22 (101)	60.30 ± 6.97 (102)	0.000*
BMI ($kg \cdot m^{-2}$)	24.2 ± 2.6 (101)	21.5 ± 2.1 (102)	0.000*
Training (y)	10.65 ± 6.82 (99)	10.31 ± 6.73 (102)	0.701
Training ($day \cdot wk^{-1}$)	4.05 ± 1.33 (99)	4.05 ± 1.25 (102)	0.925
Training ($hr \cdot wk^{-1}$)	5.57 ± 3.90 (96)	5.58 ± 2.76 (102)	0.362
Training ($km \cdot wk^{-1}$)	43.48 ± 26.31 (98)	39.36 ± 22.33 (102)	0.377
Race (y)	9.06 ± 6.79 (98)	6.65 ± 5.63 (102)	0.014*
No. races (past 2y)	11.61 ± 12.01 (72)	10.50 ± 11.15 (102)	0.479
Race dist. (past 2y, km)	21.45 ± 11.48 (72)	23.22 ± 10.71 (102)	0.323
Curr. 21km time (min)	107.25 ± 19.58 (67)	121.27 ± 18.81 (95)	0.000*
PB 21km time (min)	98.39 ± 15.38 (46)	107.86 ± 21.62 (36)	0.001*

The data are presented as mean ± SD (n). The p-value represents significance as determined by a one-way ANOVA for normally distributed data and a Mann-Whitney U test for data that were not normally distributed. * indicates statistical significance. RUNM = male runner group; RUNF = female runner group; BMI = Body mass index; No. = Number; Dist. = Distance; Curr. = Current; PB = Personal best.

2.3.6 Chronotype

All of the runners (n=203) completed the H-Ö questionnaire. Cronbach's α was 0.83, and was considered to be satisfactory. The mean H-Ö questionnaire scores for the RUNM and RUNF groups were 61.6 ± 10.6 and 65.7 ± 8.0 (mean ± SD), respectively, and were significantly different (p=0.010). The H-Ö scores ranged from 42 - 82 in the RUNF group and from 33 - 79 in the RUNM group. The H-Ö score data for all participants were significantly skewed towards the higher scores, (skewness = -0.70 ± 0.17, p<0.001). In particular, the RUNF H-Ö questionnaire scores were significantly skewed towards higher scores (RUNF: skewness = -0.80 ± 0.24, p<0.001; RUNM: skewness = -0.46 ± 0.24, p<0.001).

The chronotype frequencies of the RUNM and RUNF groups are presented in Figure 12A. As in section A, the MMT and DMT individuals were combined to form the MT group (62 males and 85 females). As no runners scored as DET, the ET group is made up of only MET individuals (n=4, all males). From Figure 12A, it is evident that there was a significant difference in the chronotype distribution (p=0.000) with regards to gender. Specifically, post-hoc analyses showed there were significantly more MT females than males (p=0.001) and more NT males than females (p=0.002). No RUNF individual was categorised as ET and only four RUNM (4%) were categorised as ET.

The chronotype frequencies of the RUNM and RUNF groups categorised by race discipline are presented in Figures 12B and 12C respectively. The male runners who regularly ran marathon or ultra-marathon distances were grouped to form the RUNM FULL group, and those who ran half-marathon races the RUNM HALF group. Similarly, the females were sub-divided into the RUNF FULL and the RUNF HALF groups. Although there was no significant difference in chronotype distribution between the RUNM FULL and RUNM HALF (B) groups, there was a trend for the RUNM FULL group to have more MT and fewer NT and ET individuals compared to the RUNM HALF group. Similarly, there was no difference in the chronotype distribution when comparing the RUNF FULL and RUNF HALF groups (C), however, the RUNF FULL group tended to have more MT and fewer NT individuals compared to the RUNF HALF group.

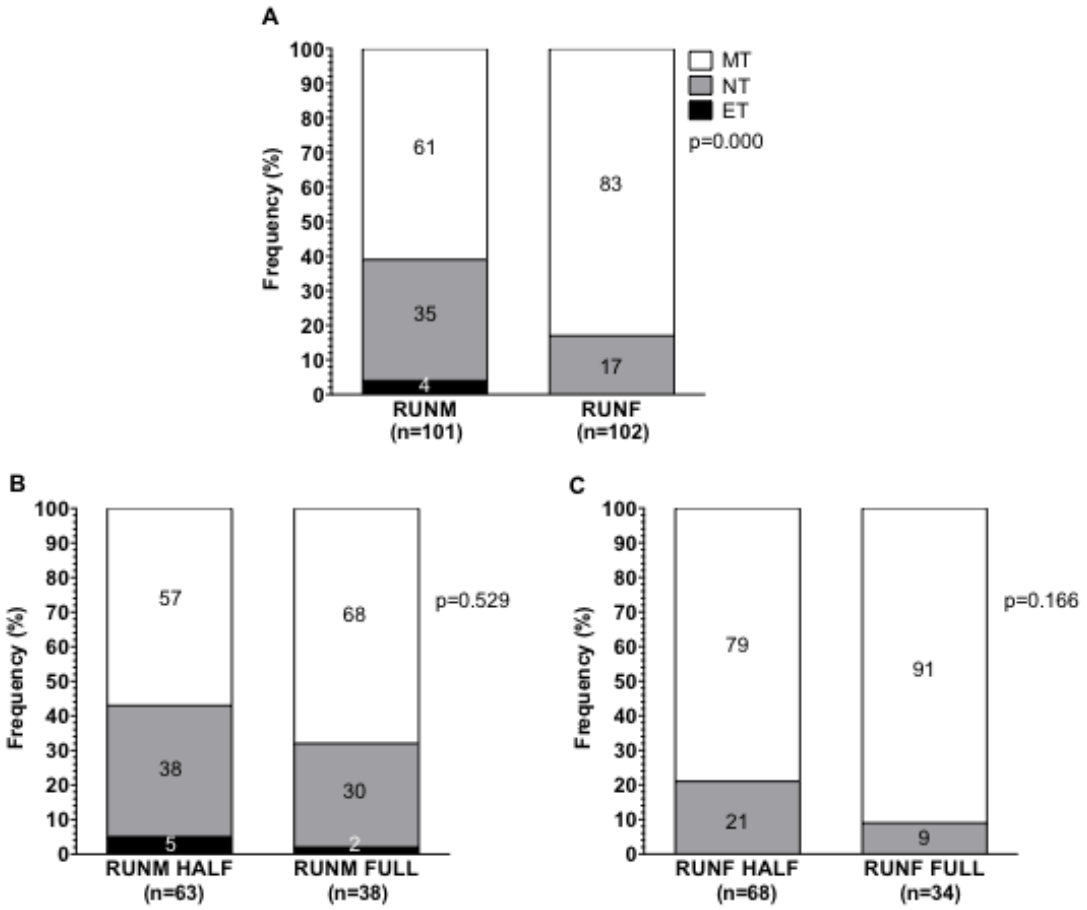

Figure 12: (A) Chronotype frequencies of the RUNM and RUNF groups, and the male (B) and female (C) runners categorised by race discipline. RUNM = male runner group; RUNF = female runner group; RUNM FULL = male marathon and ultra-marathon runners; RUNM HALF = male half marathon runners; RUNF FULL = female marathon and ultra-marathon runners; RUNF HALF = female half-marathon runners; MT = morning-type individuals; NT = neither-type individuals; ET = evening-type individuals. The p-values represent the overall frequency distribution significance as determined using the Freeman-Halton extension of the Fisher's exact chi-squared test.

2.3.6.1 Chronotype and age

Interestingly, there was a correlation between chronotype and age when all runners (n=203) were grouped together ($r= 0.316$; $p<0.05$). This finding was also true when the runners were grouped according to gender (RUNM: $r= 0.423$; $p<0.05$ and RUNF: $r=0.209$; $p<0.05$). More specifically, with an increase in age, the runners were more likely to be morning-type individuals. These results are

somewhat expected as previous research has concluded that there is a positive association between chronotype and age (Paine *et al.,* 2006; Caci *et al.,* 2009; Dijk and Archer, 2010)."

2.3.6.2 Chronotype and actual training time-of-day

The relationship between chronotype and actual training time-of-day for all runners (n=203), the RUNM (n=101) and RUNF groups (n=102) was also explored (Figure 13 A, B and C, respectively). When the runners were grouped together, there was a significant difference in the number of MT, NT and ET individuals that trained in either the mornings (before 12h00), afternoons/ evenings (after 12h00) or both (either time) times of the day ($p<0.001$, Figure 13A). More specifically, the majority of the MTs trained in the mornings (before 12h00) compared to other training times of the day (afternoons/ evenings or both times of the day), and the majority of the NTs trained in the afternoons/evenings. Similarly, when the RUNM and RUNF groups were analysed separately, there was a difference regarding chronotype and training time-of-day (Males: $p=0.001$, Females: $p=0.022$, Figure 13B and 13C, respectively). Although, the small sample size of the ET group (n=4) made it difficult for statistical comparisons to be made between chronotype groups, it was evident that all four of the ET runners trained in the afternoons/ evenings (after 12h00).

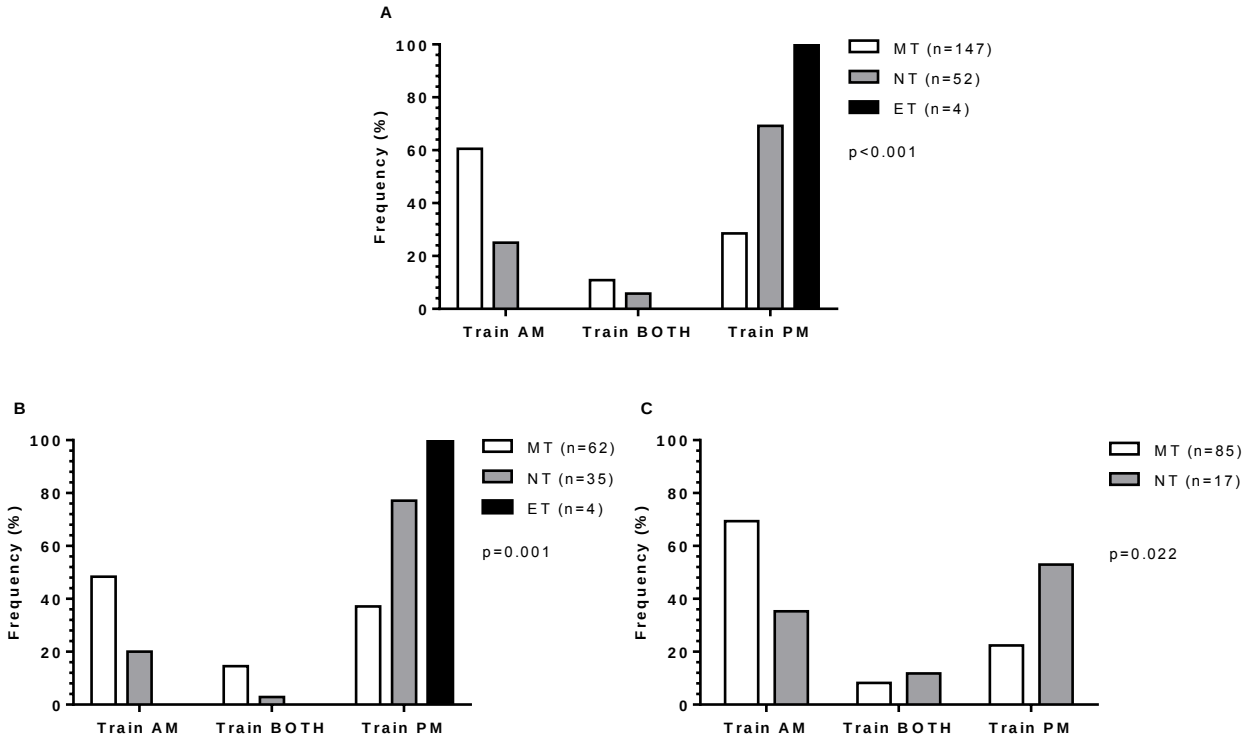

Figure 13: Actual training time-of-day reported by (A) all runners (n=203), (B) males only (n=101) and (C) females only (n=102) categorised by chronotype. RUNM = male runner group; RUNF = female runner group; MT = morning-type individuals; NT = neither-type individuals; ET = evening-type individuals; Train AM = train in the mornings (before 12h00); Train BOTH = either time; Train PM = train in the afternoon/ evenings (after 12h00). The p-values represent the overall frequency distribution significance as determined using the Chi-squared test.

2.3.7 Genotype

The *PER3* VNTR genotype distribution of the RUNM and RUNF groups combined did not deviate significantly from Hardy-Weinberg equilibrium (HWE) (X^2=6.03, p=0.551). Similarly, when the *PER3* VNTR genotype distribution of the RUNM and RUNF groups were analysed separately, the data were in HWE equilibrium (X^2=-0.177, p=0.100 and X^2=0.08, p=0.495 for the RUNM and RUNF groups respectively).

The *PER3* VNTR genotype frequencies of the RUNM and RUNF groups are presented in Figure 14A. There was a significant difference in genotype distribution when comparing the male and female runners (p=0.025). Post-hoc analyses specified that more males were genotyped as *PER3*^{45} compared

to the females (p=0.017), and a greater number of females were homozygous for the 4-repeat allele (p=0.009). Additionally, the runners genotyped as *PER3*^{44} had a lower mean training number of days per week (p=0.220), a lower weekly mileage (km.week^{-1}) (p=0.235) and spent fewer hours running per week (p=0.157) in comparison to the *PER3*^{45} and *PER3*^{55} runners.

The *PER3* VNTR genotype frequencies with regard to race discipline for the RUNM and RUNF groups are presented in Figure 14. From panel B, it is evident that there was a significant difference in genotype distribution by race discipline for the male runners (p=0.003). Post-hoc analyses showed that significantly more of the RUNM HALF group was genotyped as *PER3*^{44} (p=0.001), while more of the RUNM FULL group was genotyped as *PER3*^{45} (p=0.013).

There was no significant difference in genotype distribution when comparing the female runners by race discipline category (p=0.579) (C).

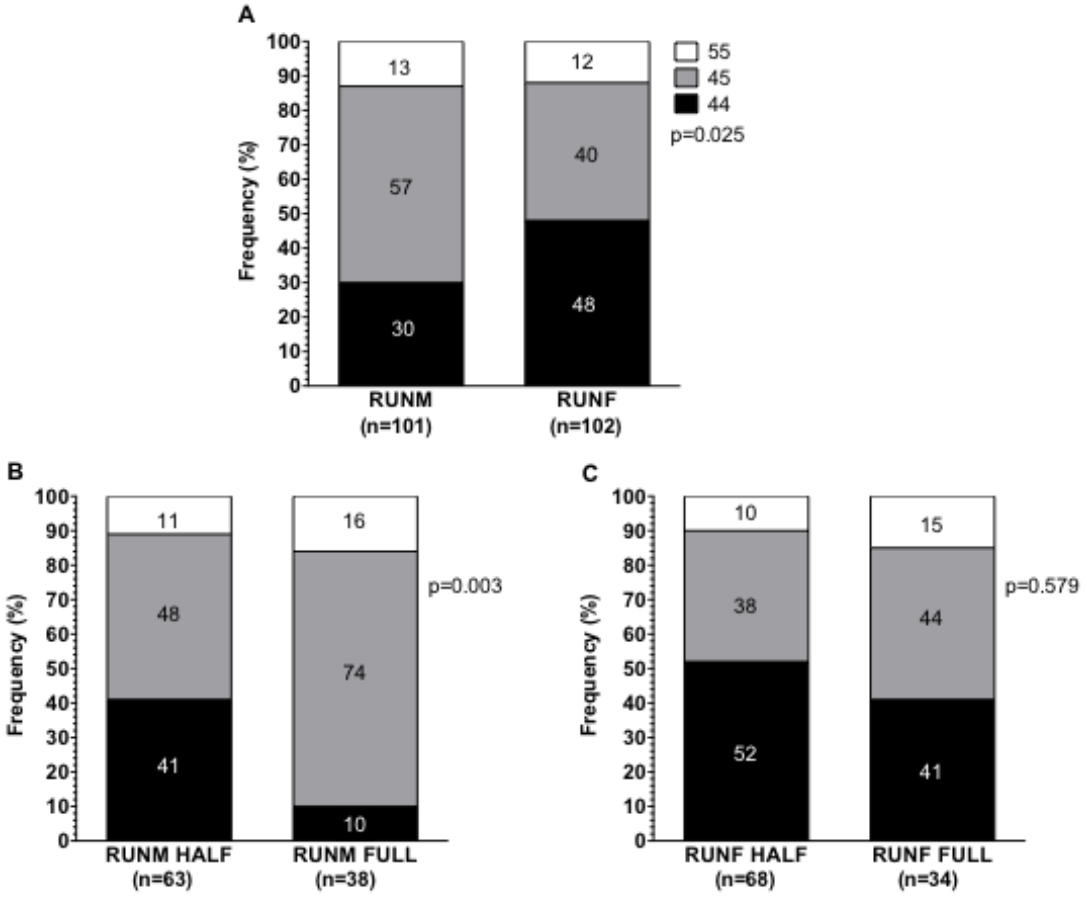

Figure 14: (A) *PER3* VNTR genotypic frequencies within the RUNM and RUNF groups and (B) the RUNM group and (C) the RUNF group categorised by race discipline. RUNM = male runner group; RUNF = female runner group; RUNM FULL = male marathon and ultra-marathon runners; RUNM HALF = male half marathon runners; RUNF FULL = female marathon and ultra-marathon runners; RUNF HALF = female half-marathon runners; 55 = *PER3*^{55}; 45 = *PER3*^{45}; 44 = *PER3*^{44}. The p-values represent the overall frequency distribution significance as determined using the Freeman-Halton extension of the Fisher's exact chi-squared test.

The *PER3* VNTR allele frequencies of the RUNM and RUNF runners are presented in Figure 15A. Although the 4-repeat allele was the dominant allele in both groups, the allele frequency distribution differed by gender (p=0.042), with more females carrying the 4-repeat allele compared to the males. Panels B and C (Figure 15) represent the *PER3* allele frequency distributions of the RUNM and RUNF runners, respectively, categorised by race discipline. A greater number of the RUNM FULL group carried the 5-repeat allele compared to the RUNM HALF group (B, p=0.013). On the other hand, there was no difference in allele frequency distribution when comparing the RUNF FULL and RUNF HALF groups (C).

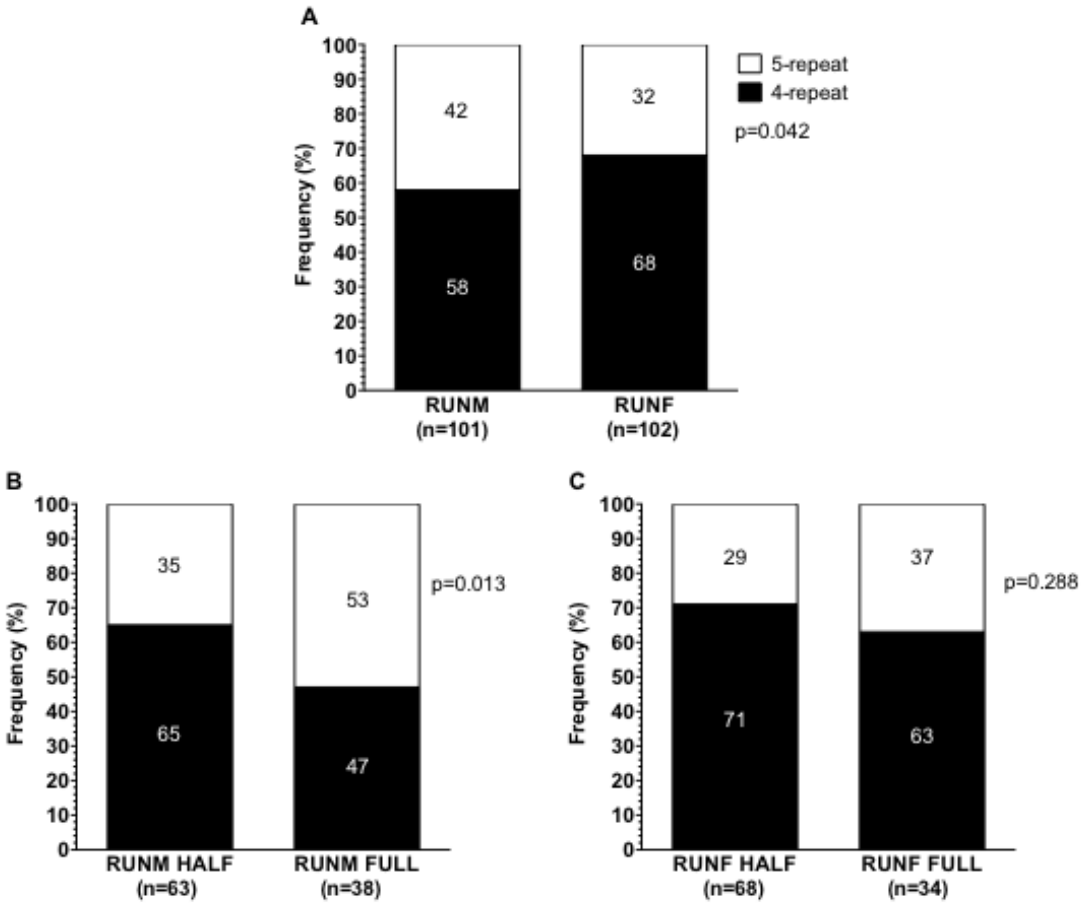

Figure 15: (A) *PER3* VNTR allele frequencies of the RUNM and RUNF groups, and (B) the RUNM runners and (C) the RUNF runners categorised by race discipline. RUNM = male runner group; RUNF = female runner group; RUNM FULL = male marathon and ultra-marathon runners; RUNM HALF = male half-marathon runners; RUNF FULL = female marathon and ultra-marathon runners; RUNF HALF = female half-marathon runners; 4-repeat = *PER3*^{4} allele; 5-repeat = *PER3*^{5} allele. The p-values represent the frequency distribution significance determined using the Pearson's chi-squared test.

2.3.8 Relationship between chronotype and genotype

Figure 16 represents the H-Ö questionnaire scores for each runner relative to his/ her genotype. From panel A it can be seen that there is no relationship between chronotype and *PER3* VNTR genotype when the male and female runners are combined into one group (p=0.383) or when the males and females are analysed separately (p=0.889 and p=0.259 respectively). All individuals genotyped as *PER3*^{55} were categorised as NT, MMT or DMT. In contrast, only one individual genotyped as *PER3*^{44} was categorised as an ET with the majority (99%; n=78) being categorised as NT, MMT and DMT.

From panel B it can be seen that there was also no correlation between H-Ö questionnaire score and chronotype, when the male runners were analysed by race discipline (FULL: p=0.813 and HALF: p=0.947). As expected, there was also no correlation in the RUNF group with regards to race discipline (panel C, FULL: p=0.300 and HALF: p=0.455).

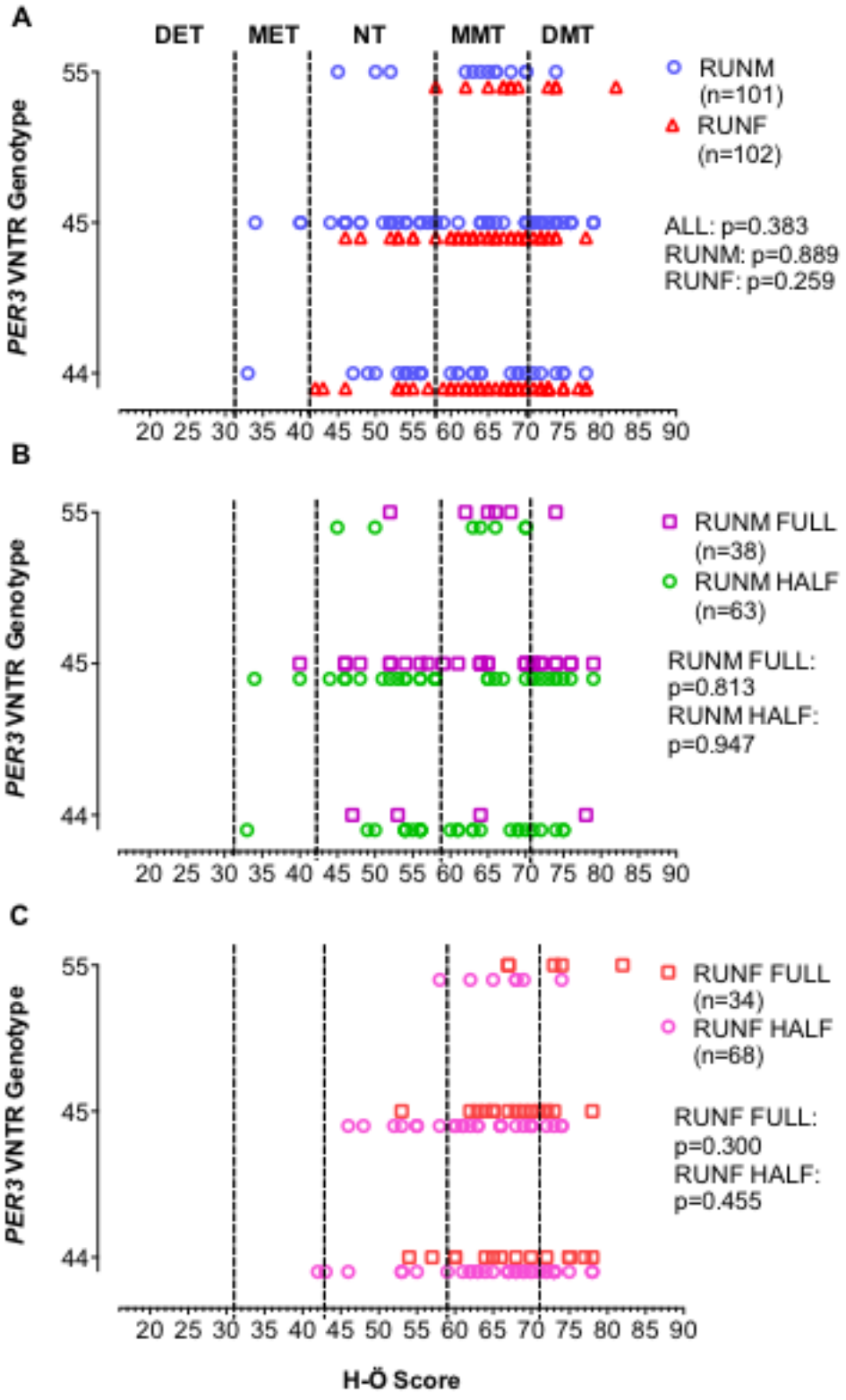

Figure 16: Relationship between the H-Ö questionnaire score and *PER3* VNTR genotype in the RUNM and RUNF groups (A), and the male (B) and female (C) runners categorised by race discipline. DET= definite evening-type; MET = moderate evening-type; NT = neither-type; MMT = moderate morning-type; DMT = definite morning-type; VNTR = variable number tandem repeat polymorphism; 44 = *PER3*^{44}; 45 = *PER3*^{45}; 55 = *PER3*^{55}; H-Ö = Horne-Östberg;

RUNM = male runner group; RUNF = female runner group; RUNM FULL = male marathon and ultra-marathon runners; RUNM HALF = male half marathon runners; RUNF FULL = female marathon and ultra-marathon runners; RUNF HALF = female half-marathon runners. The vertical dotted lines delineate the chronotype categories. The p-values represent the correlation significance as determined by a multinomial logistic regression.

2.4 DISCUSSION

The first aim of this study was to answer the question: Does physical activity level have an effect on the distribution of chronotype and *PER3* VNTR genotype within a South African population of Caucasian males?

The first important finding relates to the observed significant difference in chronotype distribution between the high physical activity (RUNM) and low physical activity (LOWM) population groups (Figure 9). The majority of the male runners were morning-type (61%) individuals, which was significantly more than the proportion of morning-types observed in the low physical activity males group (41%). The proportion of morning-types to neither-types in the LOWM group was similar (41% and 46% respectively). However, these findings are in line with those of Kunorozva *et al.* (2012), where it was shown that 72% of their runners and 42% of their controls (active but non-competitive males) were categorised as morning-type individuals. These findings are in contrast to other populations around the world (Park *et al.,* 1997; Taillard *et al.,* 1999; Ahmed *et al.,* 2011). For example Park *et al.* (1997) reported that only 13.9% of Korean students, 5.6% of Japanese students and 13.5% of Japanese workers were morning-type individuals. Additionally, 22.3% of Romanian participants and 26.9% of Saudi students (Ahmed *et al.,* 2011) were classified as evening-type individuals. Thus, based on our data it could be hypothesised that the male runners in this study may be more morning-oriented due to the early morning training and racing demands of the sport of running. Therefore, it seems possible that "conditioning" to the early morning start times of running training and racing could ultimately shift chronotype in order to enable runners to cope with the early morning demands of the sport. Additionally, maybe more morning-type individuals choose and persevere with running as the early morning start times suit their personality.

Interestingly, based on the findings of this study and those of Kunorozva *et al.* (2012) it appears that very few Caucasian male South Africans are evening-types, and specifically definite evening-type individuals, regardless of whether they are runners or inactive (Figure 9). This is in contrast to other populations described around the world (Smith *et al.,* 2002; Osland *et al.,* 2011), with the exception of New Zealanders, of which only 5.6% were classified as evening-types (Paine *et al.,* 2006). One possible explanation for the small percentage of South African evening-type individuals could be climate. Smith *et al.* (2002) reported that less temperate countries had a lower "morningness" compared to the temperate countries. Similarly, Randler (2008) concluded that students living in temperate and tropical regions displayed a greater preference for mornings compared to those living in the sub-tropics.

Previous research has concluded that evening-type individuals tend to live less-healthy lifestyles compared to morning-type individuals (Wilson, 1990; Chelminski *et al.,* 1999). For example, "eveningness" has been associated with depression (Chelminski *et al.,* 1999) and evening-type individuals were reported to drink and smoke more in comparison to morning-type individuals (Wilson, 1990). Since the LOWM group are relatively inactive it could be assumed that they live an "unhealthy" lifestyle, which may be evidenced by the heavier body mass and greater BMI of this group compared to the RUNM group (Table III), and thus the results of this study support the conclusion.

The second significant finding was that the *PER3* VNTR genotypic frequencies of the RUNM and LOWM were significantly different (Figure 10). More runners were genotyped as $PER3^{55}$ and fewer as $PER3^{44}$ in comparison to the low physical activity group. The *PER3* VNTR genotype distribution of the LOWM group was also interesting in that there were very few individuals genotyped as $PER3^{55}$, in fact only one individual, and this group had a relatively even distribution of individuals genotyped as $PER3^{44}$ and $PER3^{45}$. The *PER3* VNTR genotypic distributions of both the RUNM and LOWM groups are different to what has previously been reported. For example, only 6% of the runners in the Kunorozva *et al.* (2012) study were genotyped as $PER3^{44}$, whereas 30% of the runners in the current study were homozygous for the 4-repeat allele. Additionally, the $PER3^{44}$ (48%) and $PER3^{45}$ (51%) genotype distribution of the LOWM group was different to that of the active control group ($PER3^{44}$: 33% and $PER3^{45}$: 58%) in the Kunorozva *et al.* (2012) study. It could be hypothesised that the higher $PER3^{44}$ genotypic frequency in the LOWM group compared to the latter active control group could be linked to their lower physical activity level. The LOWM group was "inactive" in comparison to the Kunorozva *et al.* (2012) active control group. Thus, it appears that when populations are sorted according to activity level, the frequency of the $PER3^{44}$ genotype increases inversely with activity levels.

Additionally, the *PER3* VNTR genotypic distributions of the RUNM and LOWM groups were also different to those of various populations around the world (Archer *et al.,* 2003; Nadkarni *et al.,* 2005; Ciarleglio *et al.,* 2008). For example, European populations tend to have 4-repeat allele frequencies ranging from 65-69% (Nadkarni *et al.,* 2005; Osland *et al.,* 2011), whereas, Japanese (Ebisawa *et al.,* 2001) and Asian (Nadkarni *et al.,* 2005) individuals have a much higher 4-repeat allele frequency. Therefore, the RUNM and LOWM groups appear to have similar genotype distributions to populations of European descent, which is appropriate as the participants in the current study were white South Africans of European descent.

The *PER3* VNTR genotype distribution of the RUNM group raised an important question: What is different between this running population and that of the Kunorozva *et al.* (2012) study? Firstly, there was no performance or calibre-related participation criterion for the runners in the current study, except that runners must have been training two to three times per week. In contrast, the runners who participated in the Kunorozva *et al.* (2012) study had to have completed a half-marathon in under 120 minutes. This lack of cut-off criterion resulted in a large range in calibre of the runners in the current study. For example, the number of years running ranged from 1-30 years; with 32% and 44% of the RUNM group racing for less than five years, respectively. Thus, given the fact that the RUNM group in the current study was recruited at relatively "social" races and without performance-related cut-off criteria, it could be argued that they are largely recreational, and, perhaps of a lower calibre compared to the runners analysed by Kunorozva *et al.* (2012), and this may account for the observed higher *PER3*^{44} genotypic frequency.

Although the RUNM group in the current study has a different genotype distribution to the runners in the Kunorozva *et al.* (2012) study, the chronotype distribution is similar for the two running groups. Therefore, due to the fact that the RUNM group contained fewer individuals homozygous for the 4-repeat allele compared to the LOWM group, it seems that genotype may to some extent contribute to the choice of activity. However, the influence of genotype on physical activity level is not exclusive; otherwise the RUNM group would contain very few *PER3*^{44} individuals as did the well-trained runners described by Kunorozva *et al.* (2012). Additionally, it could be postulated that physical activity conditions, or influences, chronotype; which could potentially explain the higher frequency of "morning-type" individuals within the RUNM group, despite the high frequency of the 4-repeat allele (Figure 11), which has been associated with "eveningness" (Archer *et al.,* 2003; Ellis *et al.,* 2009). Theoretically, one could postulate that the *PER3*^{44} runners are not as serious or of the same calibre as the *PER3*^{45} and *PER3*^{55} runners, but are more social. Since it appears that the *PER3*^{44} runners are not yet as experienced as the *PER3*^{45} and *PER3*^{55} runners it could be questioned if, over time, they would be able to maintain early morning running training, racing and conditioning as easily or readily as the *PER3*^{45} or *PER3*^{55} runners may be able to?

Thirdly, these data show that the relationship between chronotype and the *PER3* VNTR genotype is significant when the RUNM and LOWM groups were combined (Figure 11). That is, when the male runners group and the low physical activity males group were combined, chronotype and more specifically lower H-Ö scores were associated with the *PER3*^{44} genotype; and higher scores correlated with the *PER3*^{55} genotype. This relationship did not persist when the male runner group was analysed separately (Figure 11). This is most probably due to the fact that 58% (n=17) of the

PER3^{44} male runners group scored as morning-type individuals, as per the H-Ö questionnaire. This disparity could be due to a shift in chronotype, towards a greater preference for mornings, which has resulted from conditioning to early morning training and the early start times of South African road running races, regardless of genotype. The genotype-chronotype association within the LOWM group was most likely due to the fact that there were only two "outliers" who were genotyped as *PER3*^{44} despite scoring as MMT, with the rest of the group scoring as neither-type and evening-type individuals (Figure 11). The *PER3*^{45} runners displayed a large range in H-Ö score, which was expected. Thus, despite only one LOWM being genotyped as *PER3*^{55}, the genotype-chronotype distribution of this group appears to be similar to previous findings. In conclusion, the RUNM were more morning-oriented and had a higher *PER3*^{5} allele frequency compared to the LOWM group.

The second aim of this study was to address the question: Does gender have an effect on the distribution and relationship between chronotype and *PER3* VNTR genotype within a South African running population?

When the male and female runners were combined, it was clear that they were more likely to be morning-type individuals than neither- or evening-types (Figure 12A). This finding was even more pronounced when the male and female running groups were analysed separately, with more females being morning-type (83%) compared to the males (61%). This finding is in accordance with previous findings that females are more morning-oriented than males (Adan and Natale, 2002; Randler, 2007). Interestingly, no female runners scored as evening-type whereas 4% males were evening-type (Figure 12A). As it is known that females are more morning-oriented (Adan and Natale, 2002; Randler *et al.,* 2007), and since changes during puberty have been shown to be associated with "eveningness" in adolescents (Kim *et al.,* 2002; Smith *et al.,* 2002), do hormonal differences account for the greater morning preference in females, regardless of genotype? Duffy *et al.* (2011) reported that both pre- and post-menopausal women had a shorter intrinsic circadian period compared to males. Therefore, maybe the greater "morningness" in females is not maintained by hormones but rather set by hormones at some developmental stage (Duffy *et al.,* 2011).

The second finding of this study was that there was a sex difference in the *PER3* genotype distribution, with the frequency of the *PER3*^{4} allele being more common in females than males (Figure 15A). This is in contrast to a previous study (Jones *et al.,* 2007). Although the study by Jones *et al.* (2007) had much larger sample size (707 males and 883 females), this is a really interesting finding and raises an important question: Why do the female runners behave differently in this analysis to the male runners? *PER3* is located on chromosome 1 (Ebisawa *et al.,* 2001); thus the fact that this gene is autosomal means that differences in inheritance or penetrance cannot account for

the observed differences in *PER3* VNTR genotypic frequency between the males and females in this study. Future research needs to be conducted on larger cohorts of both active and inactive females, in comparison to males, in order to confirm the findings of this study. Additionally, future research that focuses on polymorphisms in other circadian clock-genes in populations of males and females is required.

It thus seems that there must be significant factors other than *PER3* VNTR genotype that contribute to chronotype in women. Since it has been established that females are more morning-oriented (discussed above), maybe this drives more females, regardless of genotype, to select running or morning-related sports events over evening-based events? Additionally, it could be assumed or generalised that females are more likely to participate in individual sports events over team sports, such as rugby or cricket, due to the limited opportunities for female team-sports participation, which could possibly result in more females selecting early-morning individual-type sports, despite genotype.

As discussed above, the high *PER3*^{4} allelic frequency in this sample of runners (both RUNM and RUNF) is different to what has previously been observed. Since the runners of this sample were not of the same calibre compared to those in the Kunorozva *et al.* (2012) running population sample, the RUNM and RUNF running population groups were further subdivided into four groups based on race discipline: full marathon males (RUNM FULL), half-marathon males (RUNM HM), full marathon females (RUNF FULL) and half-marathon females (RUNF HM). Full-marathon runners were those that participated in marathon and ultra-marathon races and half-marathon, those that participated in half-marathon running races. The distribution of FULL and HM runners within the RUNM and RUNF group was relatively similar. (RUNM; FULL, n=38; HM, n=63 and RUNF; FULL, n=34; HM, n=68).

Interestingly, the chronotype distribution of the runners did not change when analysing chronotype by race discipline (Figures 12B and 12C). *PER3* VNTR genotype distribution was however significantly different when comparing the male full runners group and the half-marathon group (Figure 14B). Specifically, there were more *PER3*^{55}, more *PER3*^{45} and fewer *PER3*^{44} in the RUNM FULL group ($p=0.001$). Therefore, the *PER3* VNTR genotypic distribution of the RUNM FULL group was very similar to that reported by Kunorozva *et al.* (2012). Thus, since one would assume that the RUNM FULL group are more committed runners, it may explain why their *PER3* VNTR genotype distribution is similar to what has previously been reported. Race discipline thus seems to be key factor when comparing *PER3* VNTR genotypic distributions in the RUNM group. On the other hand, race discipline did not seem to have an effect on the female *PER3* VNTR genotypic distribution (Figure 14C). Nevertheless, the genotype distribution of the RUNF FULL group is going in the right "direction" as

there are more *PER3*^{55}, *PER3*^{45} and fewer *PER3*^{44} in comparison to the RUNF HM group (Figure 14C). One limitation when analysing the sub-groups categorised by race discipline was the resultant smaller sample sizes which made statistical analyses difficult.

The chronotype-genotype relationship within the RUNM group was discussed above. With regard to race discipline, from figure 16B, it is apparent that the majority of the male runners who were genotyped as *PER3*^{44} were half-marathon males, with the exception of four marathon and ultra-marathon males. Of those four FULL males, two scored as neither-type individuals and two as morning-type individuals, despite all four of them habitually training in the mornings. Thus, the two morning-type FULL runners who were genotyped as *PER3*^{44} could be viewed as "outliers" who have become morning-type due to conditioning of their training and race schedules. The RUNM HM and RUNM FULL participants genotyped as *PER3*^{45} display a wide range in H-Ö questionnaire score which was expected. Although there was no significant correlation between chronotype and *PER3* VNTR genotype when analysing the RUNM FULL or RUNM HM separately, it appears that the chronotype-genotype association of the FULL males starts to "behave" like that of the runners in the Kunorozva *et al.* (2012) study, most probably due to the higher calibre of this RUNM FULL group, making this group comparable to the running population in the latter study. However, a larger sample size is needed to confirm this observation.

Similarly as with the RUNM group, there was no association between chronotype and genotype within the RUNF population (Figure 16A). In addition, there was no hint of an association, as with the males (RUNM FULL), between chronotype and genotype, when female runners were analysed by race discipline separately (Figure 16C). Of the *PER3*^{55} RUNF, only one runner scored as neither-type and eleven as morning-type individuals (Figure 16C). As with the RUNM, the *PER3*^{45} runners display a large range in H-Ö score. What is really interesting, is that 84% (n=41) of the RUNF genotyped as *PER3*^{44} scored as morning-type individuals. This is much higher than the 57% of male *PER3*^{44} runners who scored as morning-type individuals. Thus, perhaps female runners, in particular, are more morning-oriented due to 1) the early morning training and racing demands of running and 2) as well as being more morning-oriented, compared to males, in general, which was discussed in the literature review (section 1.4.4).

It is important to note that there are strengths and limitations of this study. One of the strengths is the uniformity of the population sample ethnicity. *PER3* VNTR allelic frequency has been shown to differ when comparing populations of different ethnic origin (Nadkarni *et al.,* 2005; Ciarleglio *et al.,* 2008; Barbosa *et al.,* 2010). Therefore, all participants were Caucasian of European origin in order to eliminate the possible confounding influence of ethnicity. Additionally, the relationship between

chronotype and age has been well documented (Paine *et al.,* 2006; Dijk and Archer, 2010; Caci *et al.,* 2009), with a tendency towards evening preference around the age of 13 and then with an increase in age there is a shift towards greater morning-preference, specifically after the age of 50 years (Smith *et al.,* 2002; Cavallera and Giudici, 2008). Therefore, participants in the current study were included if they were between 25 and 50 years of age, in order to minimise the chance of chronotype being significantly influenced by age. Another limitation regarding study 1 was that the participants were only questioned verbally, regarding transmeridian travel history and shift work history. Nevertheless, participants were excluded from taking part in the study if they had travelled across more than two time-zones within the past two months prior to the study or if they were currently involved with shift work, or had been in the past. Additionally, the fact that there was no strict participation criterion for the running population groups proved to prompt an important finding. The lack of participation criterion resulted in the population samples consisting of runners of a diverse calibre and lead to the first inkling that *PER3* VNTR genotype is possibly more strongly associated with chronotype when the level of competition or the physical activity status of participants is matched. However, in future larger sample sizes of recreational versus elite athletes are required to confirm this. Finally, a further limitation regarding study 1 was that data collection (runners and low-activity male participants) took place during April 2011 to December 2012. Seasonal variations in light and temperature, which are known to have influence chronotype, could have had an influence on the results.

2.5 SUMMARY AND CONCLUSIONS

In summary, the data showed that:

(i) Very few South Africans are categorised as evening-type individuals.

(ii) Conditioning, driven by physical activity level, appears to have an influence on chronotype; with a higher level of physical activity there tends to be a greater preference for mornings.

(iii) The frequency of the *PER3* VNTR genotype distribution was different when comparing the male runners and the male low-physical activity individuals and seemed to be related to physical activity level; with the runners displaying a higher frequency of the *PER3*^{5} allele.

(iv) Female runners were more morning-oriented and the frequency of the *PER3* VNTR genotype distribution was different when comparing the male and female runners.

(v) It appears that factors other than *PER3* VNTR genotype contribute to chronotype in the female runners, as evidenced by the high *PER3*^{4} allele frequency within this group.

(vi) Runner calibre appears to be a key factor to consider when analysing the relationship between chronotype and *PER3* VNTR genotype.

Although it was evident that South Africans appeared to be more morning-oriented, this preference for mornings was skewed even further with a greater level of physical activity. Furthermore, the extent of morning preference was even greater within the female running population group. Additionally, while all three populations had a higher frequency of the *PER3*^{44} genotype than was expected, it appeared that the *PER3*^{44} genotype frequency increased inversely with the level of physical activity. It is important to note that although an association between H-Ö score and *PER3* VNTR genotype has previously been demonstrated (Archer *et al.,* 2003; Kunorozva *et al.,* 2012), *PER3* VNTR genotype does not define chronotype. Conditioning to chronotype appeared to override and have a far greater influence on an individual's diurnal preference than that of *PER3* VNTR genotype. This was evidenced by the fact that despite the runners having a high frequency of the *PER3*^{4} allele; they were skewed towards "morningness," most likely due to habituation to the demands of running training and racing. Lastly, this resultant mis-match between chronotype and *PER3* VNR genotype lead to the lack of any genotype-chronotype association. This descriptive study raises a number of interesting questions regarding the relationship between chronotype and *PER3* VNTR genotype in active and non-active individuals which need to be addressed further.

(i) What other factors contribute to the greater morning-preference in females?

(ii) Is the relationship between *PER3* VNTR genotype and chronotype stronger in elite versus recreational athletes? (To be addressed in the future).

CHAPTER 3: CIRCADIAN RHYTHM AND SWIMMING

THE INFLUENCE OF CHRONOTYPE, *PER3* AND HABITUAL TRAINING TIME-OF-DAY ON SWIMMING PERFORMANCE AND OTHER RELATED VARIABLES

3.1 INTRODUCTION

3.1.1 Rationale

The majority of studies that have simulated races and time-trials in various sports such as swimming and cycling have demonstrated that there is a diurnal variation in performance, with the trend of evening performance being superior to that of morning performance (Baxter and Reilly, 1983; Cappaert, 1999; Drust *et al.,* 2005). The findings from the previous chapter and the study conducted by Kunorozva *et al.* (2012) raise some interesting ideas regarding sports participation, chronotype, *PER3* VNTR genotype and conditioning to sport at different times of the day. Well trained South African athletes (Kunorozva *et al.,* 2012), as well as elite Australian triathletes (Lastella *et al.,* 2010) tend to be morning-oriented rather than evening-oriented. Therefore, a pertinent question to ask would be: does chronotype have an impact on performance at other times of the day?

A handful of studies have reported that certain physiological variables respond differently to the same exercise task, at different times of the day, in morning- and evening-type individuals. For example, VO_2max was higher in the evening, compared to the morning, in evening-type individuals but this diurnal variation was not present in morning-type individuals (Hill *et al.,* 1988). Similarly, heart rate recovery was faster after an evening time-trial for evening-type individuals, which was not the case for the morning-type individuals (Sugawara *et al.,* 2001). On the other hand, others have reported no chronotype effect on diurnal variation of performance and other associated physiological variables (Burgoon *et al.,* 1992; Atkinson *et al.,* 2005). One limitation of these studies is the variation in training status of the participants used, ranging from “healthy” students or untrained individuals, to well-trained athletes. Thus, essentially only two studies have assessed chronotype and performance in athletes (Atkinson *et al.,* 2005; Brown *et al.,* 2008), which highlights the fact there is a gap in the literature regarding the performance of morning- and evening-type athletes, at different times of the day. Additionally, the previous chapter and the findings reported by Kunorozva *et al.* (2012) indicate that *PER3* VNTR genotype distribution, specifically in males, is associated with

chronotype as well as potentially being related to physical activity level. Therefore, both chronotype and genotype also need to be assessed, when looking at the performance of individuals at different times of the day. To date, no study has considered the effect of circadian genotype either independently or together with chronotype, on athletic performance at different times of the day.

Another point of interest is that habitual training in the morning, or even twice a day may reduce the observed diurnal variation in superior evening performance (Arnett, 2001; Souissi *et al.,* 2002; Sedliak *et al.,* 2007; Chtourou *et al.,* 2012 a,b). Thus, another relevant question to ask would be: to what extent does habitual training time-of-day have an impact on performance, at different times of the day in morning- and evening-types? If habitual training time-of-day, as well as chronotype, can improve or reduce the reported diurnal variation in performance, then this too would be beneficial to athletes and coaches when planning training sessions, tapering periods and in preparation for competitive events.

Therefore, the main aim of this study was to assess swimming performance at different times of the day, taking into account chronotype, *PER3* VNTR genotype and habitual training time-of-day. Swimming is a useful mode of exercise for the study of athletic performance rhythmicity because the environmental conditions and water temperature can be precisely controlled (Kline *et al.,* 2007). It is also relatively easy to assess swimming performance analogous to competition (Kline *et al.,* 2007); and swimmers tend to habitually train in the mornings or evenings, and/or both. Additionally, swimming competitions are generally characterised by morning heats and evening finals; thus the ability of swimmers to swim their personal best times, or close to, in the morning may be imperative to successful swimming (Kline *et al.,* 2007).

3.1.2 Aim

The aim of this study was to compare 200 m swimming time-trial performance, RPE, mood state, stroke count and breath count at 06h30 and 18h30 in trained South African masters swimmers.

The objectives relating to this aim were:

1) To describe the chronotype and *PER3* VNTR genotype distribution of the swimmers.
2) To determine whether there is a diurnal difference in 200 m swimming time-trial performance, RPE, mood state, stroke count and breath count in this group of swimmers.
3) To compare the morning and evening time-trial performance times, RPE, mood state, stroke count and breath count of morning- and evening-type swimmers (based on chronotype).

4) To compare the morning and evening time-trial performance times, RPE, mood state, stroke count and breath count in the swimmers with the *PER3*^{4} allele compared to the swimmers with the *PER3*^{5} allele.
5) To compare the morning and evening time-trial performance times, RPE, mood state, stroke count and breath count of swimmers who habitually train in the morning to those who habitually train in the evening.
6) To assess if there is a relationship between chronotype, *PER3* VNTR genotype and habitual training time-of-day on diurnal variation in swimming time-trial performance times at 06h30 and 18h30.

3.1.3 Hypotheses

Firstly, it was hypothesised that a group of well-trained South African masters swimmers may contain higher proportions of individuals preferring the morning and individuals genotyped with the *PER3*^{5} allele (associated with a preference for mornings). Secondly, it was hypothesised that there will be a diurnal variation in swimming time-trial performance and other performance variables at 06h30 and 18h30, of the group as a whole. More specifically, it was hypothesised that the evening time-trial will be superior with regards to a faster 18h30 time-trial performance, a lower RPE, a lesser total mood disturbance, a greater stroke count and a reduced breath count. Thirdly, we hypothesised that when the swimmers were analysed according to the sub-groups chronotype, *PER3* VNTR genotype and habitual training time-of-day, the morning-type swimmers, the swimmers genotyped as *PER3*^{55} and the swimmers who habitually train in the mornings would have a faster 06h30 time-trial time, a lower RPE, a lesser total mood disturbance, a faster stroke count and a reduced breath count in the morning, compared to the evening time-trial.

3.2 MATERIALS AND METHODS

3.2.1 Participants

Caucasian South African male and female masters swimmers from Cape Town were invited to participate in this study. Masters swimmers are those swimmers who are over the age of 25 years. All participants were between 25 and 50 years in age. Participation criteria were that the swimmers completed a minimum of three training sessions per week in the pool, and that 80% of these sessions must have been at the same time-of-day, each week. This training pattern must have been followed for at least one year. The health of the swimmers was self-reported, both verbally and in written form. Prior to participation swimmers were questioned by the author regarding their current health state. Additionally, all swimmers completed Appendix 8 (page 158) which documented transmeridian travel history, shift work history, as well as current medication and supplement use. Participants that reported illness or had been on medication within the last month were excluded from the study. Participants were also excluded from taking part in the study if they had travelled across more than two time-zones within the past two months prior to the study. Moreover, if a participant was currently involved with shift work, or had been in the past, they were excluded from taking part in the study. Thus, all participants were apparently healthy, had been injury-free for the previous two months and had not taken amphetamines, pemoline, modafinil, soporific drugs or hypnotics (e.g. benzodiazepines and minor tranquilizers, diazepam, loprazolam, zopiclone) or melatonin or any selective serotonin reuptake inhibitors (SSRI's), within the previous three months.

3.2.2 Study design

Testing took place on three occasions: Visit 1, at the Sports Science Institute of South Africa (SSISA), and Visits 2 and 3, at either the Old Mutual gym or the SSISA swimming pools in Cape Town. Both pools were indoor, heated, 25 m pools, and visits 2 and 3 took place in the same pool for each participant. During the first testing session, the researcher informed the swimmers as to the purpose of the study, the testing to be undertaken and the risks and benefits relating to the study (Appendix 6). On agreeing to participate, all swimmers signed a consent form (Appendix 7) and completed questionnaires (Appendices 3 and 8). The researcher then took anthropometrical measurements and a trained phlebotomist drew a 5 ml venous blood sample from a forearm vein of each volunteer. The blood samples were collected into EDTA vacutainer tubes and stored at -20°C until subsequent DNA extraction. Each individual was genotyped for the VNTR polymorphism within the *PER3* gene (Archer *et al.,* 2003). Each participant was also given a diary (Appendix 9), in which they were asked to log training, sleep, food consumption and oral temperature (using a digital oral thermometer) 24-hours

prior to each time-trial. Testing session one also served as a verbal explanation for the subsequent testing sessions to be conducted.

During the second and third testing sessions, the swimmers performed a 200 m freestyle time-trial at either 06h30 or 18h30. While the order in which they performed these trials was randomised, the procedures remained identical. Each session was separated by a minimum of three days to allow for ample recovery. On arrival at the pool the participants completed the Profile of Mood States (POMS) questionnaire (Appendix 10). Oral temperature and mass were recorded, after which a standardised pre-time-trial snack was consumed (Appendix 11). All participants performed the same standardised 600 m warm-up immediately after which they were asked to rate their perception of effort (RPE) of the warm-up using Borg's 20-point RPE scale (Appendix 12). After 5 min of rest the swimmers performed a 200 m time-trial at race pace. Total time, 50 m split time, stroke count and breath count were recorded and the swimmers were asked to report their RPE immediately after the time-trial. DNA extraction and genotyping was performed at the UCT/MRC Research Unit for Exercise Science and Sports Medicine, Department of Human Biology, Faculty of Health Sciences, and the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town. The study was approved by the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee (Ref: 055/2012), and performed in accordance with the principles of the Declaration of Helsinki (October 2008, Seoul), the International Conference on Harmonization (ICH) and the South African Good Clinical Practice (GCP) guidelines. The protocol used in this study conformed to the international ethical standards for biological rhythm research described by Portaluppi *et al.* (2010).

3.2.3. Detailed testing procedures

3.2.3.1 Chronotype

The chronotype of each swimmer was determined using the H-Ö questionnaire, as described in Chapter 2 (section 2.2.3.1).

3.2.3.2 Anthropometry

The participant's height (cm), mass (kg) and arm span (cm) were recorded in session 1. Arm span was measured as the distance between the tips of the mid-fingers (longest fingers) in each hand, with arms stretched horizontally while in standing position and with the back against a wall (Wells and Fewtrell, 2006). Body mass index was determined from their height and mass ($kg \cdot m^{-2}$).

3.2.3.3 Laboratory analysis

The DNA extraction protocol and the *PER3* VNTR genotyping protocol, as well as PCR analyses and digestion of the PCR product, were conducted as described in Chapter 2 (section 2.2.3.3).

3.2.3.4 24-hour diary

The swimmers were asked to keep their training, work, eating and sleep patterns and habits as close to normal as possible for the duration of the study. Twenty-four hours prior to sessions 2 and 3 the swimmers were asked to log their oral temperature hourly during their waking hours in the 24-hour diary provided (Appendix 9). They were also asked to log training, sleep and food consumption during this 24-hour period (Appendix 9).

3.2.3.5 Oral temperature

To be able to document how each swimmer's body temperature changed during the 24-hour period prior to the time-trial and the phase of their temperature rhythm at the start of each time-trial, oral temperature was recorded hourly in each swimmer in the 24-hour period before each time-trial using a digital oral thermometer (Microlife, MT 1681, Medical Science Ltd., Sweden). Since fluid and food may alter temperature in the mouth, the swimmers were asked to measure temperature prior to eating/drinking and to diarise their fluid and food intake during this period in the 24-hour diary (Appendix 9). Oral temperature was also recorded on arrival at the pool at 06h00 and 18h00, prior to the warm-up and 200 m freestyle time-trial.

3.2.3.6 Profile of Mood States (POMS) questionnaire

Participants completed the POMS questionnaire (Appendix 10) immediately prior to each time-trial. The aim of the questionnaire was to assess the affective mental/mood state of the participants at 06h00 and 18h00. The questionnaire contains 65 self-report items and scores individuals on tension, anxiety, vigour, activity, depression, dejection, fatigue, inertia, anger, hostility, confusion and bewilderment, using the 5-point Likert Scale (McNair *et al*., 1971). Participants can choose from not at all (0), a little (1), moderately (2), quite a lot (3) to extremely (4), for each question. The test takes approximately three to 7 min for healthy participants to complete (McNair *et al*., 1971).

3.2.3.7 Standardised pre- time-trial snack

Participants fasted from 22h00 the night before the 06h30 time-trial and consumed a standard pre-time-trial snack 30 min prior to the time-trial. Participants were fasted for four hours prior to the 18h30 time trial, and were allowed the same pre-time trial snack at 18h00. This standardised pre-time-trial snack was given to the swimmers to ensure that they were not hypoglycaemic while swimming, especially for the morning time-trial session. The snack was a carbohydrate (CHO) drink (Refuel Glucose Polymer, Refuel, South Africa) that was normalised according to body mass for each participant. The concentration of the carbohydrate drink was 7% (w/v) and each participant consumed a calculated volume of the CHO drink that provided approximately 0.3 g CHO.kg body mass^{-1}. For example, for an 80 kg male: 0.3 g CHO × 80 kg = 24 g CHO. To calculate the volume of water to be added to the CHO powder: volume = (v) =mass/concentration, therefore (v) = 24 g /0.07 = 340 ml water.

3.2.3.8 Rating of perceived exertion (RPE)

RPE was noted immediately after the standardised warm-up and after the time-trial using Borg's 20-point scale (Borg, 1976) (Appendix 12). Borg's RPE scale is a widely accepted psychophysical scale that is used to assess how subjective intensity varies with physical intensity (Borg, 1990).

3.2.3.9 Standardised warm-up and 200 m freestyle time-trial

WARM-UP

Prior to each time-trial the swimmers completed a standardised 600 m warm-up swim consisting of a 300 m swim at recovery pace, 100 m drill, 100 m kick with a board and 100 m swim at recovery pace. Immediately after the 600 m warm-up, participants were asked to rate their RPE and had a rest period of 5 min before diving in for the time-trial. Other researchers who have focussed on swimming performance and biomechanical parameters have used similar standardised warm-up protocols ranging from 400 m to 1500 m (Baxter and Reilly, 1983; Martin and Thompson, 2000; Sinnerton and Reilly, 2002; Pedersen and Kjendlie, 2006).

TIME-TRIAL

The time-trial was performed at either the Old Mutual swimming pool or the SSISA swimming pool. Both swimming pools are indoor, short course (25 m) and heated. Table V displays the descriptive characteristics of the two swimming pools. With the exception of water temperature, there were no differences in any of the environmental variables between the two swimming pools.

Table V: Descriptive characteristics of the OM and SSISA swimming pools.

	OM pool (n=36 TT sessions)	**SSISA pool (n=16 TT sessions)**	**p-value**
Air temperature (°C)	20.22 ± 1.40	20.04 ± 1.93	0.698
Humidity (%)	70.31 ± 6.55	71.50 ± 7.13	0.557
Water temperature (°C)	27.42 ± 0.50	27.88 ± 0.34	0.002*

Data are presented as the mean ± SD. OM = Old Mutual; SSISA = Sports Science Institute of South Africa; TT = time-trial. The p-value represents significance as determined by an independent t-test. * indicates statistical significance.

Swimmers started on a starter's mark from a dive. The time-trial consisted of a 200 m freestyle maximal effort sprint. Each swimmer completed the time-trial in exactly the same manner for both the morning and evening time-trials. For example, if the swimmer swam alone in the morning time-trial, with no competition or pacing, this was repeated for the evening time-trial. Total time taken to complete the time-trial as well as 50 m split times were recorded using a standard stopwatch. Immediately on finishing the time-trial, RPE was recorded.

STROKE COUNT (SC) AND BREATH COUNT (BC)

Each time-trial was recorded using a digital camera (Casio, EX-FH-20 Exilm). The footage was subsequently used to determine SC and BC for each 25 m lap and over the 200 m distance. SC was defined as the number of times one of the swimmer's arm made a full rotation from when the arm entered the water to pull until it entered the next pull. (Pyne *et al.,* 2001; Polaha *et al.,* 2004). Each full arm rotation was counted as one stroke (Pyne *et al.,* 2001; Polaha *et al.,* 2004). BC was defined as the number of breaths that the swimmer took over each length (25 m) of the 200 m time-trial. Since Pedersen and Kjendlie (2006) concluded that swimmers competing at faster speeds take fewer breaths over the given race distance, it was anticipated that a faster time-trial at a given time-of-day should result in a lower BC.

3.2.4 Statistical analyses

All data were expressed as the mean ± standard deviation. The Shapiro-Wilks test was used to determine if the data were normally distributed. Characteristics of the male and female swimmers were compared using a one-way analysis of variance (ANOVA) for normally distributed data, and a Mann-Whitney U test for data that were not normally distributed. Post hoc analyses were performed used Scheffe's post hoc test. Cronbach's α (George and Mallery, 2003) was used to determine the internal consistency of the H-Ö questionnaire within the swimming group. Correlations were performed using Pearson's product-moment correlation or a multinomial logistic regression. Fisher's exact two tailed tests and Pearson's Chi-squared tests were used to compare the chronotype, genotype and allelic frequencies and the habitual training time-of-day frequencies between the male and female swimmers, due to the small sample sizes. Paired t-tests were used to

compare swimming performance of the swimming in the morning and the evening. Various data are presented as the difference from the morning to the evening time-trial which was determined by subtracting the evening time-trial value from the morning time-trial value. Independent t-tests and Kruskal-Wallis ANOVA were used to compare the difference in swimming performance from the morning to the evening within the sub-groups of chronotype, *PER3* VNTR genotype and habitual training time-of-day. Depending on the data; Wilcoxon matched pairs tests, Mann- Whitney U tests, paired t-tests and independent t-tests were used to compare RPE, mood state, stroke count and breath count of the swimming group in the morning and the evening and to determine the differences in RPE and mood state from the morning to the evening within the sub-groups of chronotype, *PER3* VNTR genotype and habitual training time-of-day. Data were analysed using STATISTICA version 10 (Soft Inc., Tulsa Oklahoma, USA). Statistical significance was accepted when $p<0.05$.

3.3 RESULTS

3.3.1 Participant characteristics

The descriptive characteristics of the swimmers are presented in Table VI. The male swimmers were significantly taller (p<0.001), heavier (p<0.001) and had a greater body mass index (BMI) (p<0.001) and arm span (p<0.001) compared to the female swimmers.

Table VI: Descriptive characteristics of the swimming group as a whole, the male swimmers and the female swimmers.

	Group (n=26)	Males (n=18)	Females (n=8)	p-value
Age (y)	32.6 ± 5.7	33.6 ± 5.8	30.3 ± 5.1	0.169
Height (m)	1.79 ± 0.10	1.83 ± 0.07	1.69 ± 0.08	<0.001*
Body mass (kg)	78.88 ± 11.37	84.17 ± 7.95	66.98 ± 8.62	<0.001*
BMI ($kg \cdot m^{-2}$)	22.07 ± 2.31	23.03 ± 1.82	19.90 ± 1.83	<0.001*
Arm Span (cm)	178.47 ± 12.10	184.11 ± 8.71	165.79 ± 8.54	<0.001*
Training (y)	12.75 ± 9.02	12.64 ± 9.96	13.0 ± 7.07	0.927

The data are presented as mean ± SD. The p-value represents significance for a gender comparison as determined by a one-way ANOVA for normally distributed data and a Mann-Whitney U test for data that were not normally distributed. BMI = body mass index. * indicates statistical significance.

3.3.2 Chronotype

The mean (± SD) H-Ö questionnaire score for the swimmers was 62.69 ± 10.32, ranging from 42 to 82. The mean (± SD) H-Ö questionnaire scores for the males and females were 61.94 ± 10.26 and 64.38 ± 10.97, respectively, and were not significantly different (p=0.590). The H-Ö score data for all participants were significantly skewed towards the higher scores (skewness = -0.34 ± 0.46; p<0.001).

The chronotype characteristics of the swimming group as a whole, the male swimmers and the female swimmers are presented in Table VII. There was no difference in chronotype distribution when comparing the male and female swimmers (p=0.395). Cronbach's α (George and Mallery,

2003) was used to determine the internal consistency of the H-Ö questionnaire within the swimming group and was considered to be satisfactory (Cronbach's α=0.88).

Table VII: Chronotype characteristics of the swimmers

Chronotype	All (n=26)	Males (n=18)	Females (n=8)	p-value
MT	15 (58)	9 (50)	6 (75)	
NT	11 (42)	9 (50)	2 (25)	0.395
ET	0 (0)	0 (0)	0 (0)	

Data are presented as the number of observations with the percentage in parentheses. MT = morning-type; NT = neither-type; ET = evening-type. The p-value represents the frequency analysis significance for a gender comparison, as determined using the Fisher's exact two-tailed test.

3.3.2.1 Chronotype and age

There was no correlation between chronotype and age for the swimming group as a whole (r=0.331, p>0.05) or when the swimmers were grouped according to gender (males: r=0.420; p>0.05; females: r=0.169, p>0.05). This finding was most likely due to the small sample size of the swimming population assessed in this study, which made correlations difficult.

3.3.3 *PER3* VNTR genotype

The *PER3* VNTR genotypic and allelic frequencies for all swimmers are presented in Table VIII. As only two individuals were homozygous for the $PER3^{5}$ allele, the $PER3^{55}$ genotype was omitted from the analysis of genotype frequency in males and females. There were no differences in *PER3* VNTR genotype or allele distributions when comparing the male and female swimmers. Nevertheless, the frequency of the 4-repeat allele was higher for the swimming group as a whole (65%) as well as for the male (67%) and female (63%) swimmer groups.

Table VIII: *PER3* VNTR polymorphism characteristics of the swimmers

		All (n=26)	Males (n=18)	Females (n=8)	p-value
Genotype	**44**	10 (38)	6 (33)	4 (50)	0.192
	45	14 (54)	12 (67)	2 (25)	
	55	2 (8)	0 (0)	2 (25)	
Allele	**4-repeat**	34 (65)	24 (67)	10 (63)	0.771
	5-repeat	18 (35)	12 (33)	6 (37)	

Data are presented as the number of observations with the percentage in parentheses. 44 = *PER3*^{44}; 45 = *PER3*^{45}; 55 = *PER3*^{55}. The p-values represent the frequency analysis significance for a gender comparison, as determined using Fisher's exact two-tailed test (genotype) and the Pearson's chi-squared test (allele). The frequency of the *PER3*^{55} genotype was not included in the genotypic frequency analysis.

3.3.4 Habitual training time-of-day

The habitual training characteristics of the swimmers are presented in Table IX. There was a relatively even distribution regarding habitual training time-of-day, with 54% of the swimmers habitually training in the morning and 46% in the evening. There was no significant difference in habitual training time-of-day between the male and female swimming groups (p=1.000).

Table IX: Habitual training characteristics of the swimmers

	All (n=26)	Males (n=18)	Females (n=8)	p-value
Train AM	14 (54)	10 (56)	4 (50)	1.000
Train PM	12 (46)	8 (44)	4 (50)	

Data are presented as the number of observations with the percentage in parentheses. Train AM = habitual morning training; Train PM = habitual evening training. The p-value represents the frequency analysis significance for a gender comparison as determined using the Fisher's exact two-tailed test.

Table X represents the frequency (percentage) of each chronotype group that habitually trains in either the morning (Train AM) or in the evening (Train PM). Although no significant difference (p=0.233) was observed, it appears that the majority of morning-type individuals (71%) habitually train in the mornings, and almost 60% of the NT swimmers train in the evenings. Additionally, when the male and female swimmers were analysed separately, there was no significant difference between chronotype and habitual training time-of-day of the swimmers (data not shown). Moreover, there was a correlation between H-Ö questionnaire score and habitual training time-of-day (n=26, r=-0.469, p=0.016). That is, the swimmers with higher H-Ö questionnaire scores generally habitually trained in the mornings.

Table X: Chronotype and habitual training time-of-day of the swimmers

	Train AM (n=14)	Train PM (n=12)	p-value
MT	10 (71)	5 (42)	0.233
NT	4 (29)	7 (58)	

Data are presented as the number of observations with the percentage in parentheses. MT = morning-type; NT = neither-type; Train AM = habitual morning training; Train PM = habitual evening training. The p-value represents significance as determined using the Fisher's exact two-tailed test.

3.3.5 Time-trial (TT)

3.3.5.1 Oral temperature

There was a significant difference (p=0.004) in oral temperature of the swimmers prior to the morning (mean ± SD: 35.98 ± 0.64°C) and the evening TT's (mean ± SD: 36.36 ± 0.65°C), when the swimmers were pooled into one group. Additionally, when the male and female swimmers were analysed separately, there was only a significant difference (p=0.001) in oral body temperature prior to the two time-trials for the male swimmers (female swimmers: p=0.839), with oral body temperature being higher prior to the evening TT (mean ± SD: 36.36 ± 0.70°C) compared to the morning TT (mean ± SD: 35.83 ± 0.70°C). Analysis of variance with repeated measures showed that

there were no time-by-group interaction or group effects for oral temperature prior to the two TT's with respect to chronotype, *PER3* VNTR genotype and habitual training time-of-day (Table XI). However, there were time-of-day effects (p=0.004, p=0.004 and p=0.005, respectively). In addition, post-hoc analyses indicated that the time-of-day effect with regard to *PER3* VNTR genotype was between the oral body temperature prior to the morning (mean ± SD: 35.93 ± 0.68°C) and evening (mean ± SD: 36.48 ± 0.72°C) TT for the *PER3*^{45} group (p=0.014). When gender was analysed separately, there were no time time-by-group interaction, group or time-of-day effects for oral body temperature prior to the two TT's with respect to chronotype, *PER3* VNTR genotype and habitual training time-of-day.

Table XI: ANOVA with repeated measures results for oral temperature prior to the morning and evening TT, when swimmers were grouped by chronotype, *PER3* VNTR genotype and habitual training time-of-day.

	Chronotype	*PER3* VNTR genotype	Habitual training time-of-day
Interaction effect	0.574	0.135	0.620
Group effect	0.212	0.755	0.486
Time-of-day effect	0.004*	0.004*	0.005*

Data are the p-values obtained from an ANOVA with repeated measures. Interaction effect = differences in oral temperature prior to the TT's due to a time-by-group effect; group effect = differences in oral temperature prior to the TT's due to an effect associated with either chronotype, *PER3* VNTR genotype or habitual training time-of-day group; time-of-day effect = differences in oral temperature prior to the TT's due to the time-of-day (morning or evening); * indicates statistical significance.

3.3.5.2 Sleep

The swimmers had less sleep prior to the morning TT (mean ± SD: 6.75 ± 1.12h) compared to the evening TT (mean ± SD: 7.63 ± 0.79h, p<0.001). However, when the male and female swimmers were analysed separately, there were no differences regarding the amount of sleep prior to the two TT's (data not shown). There were no time-by-group interaction effects in the amount of sleep obtained prior to each of the two time-trials, when sleep-time was analysed according to the sub-groups of chronotype (p=0.278), *PER3* VNTR genotype (p=0.068) and habitual training time-of-day (p=0.172)

(data not shown). However, there were time-of-day effects when comparing sleep-time prior to the morning and evening TT's with regard to chronotype (p=0.001), *PER3* VNTR genotype (p=0.000) and habitual training time-of-day (p=0.001) (data not shown). More specifically, the MT swimmers, *PER3*^{55} swimmers and those swimmers that habitually trained in the morning had a greater amount of sleep prior to the two time-trials. There were also no differences regarding sleep prior to the two TT's with regard to chronotype, *PER3* VNTR genotype and habitual training time-of-day (data not shown) when the male and female swimmers were analysed separately.

3.3.5.3 200 m TT swimming performance

Figure 17 represents the mean 200 m swimming performance for the morning (06h30) TT versus the evening (18h30) TT for all the swimmers (A), as well as the males (B) and the females (C). There was no significant difference when comparing 200 m swimming performance at 06h30 and 18h30 when the data for all the swimmers were pooled (p=0.590). Similarly, there was no diurnal variation in swimming performance for the males (p=0.653) or the females (p=0.775).

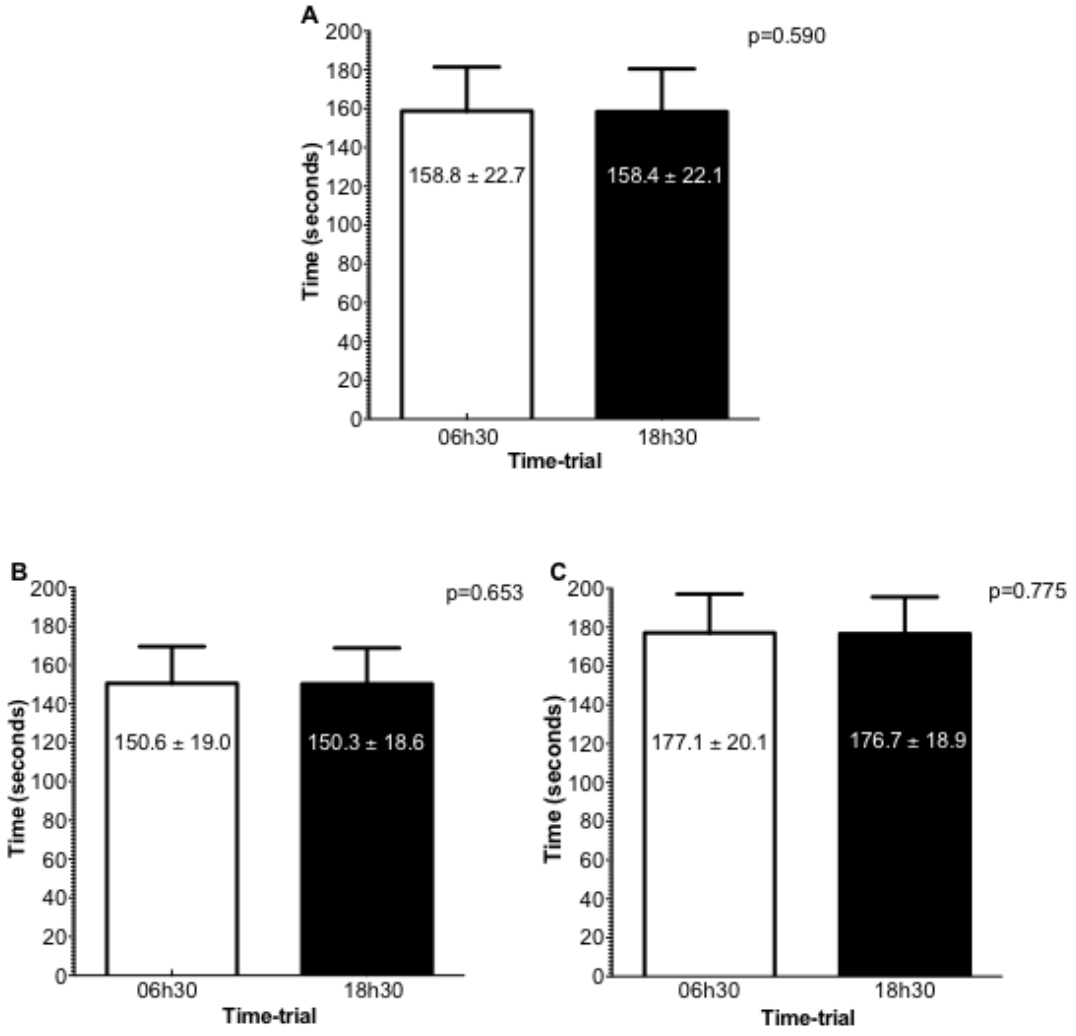

Figure 17: 200 m swimming performance in the morning (06h30) and the evening (18h30) time-trials for (A) all the swimmers (n= 26), (B) the males (n=18) and (C) the females (n=8). The data are presented as mean ± SD. The p-values represent significance as determined by paired t-tests.

To confirm that there was no order or learning effect, swimming performance time of each swimmer's first TT was compared to his/her second TT, regardless of the time-of-day (i.e. 06h30 or 18h30). Performance for TT1 (mean ± SD: 158.9 ± 22.7s) was not significantly different to that of time-trial 2 (mean ± SD: 158.2 ± 22.1s, p=0.374).

Figure 18 represents the difference in 200 m swimming performance between the morning (06h30) and evening (18h30) TTs when the swimmers were grouped by (A) chronotype, (B) *PER3* VNTR

genotype and (C) habitual training time-of-day. There was a significant diurnal variation in 200 m swimming performance with regard to chronotype (p=0.032, A). The morning-type (MT) swimmers swam faster in the morning TT and the neither-type (NT) swimmers in the evening TT. More specifically, 67% of the MT swimmers were faster at 06h30 and 64% of the NT swimmers were faster at 18h30. In addition, there was a correlation between H-Ö questionnaire score and the difference in swimming performance between the morning and the evening TT's (n=26, r=0.510, p=0.008). That is, the swimmers with higher H-Ö questionnaire scores tended to swim faster in the morning TT. In contrast, there was no diurnal variation in swimming performance with regard to *PER3* VNTR genotype (Figure 18B). Although the two swimmers genotyped as *PER3*^{55} were not included in the statistical analyses due to insufficient power, their data suggest that they were faster in the morning.

There was, however, diurnal variation in swimming performance with regard to habitual training time-of-day (p=0.012, C). Specifically, the swimmers who habitually trained in the morning swam faster in the morning TT. Seventy-one percent (n=10) of the swimmers who trained in the morning were faster at 06h30 and 67% (n=8) of those who trained in the evening swam faster at 18h30. Very small effect sizes regarding the difference in performance were found for all groups (A, B and C). In addition, when the male and female swimmers were analysed separately, there was only a significant diurnal variation in swimming performance with regard to habitual training time-of-day for the male swimmers (males: p=0.029; females: p=0.280).

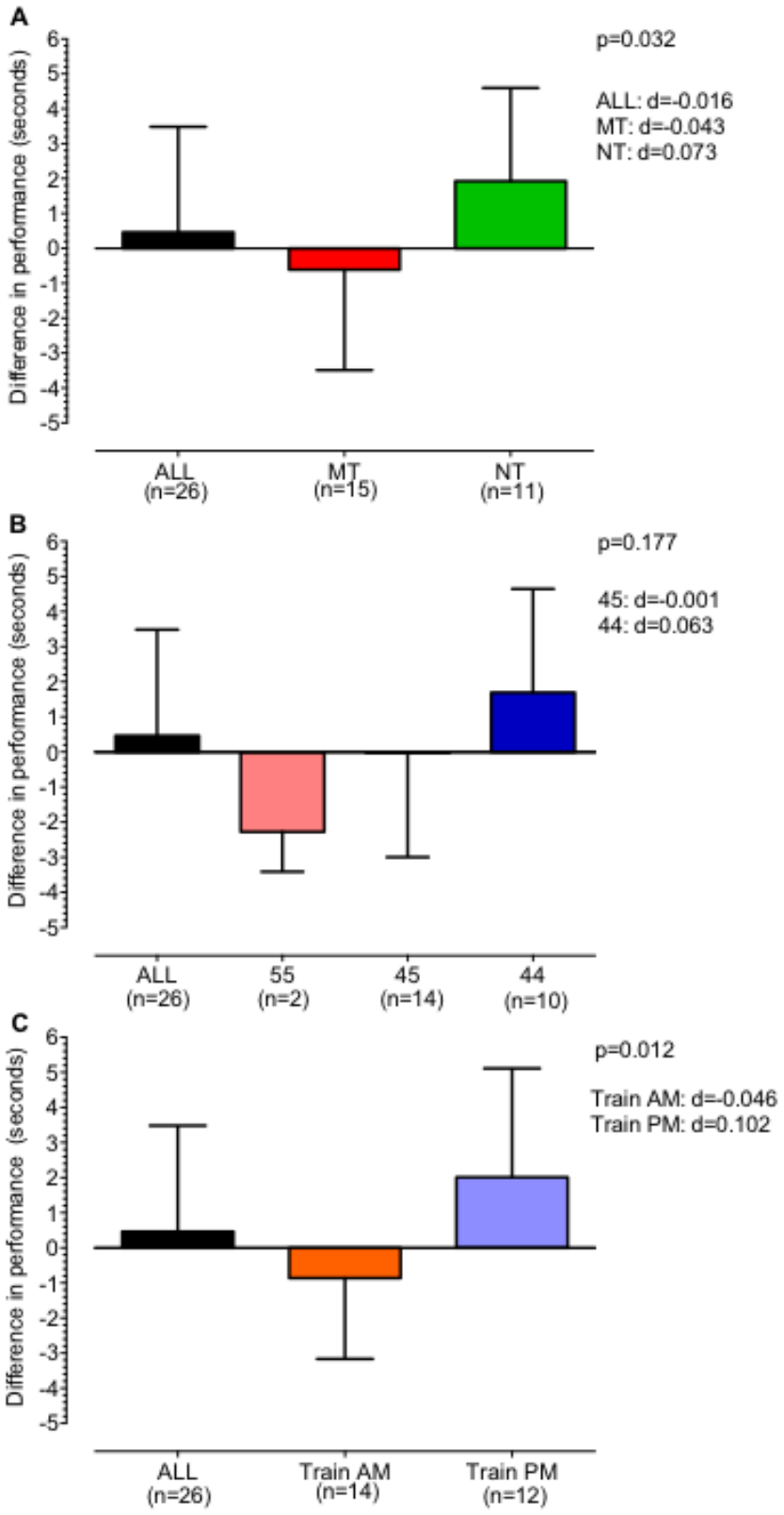

Figure 18: 200 m swimming performance difference between the morning (06h30) and evening (18h30) time-trials, with swimmers grouped by (A) chronotype, (B) *PER3* VNTR genotype and (C) habitual training time-of-day. ALL = all swimmers (data not included in statistical analyses); MT = morning-type; NT = neither-type; 44 = *PER3*^{44}; 45 = *PER3*^{45}; 55 =

PER3^{55}; Train AM = habitual morning training; Train PM = habitual evening training; d = effect size. Data are presented as the mean ± SD. The p-values represent significance as determined by an independent t-test. Cohen's d was used to determine effect size. Statistical analyses were not conducted on *PER3*^{55} due to the small sample size.

Figure 19 displays the difference in swimming performance between the morning and evening TTs when swimmers were grouped by combinations of chronotype, *PER3* VNTR genotype and habitual training time-of-day. There was no significant difference in performance when swimmers were grouped by (A) chronotype and *PER3* VNTR genotype or (C) *PER3* VNTR genotype and habitual training time-of-day. On the other hand, there was a significant difference in performance when the swimmers were grouped according to chronotype and habitual training time-of-day (p=0.041, B). More specifically, 80% (n=8) of the MT swimmers who trained in the morning swam faster at 06h30, whereas 60% (n=3) of the MT and 71% (n=7) of the NT swimmers who trained in the evening swam faster at 18h30. In addition, very small effect sizes regarding the difference in performance were found for all sub-groups (A, B and C).

With regard to Figure 19A, the two MT swimmers genotyped as *PER3*^{55} swam faster at 06h30 while 50% of the MT (n=2) and 67% of NT (n=4) swimmers genotyped as *PER*^{44} swam faster at 18h30 (Figure 19A). In addition, of the swimmers genotyped as *PER3*^{45}, 67% of the MT swimmers (n=6) were faster at 06h30 and 60% of the NT swimmers (n=3) were faster at 18h30 (Figure 19A). With regard to the combination of genotype and chronotype, the two swimmers genotyped as *PER3*^{55} who habitually trained in the mornings swam faster at 06h30 (Figure 19C). In contrast, 50% of the swimmers genotyped as *PER3*^{44}, who trained in the morning (n=2) and 67% of those who trained in the evening (n=4), were faster at 18h30. Lastly, 75% of the swimmers genotyped as *PER3*^{45} who trained in the morning (n=6) were faster at 06h30 and 67% who trained in the evening (n=2) were faster at 18h30.

Additionally, there was no diurnal variation in performance for any of the combinations of chronotype, *PER3* VNTR genotype and habitual training time-of-day (data not shown), when the male and female swimmers were analysed separately.

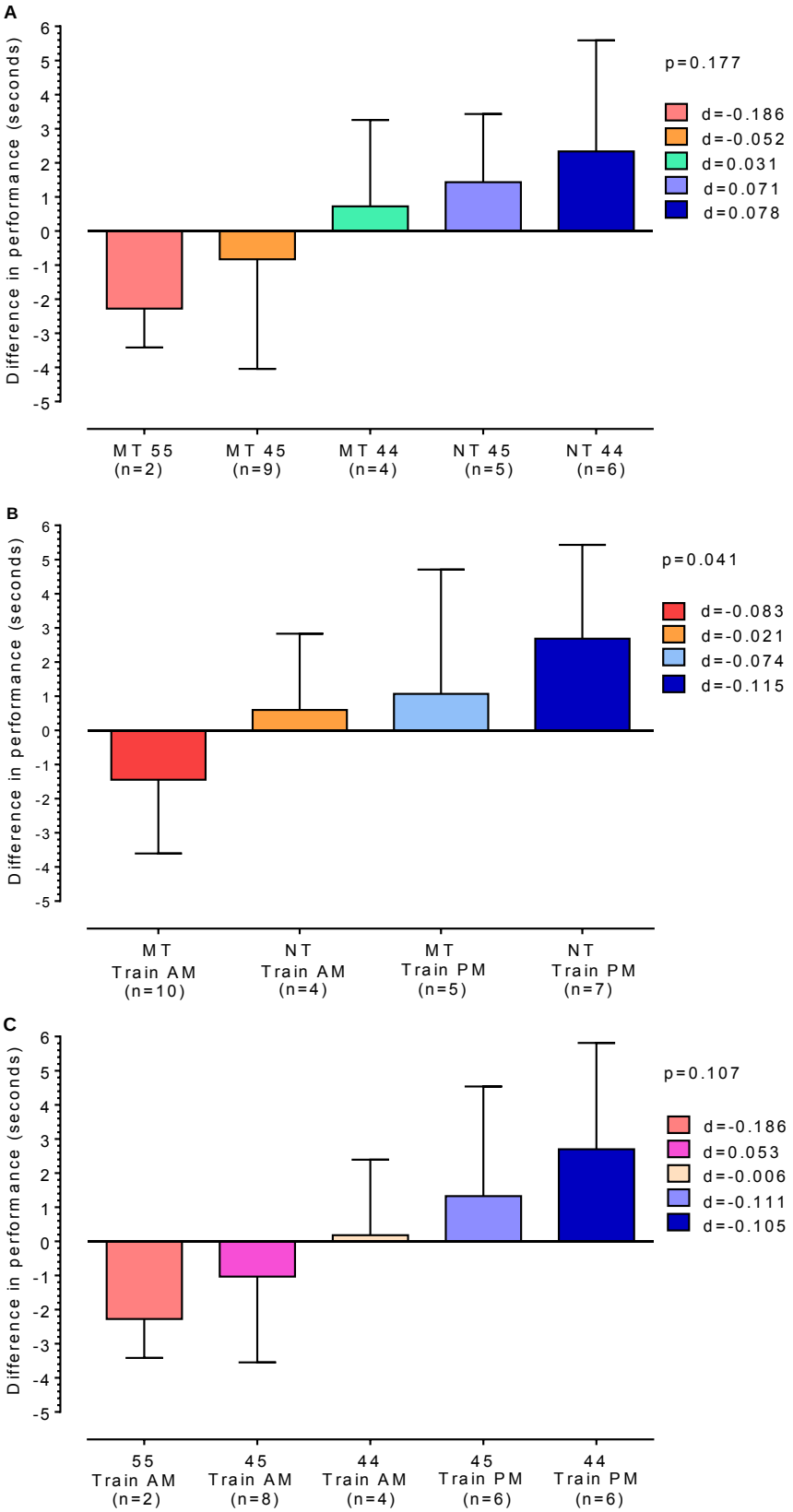

Figure 19: 200m swimming performance difference between the morning and evening time-trials in swimmers grouped by (A) chronotype and *PER3* VNTR genotype, (B)

chronotype and habitual training time-of-day and (C) *PER3* VNTR genotype and habitual training time-of-day. MT = morning-type; NT = neither-type; 44 = *PER3*^{44}; 45 = *PER3*^{45}; 55 = *PER3*^{55}; Train AM = habitual morning training; Train PM = habitual evening training; d = effect size. Data are presented as mean ± SD. The p-values represent significance as determined by a Kruskal-Wallis ANOVA. Cohen's d was used to determine effect size. Statistical analyses were not conducted on *PER3*^{55} due to the small sample size.

Figure 20 represents the difference in performance between the morning and evening TTs for the swimmers grouped by chronotype, *PER3* VNTR genotype and habitual training time-of-day. The data were plotted merely for a visual comparison since the very small sample sizes of the sub-groups precluded statistical analyses. However, interesting trends regarding diurnal variation in performance were revealed. For example, all of the MT swimmers genotyped as *PER3*^{55} who trained in the morning (n=2) and 83% of the MT swimmers genotyped as *PER3*^{45} who trained in the morning (n=5) were faster at 06h30. Similarly, 75% of the NT swimmers genotyped as *PER3*^{44} who trained in the evening (n=3) swam faster at 18h30.

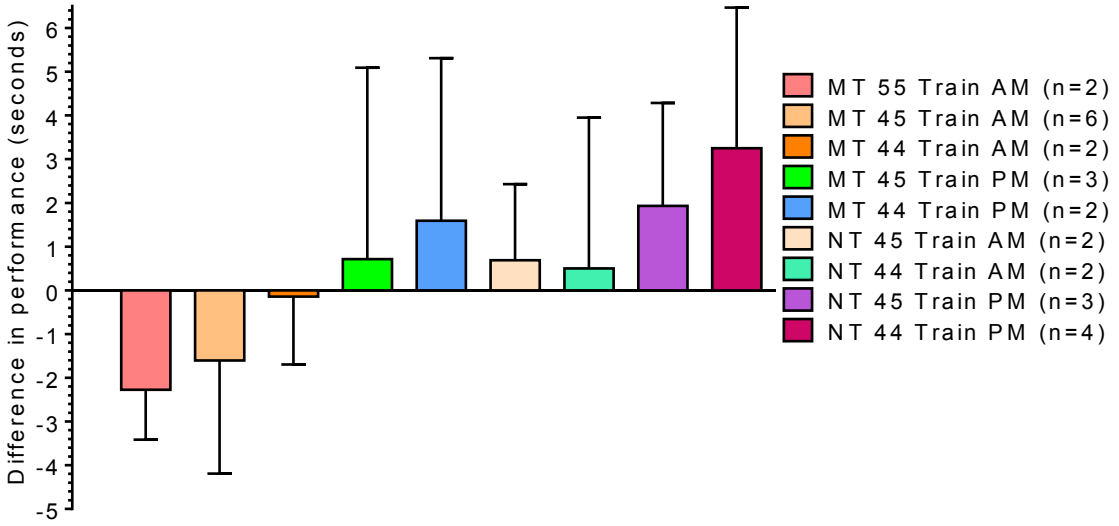

Figure 20: 200m swimming performance difference between the morning and evening time-trials in swimmers grouped by a combination of their chronotype, *PER3* VNTR genotype and habitual training time-of-day. MT = morning-type; NT = neither-type; 44 = *PER3*^{44}; 45 = *PER3*^{45}; 55 = *PER3*^{55}; Train AM = habitual morning training; Train PM = habitual evening training. Data are presented as mean ± SD. Statistical analyses were not conducted due to the small sample sizes of the respective sub-groups.

3.3.6 Rating of perceived exertion (RPE)

Figure 21 represents the mean RPE scores post warm-up (A) and post 200 m TT (B) for all of the swimmers. There was no diurnal variation in mean RPE score post warm-up (p=0.420, A) and post 200 m time-trial (p=0.276, B). The difference in RPE between the morning and evening sessions, measured at both the end of the warm-up (C) and after the TT (D), was not different for swimmers categorized as morning-types and neither-types. There was, however, a moderate effect size for the change in RPE of the morning-type swimmers after the warm-up (d=-0.480). Similarly, there was no difference in RPE between the morning and evening sessions after both the warm-up and the TT for either of the *PER3* VNTR genotype groups (E and F respectively). On the other hand, there was a diurnal variation in RPE post warm-up when comparing the swimmers who habitually trained in the morning to those who habitually trained in the evening (p=0.020, G). More specifically, the swimmers who trained in the morning had a lower RPE score post warm-up at 06h30 and those who trained in the evening had a lower RPE score at 18h30 post warm-up. The effect sizes were relatively large for both the morning (d=-0.689) and evening (d=0.538) training groups.

Additionally, when the male and female swimmers were analysed separately, there was a difference in RPE post warm-up with regard to habitual training time-of-day for the female swimmers only (males: p=0.138; females: p=0.022). Specifically, of the four females, who trained in the morning all reported a lower RPE after the warm-up at 06h30, and of the four who trained in the evening; all reported a lower RPE after the warm-up at 18h30. On the other hand, only 60% of the males who trained in the morning reported a lower 06h30 RPE and 63% who trained in the evening had a lower 18h30 RPE.

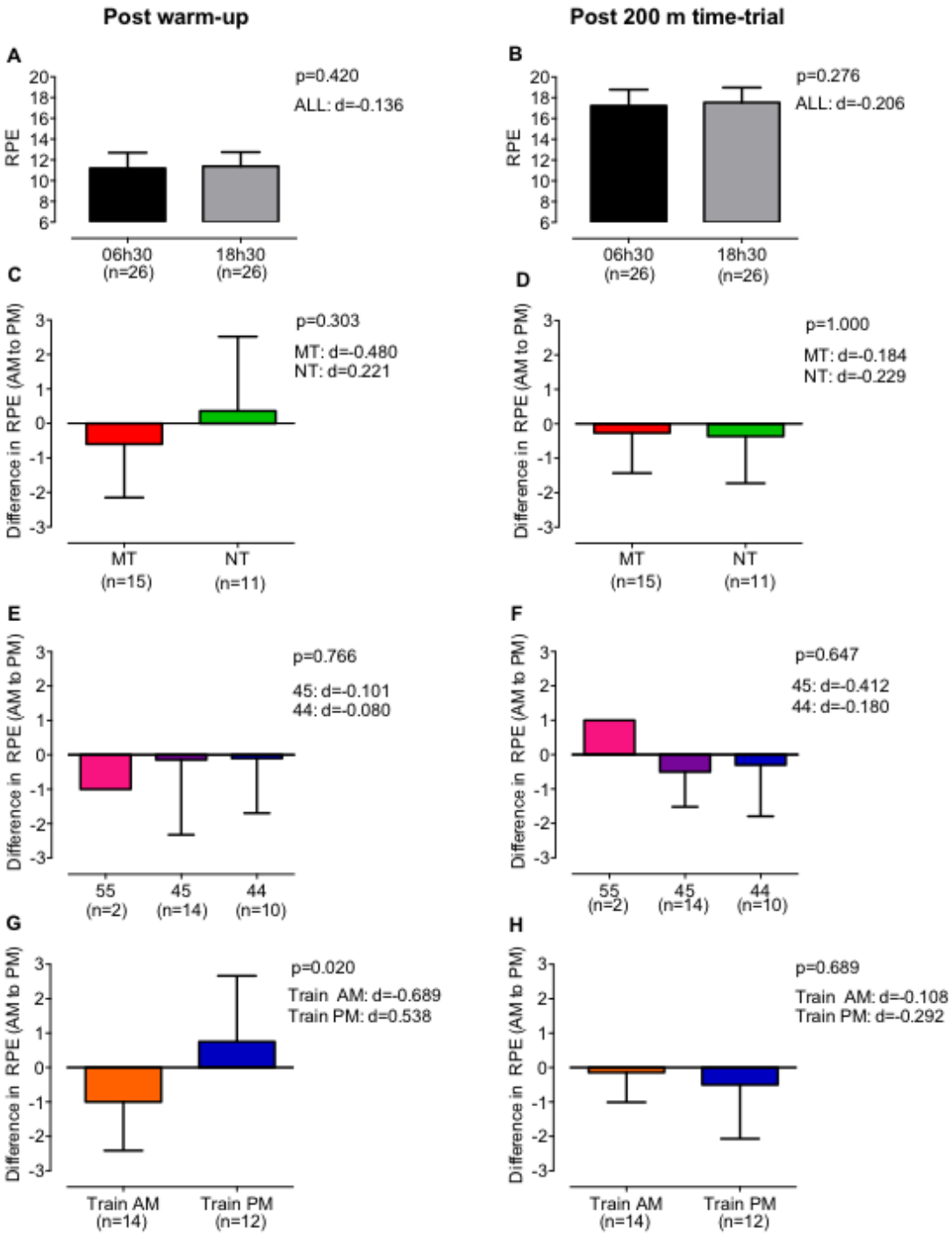

Figure 21: **Time-of-day variation in RPE for all swimmers (A, B) as well as differences in RPE between the morning and evening sessions (C, D, E, F, G and H).** RPE was measured after the warm-up (A, C, E and G) and at the end of the 200 m TT (B, D, F and H). The swimmers were grouped by chronotype (C, D), genotype (E, F,) and habitual training time-of-day (G and H). RPE = rating of perceived exertion; MT = morning-type; NT = neither-type; 44 = *PER3*^{44}; 45 = *PER3*^{45}; 55 = *PER3*^{55}; Train AM = habitual morning training; Train PM = habitual evening training; d = effect size. Data are presented as the mean ± SD. The p-values for A and B represent significance as determined using a Wilcoxon matched pairs test, and the p-values

for C, D, E, F, G and H represent significance as determined using a Mann-Whitney U test. Effect size was determined using Cohen's d statistic. Statistical analyses were not conducted on *PER3*^{55} due to the small sample size.

3.3.7 Mood state

The Profile of Mood States (POMS) questionnaire was used to analyse the mood state of the swimmers at 06h30 and 18h30. Swimming performance at both 06h30 and 18h30 was not significantly associated with any of the mood state variables ($p>0.050$, data not shown). From Figure 22 it is evident that the swimmers had a greater total mood disturbance (TMD, $p=0.022$), as well as greater states of depression ($p=0.004$), anger ($p=0.008$) and tension ($p=0.024$) before the evening 200 m TT. When the male and female swimmers were analysed separately, there was no diurnal variation in any of the mood state variables (data not shown).

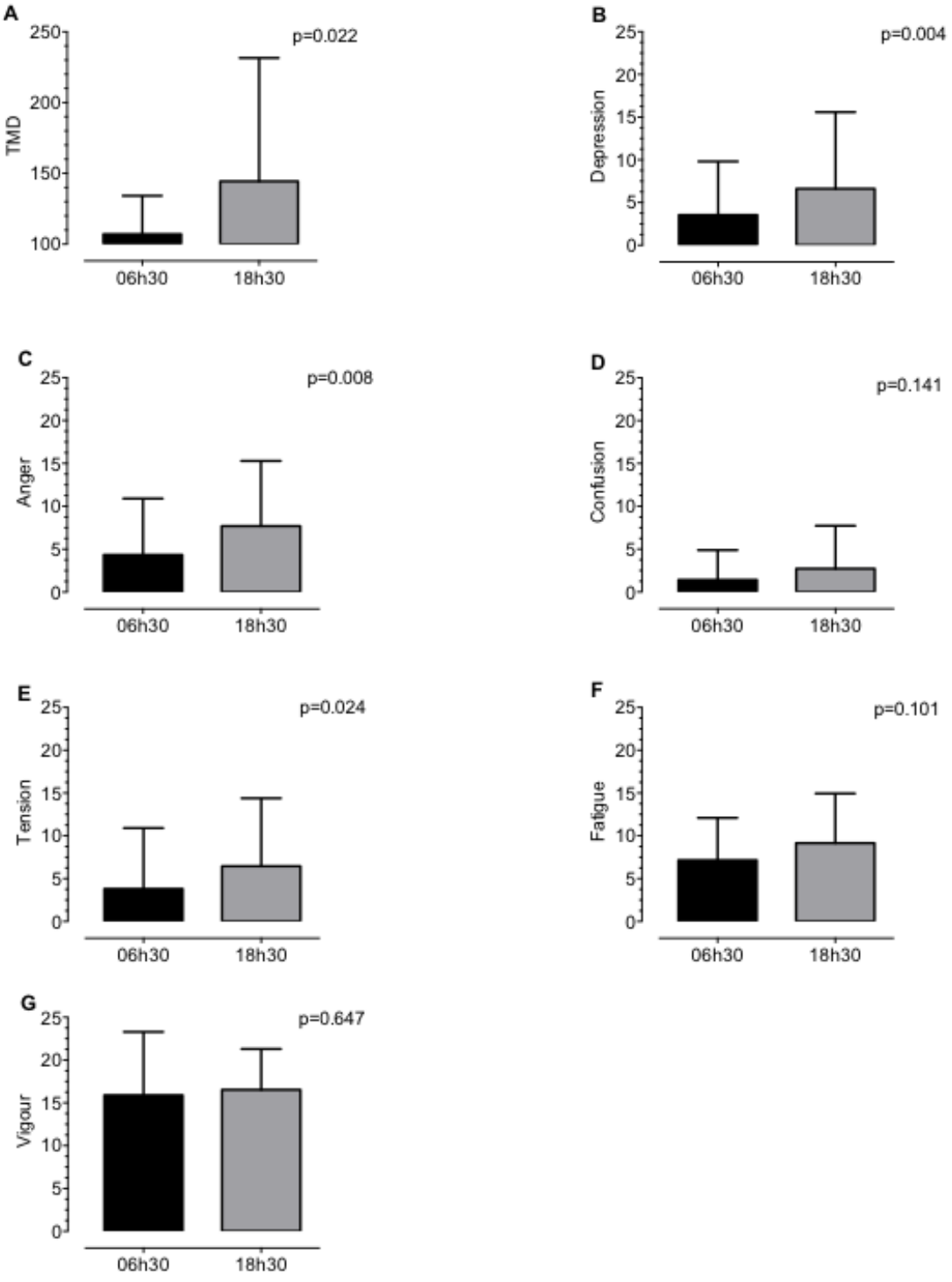

Figure 22: Profile of Mood States (POMS) scores for the swimmers prior to morning (06h30) and evening (18h30) time-trials. A: Total mood disturbance, B: Depression; C: Anger; D: Confusion; E: Tension; F: Fatigue and G: Vigour. Data are presented as the mean ± SD. The p-values for A, B, C, D, E and F represent significance as determined by a Wilcoxon matched pairs test, and the p-value for G represents significance as determined by a paired t-test.

The POMS data was further analysed by comparing the difference in the respective mood state variables between the morning and evening TTs within the sub-groups of chronotype, *PER3* VNTR genotype and habitual training time-of-day.

When the diurnal variation of mood state of the swimmers was assessed according to chronotype (Table XII), the difference in fatigue between the morning and evening was different for the MT and NT swimmers (p=0.012). More specifically, the MT swimmers had a lower fatigue in the morning (difference between morning and evening = -4.27 ± 5.8) and the NT swimmers had a lower fatigue in the evening (difference between morning and evening = 1.09 ± 3.42). Moreover, only the male swimmers displayed a difference in fatigue with regard to chronotype (p=0.041). Of the MT male swimmers, 78% (n=7) reported lower levels of fatigue at 06h30 and 67% (n=6) of the NT male swimmers a lower fatigue at 18h30.

In addition, the MT swimmers had greater vigour in the morning (difference between morning and evening = 2.33 ± 5.38) and the NT swimmers greater vigour in the evening (difference between morning and evening = -4.73 ± 7.55, p=0.010) (Table XII).

The difference in POMS scores between the 06h30 and 18h30 TT's for the respective genotypes are also presented in Table XII. The *PER3*^{55} genotype was excluded from the analysis given the small sample size. There was no diurnal variation in any of the mood state variables when the swimmers were grouped according to *PER3* VNTR genotype group.

When the swimmers were grouped by habitual training time-of-day there was a significant diurnal variation in vigour (p=0.007) (Table XII). More specifically, the swimmers who habitually trained in the morning had a higher mean vigour score at 06h30 (difference between morning and evening = 2.71 ± 5.05) compared to those who trained in the evening (difference between morning and evening = -4.58 ± 7.49). Furthermore, only the male swimmers who trained in the morning had a significantly greater vigour at 06h30 compared to those who trained in the evening (males: p=0.007; females: p=0.423). Specifically, 70% of the males who trained in the morning displayed a higher vigour at 06h30 and 63% who trained in the evening reported a higher vigour at 18h30.

Additionally, there was no association between morning and evening TT performance and any of the mood state variables when the swimmers were categorised according to sub-groups of chronotype, *PER3* VNTR genotype and habitual training time-of-day (p>0.050, data not shown).

Table XII: P-values for the difference in mood state variables between the morning and evening TT's within the sub-groups of chronotype, *PER3* VNTR genotype and habitual training time-of-day.

Mood state	Chronotype	*PER3* VNTR genotype	Habitual training time-of-day
TMD	0.232	0.703	0.410
Depression	1.000	0.790	0.855
Anger	0.566	0.837	0.776
Confusion	0.230	0.724	0.717
Tension	0.450	0.362	0.979
Fatigue	0.012*	0.930	0.236
Vigour	0.010*	0.205	0.007*

Data are the p-values obtained from Mann-Whitney U tests for TMD, Depression, Anger, Confusion, Tension and Fatigue and the p-values obtained from independent t-tests for Vigour. TMD = total mood disturbance. *PER3*^{55} was not included in the statistical analyses due to the small sample size. * indicates statistical significance.

3.3.8 Stroke count (SC) and breath count (BC)

SC and BC data were collected for 23 of the 26 swimmers due to technical problems with the digital video camera. Mean stroke and breath count for all swimmers (A and B respectively) and the difference in stroke (C, E and G) and breath (D, F and H) counts between the morning and evening TTs, for the swimmers grouped by chronotype (C and D), *PER3* VNTR genotype (E and F) and habitual training time-of-day (G and H) are presented in Figure 23. There was a diurnal variation in stroke count when the swimmers were grouped according to their habitual training time-of-day (G, p=0.012). More specifically the swimmers who trained in the morning had a greater SC at 06h30 and those who trained in the evening a greater SC at 18h30. Additionally, the effect size for SC was large for the swimmers who trained in the morning (G, d=1.068) and there was a moderate effect size for those who trained in the evening (G, d=-0.490). Although there was no diurnal variation in stroke count with regard to *PER3* VNTR genotype, a large effect size for SC for the *PER3*^{45} swimmers was also evident (E, d=0.699). Finally, SC was only different from the morning to the evening for the male swimmers regarding habitual training time-of-day (males: p=0.048; females: p=0.170). That is, 86% of the males who trained in the morning (n=7) had a higher SC at 06h30. Similarly, 65% of the males who trained in the evening had a higher SC at 18h30.

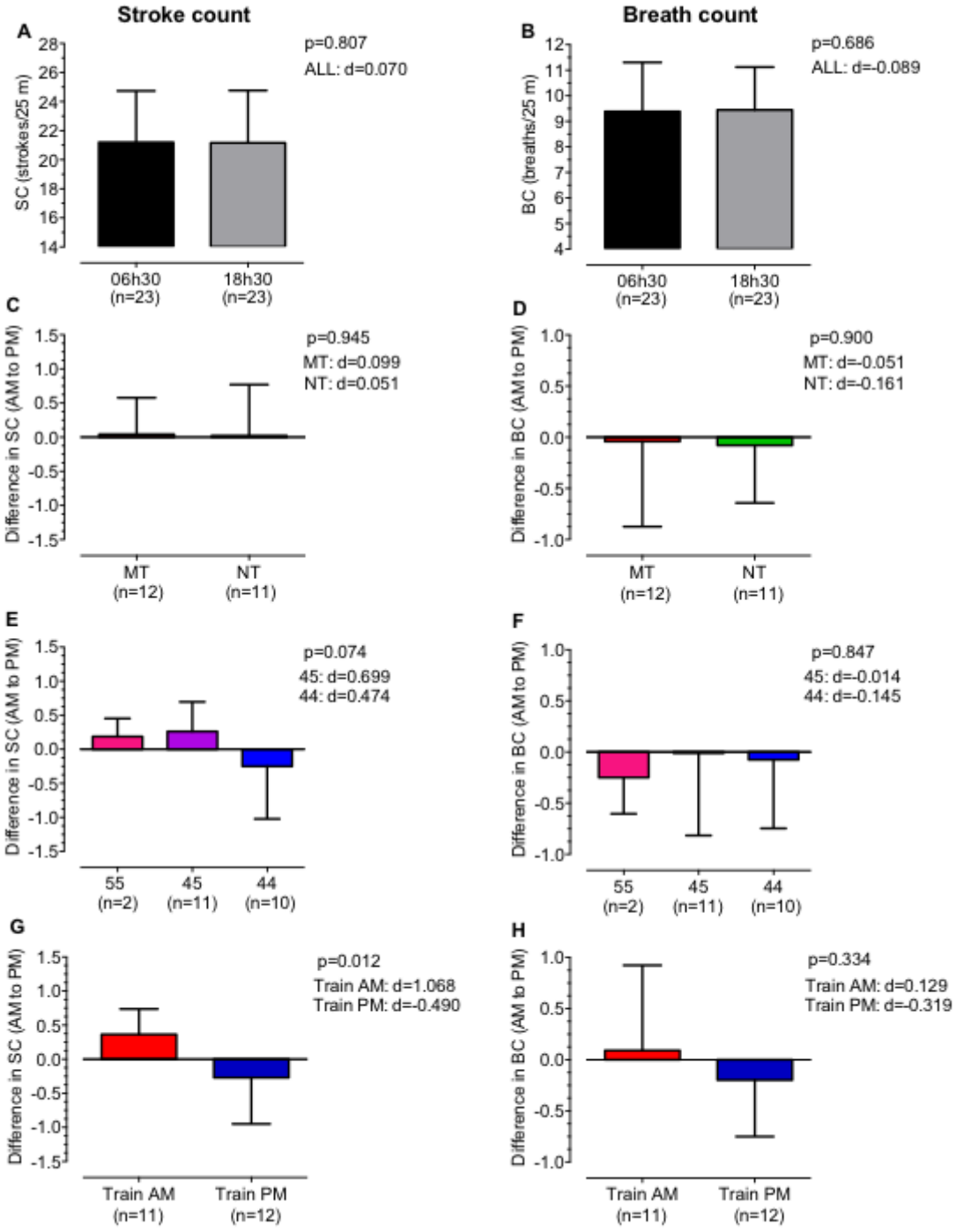

Figure 23: Stroke count and breath count for all swimmers (A and B) and the difference in stroke and breath count between the morning and evening TTs, when swimmers were grouped by chronotype (C and D), *PER3* VNTR genotype (E and F) and training time-of-day (G and H). SC = stroke count; BC = breath count; MT = morning-type; NT = neither-type; 44 = *PER3*^{44}; 45 = *PER3*^{45}; 55 = *PER3*^{55}; Train AM = habitual morning training; Train PM = habitual evening training; d = effect size. Data are presented as the mean ± SD**.** The p-values for A and B represent significance as determined by a paired t-test and the p-values for C, D, E, F, G

and H represent significance as determined by an independent t-test. Effect size was determined using Cohen's d statistic.

3.4 DISCUSSION

The main aim of this study was to describe diurnal variation in 200 m swimming time-trial performance, RPE, mood state as well as stroke count and breath count in trained South African masters swimmers. This study was novel in that it took into account the chronotype, *PER3* VNTR genotype and habitual training time-of-day of the swimmers analysed.

The first finding of this study was that there was no diurnal variation in 200 m swimming time-trial performance when this group of masters swimmers was examined as a whole (Figure 17A). This is a really interesting finding and was not expected as the majority of swimming-specific studies (Baxter and Reilly, 1983; Deschodt and Arsac, 2004; Kline *et al.,* 2007) have reported a significant diurnal variation in swimming performance, whereas, very few have reported an absence of diurnal variation (Martin and Thompson, 2000). A time-of-day effect in time trial performance in other sports has also been reported. For example, Atkinson *et al.* (2005) and Brown *et al.* (2008) both showed that there was a diurnal variation in a 16.1km cycling time-trial and a 2000 m rowing ergometer time-trial, respectively.

The lack of a diurnal variation in 200 m swimming performance in these 26 South African masters swimmers raised an important question. Are the swimmers analysed in this study somehow different to those assessed by others, and could this have accounted for the observed lack of diurnal variation? For example, this group of swimmers contained both males and females, so perhaps gender played a role. Although the mean swimming time-trial performance of the male swimming group was approximately 18% faster than that of the female group, when the males and females were analysed separately there was also no diurnal variation in performance within both the male (Figure 17B) and the female (Figure 17C) groups. Therefore, gender was unlikely to have accounted for the observed lack of diurnal variation in performance.

One explanation for the lack of diurnal variation may be that the chronotype distribution of the swimmers who participated in this study was skewed towards “morningness”. Perhaps the greater prevalence of morning-types in this group reduced the diurnal variation in swimming performance of the group as a whole. This relates to the second important finding of this study; when the swimmers were categorised according to chronotype, there was a significant diurnal variation in performance (Figure 18A). That is, the morning-type swimmers were 0.5% faster at 06h30 and the neither-type group swam 1.2% faster at 18h30.

This is a really interesting finding as performance has traditionally been shown to be superior in the evening (Cappaert, 1999; Drust *et al.,* 2005). The results from this study are in line with that of Brown *et al.* (2008) who also reported a "reversed" diurnal variation for morning-types when chronotype was considered. More specifically, morning-type collegiate rowers were significantly faster in a morning 2000 m rowing ergometer time-trial compared to the evening time-trial, and the neither- and evening-type rowers were faster in the evening (Brown *et al.,* 2008). Therefore, perhaps the evening bias of the neither-types is balanced out by the morning bias of the morning-types resulting in an apparent lack of diurnal variation in performance when the group was examined as a whole.

On the other hand, chronotype has also been shown to dampen diurnal variation (Hill *et al.,* 1988; Tamm *et al.,* 2009). For example, Tamm *et al.* (2009) reported that maximal torque production was the same in the morning and the evening for morning-type individuals, whereas torque production was greatest in the evening for evening-type individuals. In addition, Hill *et al*. (1988) showed that during a cycling time-trial, the 14 evening-types had a higher VO_2max in the evening compared to the morning whereas the VO_2max of the eleven morning-types remained the same, during the morning and evening time-trial (Hill *et al.,* 1988). However, the jury is still out as other studies have reported no chronotype effects on performance at different times of the day. For example, Burgoon *et al.* (1992) reported that there were no chronotype effects on treadmill performance in untrained participants. Lastly, in the Atkinson *et al.* (2005) 16.1km cycling time-trial study, seven participants were classified as morning-type and one as evening-type. Despite the group being skewed towards a greater preference for mornings, cycling time-trial performance was still superior in the evening (Atkinson *et al.,* 2005). Therefore, perhaps the relative proportions of chronotypes within studies could explain this lack of consensus. Nevertheless, the fact that this study has shown that chronotype has an influence on performance at different times of the day is a key finding.

Although *PER3* VNTR genotype does not significantly appear to help in explaining why there was a lack of diurnal variation in this group of swimmers as a whole, interesting trends were observed (Figure 18B). For example, mean swimming performance of the two swimmers genotyped as *PER3*^{55}, which is the genotype associated with "morningness" (Archer *et al.,* 2003; Pereira *et al.,* 2005; Jones *et al.,* 2007; Kunorozva *et al.,* 2012) was faster at 06h30, whereas 60% of the swimmers genotyped as *PER3*^{44}, the genotype associated with "eveningness" (Archer *et al.,* 2003; Pereira *et al.,* 2005; Jones *et al.,* 2007), swam faster at 18h30. With regard to the heterozygous *PER3*^{45} group, 57% of the swimmers swam faster at 06h30 and 43% at 18h30; with this genotype group showing no apparent difference in performance between the morning and evening. Thus is appears that the relationship

between swimming performance and genotype seems to be going in the right "direction". Despite the non-significance, this is still a valuable finding because, to date, this is the only study to have analysed athletic performance with respect to *PER3* VNTR genotype.

Another explanation for the lack of diurnal variation in performance observed in this group of swimmers could be the habitual training time-of-day of the swimmers; which translates to the third notable finding of this study. There was a significant diurnal variation in swimming performance when the swimmers were categorised according to habitual training time-of-day. More specifically, the swimmers who habitually trained in the morning swam 0.7% faster at 06h30 and those who trained in the evening were 1.2% faster at 18h30 (Figure 18C).

Based on these findings, it seems that the concept of diurnal variation is strongly related to habitual training time-of-day. This reasoning seems feasible as studies that have focussed on strength training and power output reported that when comparing participants who underwent morning training to those who underwent evening training, diurnal variation in performance was abolished for the groups that trained in the morning but remained unchanged for the evening training groups (Souissi *et al.,* 2002; Sedliak *et al.,* 2007; Chtourou *et al.,* 2012 a,b). Additionally, Arnett (2001) showed that there was a diurnal variation in swimming performance prior to a training intervention in which the swimmers were required to train in both the mornings and evenings for four months. After the four month training protocol it was reported that the diurnal variation in swimming time-trial performance was eliminated. In contrast, other studies have reported that training time-of-day has no influence on diurnal variation in performance. For example, Martin *et al.* (2007) reported no such effect of morning-evening training on diurnal variation in swimming performance when comparing a squad who trained in both the mornings and evenings to a squad who trained in the evenings only. However, as discussed in the literature review, the latter study design was amiss in that not only were two different swimming squads with very different training loads compared, but the morning and evening time-trial schedules were different for the two squads.

Therefore, based on the majority of the studies it appears whilst peak performance has been shown to traditionally occur in the evening, the inclusion of morning training reduces this diurnal variation in performance (Arnett, 2001; Souissi *et al.,* 2002; Sedliak *et al.,* 2007; Chtourou *et al.,* 2012a,b). This seems applicable within the context of the current study since diurnal variation in performance was observed once the swimmers were grouped according to training time-of-day, in addition to when they were grouped by chronotype. It could thus be speculated that since 71% of the swimmers who train in morning were faster at 06h30 and 67% who train in evening were faster at 18h30, the net

observation is an apparent lack of diurnal variation in performance when the group was examined as a whole.

Despite similar numbers of males training in either the morning or evening compared to the females (p=1.000), a diurnal variation in performance was only observed in the male swimmers with regard to habitual training time-of-day. Specifically, 70% of the males who trained in the morning (n=7) swam faster in the morning and 63% of the males who trained in the evening (n=5) swam faster at 18h30. It is likely that the small sample size of the female group (n=8) was responsible for the lack of significance.

Based on the findings discussed above it seems that taking into account inter-individual differences between athletes may be fundamental to understanding performance. It appears that one such way to do this is to account for inter-individual differences in circadian rhythms. This is reiterated by the fact that when the swimmers were grouped by a combination of both chronotype and habitual training time-of-day, there was a significant diurnal variation in performance (Figure 19B). On the other hand, there was no diurnal variation in performance regarding the combinations of chronotype and genotype (Figure 19A) and genotype and habitual training time-of-day (Figure 19C). The findings presented in Figure 19A and C should be interpreted with caution as statistical analyses were hampered by the fact that there were only two individuals genotyped as *PER3*^{55}. Nevertheless, the fact that genotype and habitual training time-of-day display trends going in the "right" direction with regard to a "reversed" diurnal variation, re-iterates the fact that chronotype, *PER3* VNTR genotype and habitual training time-of-day may be related to the time-of-day at which performance peaks for certain athletes.

Additionally, this is the first study to have reported such data. Bearing in mind that the sample size of the respective sub-groups represented in Figure 20 are underpowered, this is the first clue that a combination of chronotype, *PER3* VNTR genotype and habitual training time-of-day may help explain diurnal variation in performance. However, a larger sample size is needed to confirm these findings. Thus, it appears that both chronotype and habitual training time-of-day play an important role in contributing to swimming performance at different times of the day. On the other hand, no conclusion on the role of the *PER3* VNTR genotype could be drawn in this study given the very small sample size of the three genotype groups and the weak trend for diurnal variation to be related to genotype, thus further research in this area is required.

The findings of this study are in line with the findings reported in Chapter 2 where the male and female runners were significantly skewed towards early morning preference despite *PER3* VNTR

genotype. The fact that differences in circadian rhythms, in terms of chronotype, *PER3* VNTR genotype and habitual training time-of-day, may contribute to diurnal variation in performance is an imperative finding as this knowledge may enable coaches and athletes to improve an individual's performance at various times of the day, by focusing on these components of circadian rhythm.

The fourth finding of this study was that there was no diurnal variation in RPE after both the warm-up and immediately after the 200 m time-trial for this group of masters swimmers. Additionally, when the swimmers were grouped according to chronotype, *PER3* VNTR genotype and habitual training time-of-day there were still no differences in RPE after the 200 m time-trial between the morning and evening sessions. This finding is in line with various other studies that have also reported non-significant differences in RPE when comparing time-trial and maximal performance in the morning to that in the evening (Deschenes *et al.,* 1998; Dimitriou *et al.,* 2002; Arnett, 2002; Kline *et al.,* 2007). This was not a surprising finding since it was anticipated that RPE post-time-trial should be maximal, given that the time trial was an all out sprint and not a graded exercise protocol. Thus one would assume a maximal perception of effort in both the morning and evening. This is in agreement with the findings of Hill *et al*. (1989) who showed that there was a time-of-day effect for RPE during cycling intensities above the ventilatory threshold; however this effect did not persist into maximal exercise.

On the other hand, a significant diurnal variation in RPE after the warm-up, which was essentially sub-maximal exercise, was observed when habitual training time-of-day was taken into account (Figure 21G). More specifically, RPE post-warm-up was 9% lower at 06h30 for the swimmers who trained in the morning and 6.5% lower at 18h30 for the swimmers who trained in the evening. Since, 54% of the swimmers in this group trained in the morning and 46% in the evening, conditioning to a particular training time-of-day may have resulted in the swimmers perceiving either the 06h30 or 18h30 warm-up as "easier". This is supported by the large effect sizes in RPE post warm-up, evident in both of the training groups. However, the diurnal variation in RPE accounting for training time-of-day seems to have been caused by the female swimmers because this diurnal variation was only observed in the females and not in the males. This was an odd finding due to the small proportion of female to male swimmers and the fact that there was no gender difference in training time-of-day distribution (Table IX).

Of the studies that have measured diurnal variation in RPE during sub-maximal exercise some have shown that there is a diurnal variation (Martin *et al.,* 2001) and others that there is no diurnal variation (Arnett, 2002; Martin *et al.,* 2007). For example, Martin *et al.* (2001) assessed physiological responses to submaximal running in males and observed that RPE was significantly higher during the

morning session. In contrast, Arnett (2002) showed that there were no significant differences in RPE in response to a submaximal morning and evening training protocol within a group of male and female swimmers. Martin *et al.* (2007) took training time-of-day into account by comparing a squad that trained in the morning and evening to a squad that trained in the evening only and reported that RPE was similar during morning and evening time-trials for the two squads. However, as previously mentioned, the latter study design was faulty and the findings should be interpreted with caution. Nevertheless, what these studies have not considered is chronotype, *PER3* VNTR genotype or habitual training time-of-day, with the exception of the latter study. This study is therefore unique in that it compares a group of swimmers who trained exclusively in the mornings to a group who trained in the evenings. Therefore, due to the fact that there is minimal literature that has focussed on diurnal variation in RPE with respect to habitual training time-of-day, the findings of this study are important and need to be investigated further in the future.

The fifth finding of this study was that the swimmers as a whole had higher states of total mood disturbance, depression, anger and tension before the 18h30 time trial (Figure 22). This is in line with the findings of Dimitriou *et al.* (2002) who also reported the total mood disturbance was lower in the morning in 14 male swimmers, before a submaximal performance protocol. This is an interesting finding as performance has been shown to peak in the evening (Cappaert*,* 1999; Drust *et al.,* 2005), thus one would expect mood state to be more "stable" during the evening hours.

However, since 58% of the swimmers in the current study were morning-type individuals, perhaps chronotype skewed the mood distribution of this group, resulting in the greater disturbance at 18h30. This seems feasible because when the mood state of the swimmers was assessed according to chronotype, the MT swimmers had a lower state of fatigue and a higher vigour at 06h30 compared to the NT swimmers (Table XII), the time at which they performed better. Similarly, Kerkhof (1998) reported that there was a diurnal variation in mood disturbance between morning and evening-types, with the evening types showing a diurnal increase in mood and alertness. This intuitively makes sense since the behaviour of morning-types is such that they tend go to bed earlier and prefer waking up earlier in the mornings (Vink *et al.,* 2001; Cavallera and Giudici, 2008). Thus, chronotype does seem to have on influence on mood state at different times of the day, which could ultimately have an influence on an individual's performance at certain times of the day.

With regard to gender, the male swimmers grouped by chronotype, displayed a difference in fatigue before the morning and evening time-trials, with the morning-types having a lower fatigue prior to the morning time-trial (Table XII). Sleep and wake times have been shown to be related to chronotype (Vink *et al.,* 2001; Roenneberg and Merrow, 2003) and *PER3* VNTR genotype (Lázar *et al.,*

2012). For example, it has been demonstrated that MT individuals and individuals homozygous for the *PER3*^{5} allele, have earlier awakening and bed-time times (Lázar *et al.,* 2012). Perhaps the difference in fatigue with regard to chronotype was due to the amount of sleep prior to the two time-trials when comparing the morning- and neither-types. It is interesting to observe that this same observation was not present in the female swimmers, who were skewed towards a greater morning-preference (Adan and Natale, 2002; Randler*,* 2007), since one would expect chronotype in females to have a significant effect on mood state when comparing morning to evening. However, again, the small sample size of the females most probably resulted in the observed lack of significance. Additionally, the current study demonstrated that there were time-of-day effects when comparing sleep-time prior to the morning and evening time-trials with regard to chronotype, *PER3* VNTR genotype and habitual training time-of-day; which relates to the findings reported by Lázar *et al.* 2012.

In addition, the current study observed that habitual training time-of-day was also related to mood state at different times of the day. The swimmers who habitually trained in the morning had a higher state of vigour at 06h30 compared to those swimmers who trained in the evening (Table XII). This could be due to conditioning and the fact that these swimmers are "used to" swimming in the early mornings whereas the swimmers who habitually train in the evenings are not familiar with early mornings. The fact that this observation was gender specific was interesting because a similar trend was observed for the females however the small sample size of the female swimmers group most likely limited the analysis. Therefore, since the mood state of swimmers was affected by chronotype and habitual training time-of-day at different times of the day, interventions could be put in place in order to ensure that mood state does not potentially impact negatively on performance or training.

Interestingly, in this study there was no correlation between swimming time-trial performance in the morning and evening and any of the mood state variables. In contrast, a study conducted on professional male basketball players showed that with a decrease in performance, there was a decrease in vigour and an increase in anger (Hoffman *et al.,* 1999). Similarly, Kline *et al.* (2007) reported that faster 200 m swimming performance times were associated with decreased levels of TMD, anger, fatigue and confusion and an increased vigour. Similarly, Hill and Smith (1991) noted that intra-individual differences in mood state were related to performance, with positive mood states being associated with increased anaerobic power and capacity. Lastly, Atkinson and Reilly (1996) mentioned that mood states may alter an individual's predisposition for performance. However, since no correlation was reported between mood state and performance in the current study, this raised an interesting thought. Perhaps chronotype and training time-of-day are important

factors to consider in understanding diurnal variation in performance. For example, it appears that POMS scores may reflect or confirm "conditioning" in this study. Thus, the monitoring of mood states could be useful in understanding chronotype-related inter-individual differences in athletes.

The last noteworthy finding of this study was that, despite there being no diurnal variation in stroke count (SC) when the swimmers were looked at as a whole, there was a diurnal variation in SC when swimmers were grouped by training time-of-day (Figure 23). That is those who trained in the morning had a higher SC in the morning and those who trained in the evening had a higher stroke count at 18h30, with the effect size being large for both training groups. What is interesting to note is that this observation only held true for the male swimmers, and not the females. However, this was probably due to the fact that data was only available for seven females, therefore the small sample size probably resulted in a lack of significance. Stroke count is a quick and efficient method commonly used to monitor stroke parameters, training intensity and critical speed (Barden and Kell, 2009). Additionally, stroke count has been shown to increase as swimming speed increases (Pyne *et al.,* 2001). Therefore, the fact that stroke count is altered with regard to training time-of-day supports a diurnal difference in performance.

The findings from other studies concur that in general stroke count does not show diurnal variation. For example, Martin and Thompson (2000) reported that there was no diurnal variation in stroke rate during a sub-maximal swimming protocol. Similarly, Zochowski *et al.* (2007) also reported that there were no differences in stroke count in 200 m swimming performance at different times of the day. In addition, Martin *et al.* (2007) reported that there was no intra-daily variation in stroke rate and stroke count during time-trial performance, for both the squad that trained in the mornings and evenings and the squad that trained in the evenings only. Thus, Martin *et al.* (2007) observed that habitual training time-of-day had a minimal effect on intradaily variation. However, as mentioned above, the findings of the latter study should be interpreted with caution due to the nature of their study design.

On the other hand, our results show that training-time of day is an important factor to consider. Martin *et al*. (2007) did, however, note that there was an interaction between the time-of-day and the training group (morning and evening training group versus evening only training group) for stroke rate, with the group that trained in both the mornings and evenings having a higher stroke rate during the evening time-trial (Martin *et al.,* 2007). Therefore, the fact that a significant diurnal variation in stroke count was shown when the swimmers were grouped according to habitual training time-of-day essentially indicates that stroke count is more efficient during the time at which training is routinely undertaken. Based on these findings, it appears that stroke count is influenced

or altered by temporal specificity to habitual training time-of-day. Future research is required regarding temporal effects on stroke mechanics. However, it is important to note that the measurement of stroke parameters is insensitive and changes in stroke count and stroke rate are exceptionally subtle even when slight improvements in performance occur (Martin *et al.,* 2007). Lastly, the lack of any observed diurnal variation in breath count may be due to the fact that the 200 m time-trial was a maximal sprint and thus any differences in breath count would be difficult to distinguish.

The fact that gender had an effect on performance and other variables, when the swimmers were categorised according to chronotype and habitual training time-of-day, highlights the fact that this finding needs to be confirmed with a larger and equal sample size of both males and females. Since study 1 showed that the distributions of chronotype and *PER3* VNTR genotype were significantly different for male and female runners, a larger sample size comparing male and female swimmers may result in agreement with these findings.

Finally, this study was unique for a number of reasons. Firstly, only a handful of studies (Hill *et al.,* 1988; Burgoon *et al.,* 1992; Atkinson *et al.,* 2005; Brown *et al.,* 2008) and two swimming-specific studies (Martin *et al.,* 2007; Kline *et al.,* 2007) have incorporated chronotype into the assessment of performance at different times of the day. However, in the two swimming studies, chronotype was not analysed with respect to swimming performance or time-of-day but merely reported by Martin *et al.* (2007) and used as a measure to exclude extreme chronotypes by Kline *et al.* (2007). Secondly, to the best of my knowledge, no studies have reported *PER3* VNTR genotype with regard to sports performance at different times of the day, and especially no studies focussing on swimming performance. Since there is a known association between chronotype and *PER3* VNTR genotype (Ebisawa *et al.,* 2001; Archer *et al.,* 2003; Kunorozva *et al.,* 2012), it is important to analyse swimming, or any sport for that matter, at different times of the day taking into account chronotype and *PER3* VNTR genotype. Lastly, masters swimmers were chosen because they are over the age of 25 years, which thus minimises the influence of age on chronotype. In contrast, other studies that have assessed diurnal variation in swimming performance have all used participants that had mean ages ranging from 15 to 22 years and were mostly college or university students (Baxter and Reily, 1983; Martin and Thompson, 2000; Arnett, 2001; Deschodt and Arsac, 2004; Kline *et al.,* 2007; Martin *et al.,* 2007). Therefore, since it is known that adolescents are more evening-oriented (as discussed in the literature review, section 1.3.3), it is possible that a greater preference for evenings could potentially be contributing to the diurnal variation reported by the latter studies.

Limitations

A limitation to this study is the relatively small sample size, which made within sub-group comparisons difficult. Secondly, both male and female swimmers were recruited for the study which resulted in large standard deviations in performance, as the female swimmers were slower than the male swimmers. In addition, the relatively small sample size precluded single-gender comparisons of the effects of chronotype, genotype and training time-of-day. However, other similar studies analysing diurnal variation in swimming performance have also recruited both male and female swimmers (Arnett, 2002; Kline *et al.,* 2007). One of the probable complications in studying females is the potential effect of their menstrual cycle on performance (Lebrun *et al.,* 1995; Frankovich and Lebrun, 2000). The menstrual cycle phase of the female swimmers in this study was recorded, and none were menstruating when they swam the 200 m time-trials. Another limitation regarding the current study is the possible influence that the menstrual cycle phase may have had on chronotype, as this could potentially have had an impact on the interpretation of the female data. Although menstrual cycle phase was not precisely controlled, time-trial performance was not assessed during menstruation for any of the female participants, as mentioned above. Nevertheless, studies have shown that a preference for "eveningness" has been associated with menstrual pain (Takeuchi *et al.,* 2005) and longer menstrual cycles than in other chronotypes (Toffol *et al.,* 2013). This could ultimately have an impact on performance and should be addressed in future studies involving female participants. Thirdly, data collection spanned over 12 months, from April 2012 – April 2013, thus seasonal variations in light and temperature, known to influence chronotype, may have affected the results. Fourth, the swimmers did not perform a familiarisation 200 m time-trial since the distance and 25m pool where standard components of their regular training and competition schedules. Nevertheless, the order in which the swimmers performed the morning and evening time-trials was randomised and counter-balanced, and there was no order effect of the time-trials. In the future it may be beneficial to analyse swimming performance, with regard to chonotype, *PER3* VNTR genotype and habitual training time-of-day, in a homogenous swimming squad rather than analysing swimmers who have varying training loads and schedules.

3.5 SUMMARY AND CONCLUSIONS

In summary, the main findings of this study were:

(i) When the swimmers were analysed as a single group, no diurnal variation in performance was observed.

(ii) Diurnal variation in performance was observed when the swimmers were grouped by chronotype. i.e. the majority of morning-type individuals swam faster in the morning time-trial and similarly, the majority of neither-type individuals were faster in the evening time-trial.

(iii) Habitual training time-of-day also had a significant influence on diurnal variation in performance. Swimming performance was generally being better at the time-of-day during which training habitually took place (i.e. morning vs. evening).

(iv) Rating of perceived exertion after the warm-up was lower at the time-of-day at which the swimmers habitually trained (i.e. morning vs. evening).

(v) Lower fatigue and higher vigour levels were observed during the morning time-trial for both the morning-types and the swimmers who habitually trained in the morning. Similarly, the neither-types and the swimmers who habitually trained in the evening had lower fatigue and higher vigour levels during the evening time-trial.

(vi) Individuals who trained in the morning tended to have a higher stroke count in the morning time-trial.

The fact that there was no diurnal variation in performance with regard to this group of masters swimmers is at odds with the "traditional" research regarding circadian rhythm and sport performance in that "performance is best in the afternoon/ early evening". This observation is key and interesting as it adds one more nail to the coffin for the "blanket" analysis of athletes, without taking into account their inter-individual differences. The findings of this study reiterate the importance of taking inter-individual differences in circadian rhythmicity into account in order to understand and interpret performance, as well as to approaching training.

In conclusion, these data suggest that chronotype and habitual training time-of-day are two key factors to consider when assessing swimming performance at different times of the day. Both chronotype and habitual training time-of-day significantly contributed to intra-daily variation in performance, which could ultimately affect swimmers both negatively and positively. *PER3* VNTR genotype did show a weak trend towards diurnal variation in performance, thus future research of a

larger sample size is required in order to confirm these findings. Other variables such as RPE and mood state also vary at different times of the day with regard to chronotype and habitual training time-of-day. This finding could be imperative for coaches and translated into practice when planning training sessions and competitions.

CHAPTER 4: SUMMARY AND CONCLUSIONS

4.1 OVERVIEW

There is minimal descriptive data available regarding the relationship between physical activity level and chronotype. Moreover, only one published study has analysed the relationship between activity level and *PER3* VNTR genotype, albeit in a South African population. Additionally, the literature regarding the distributions of *PER3* VNTR genotype and chronotype in females is somewhat scanty, despite a relatively large volume of published research regarding *PER3* VNTR genotype in various populations. Therefore, the main aim of Chapter 2 was two-fold. The first aim was to examine the relationship between activity level and *PER3* VNTR genotype and chronotype, in South African populations of active and inactive Caucasian males. The second aim of the study was to describe the chronotype and *PER3* VNTR genotype distributions within a South African population of active Caucasian females and to compare this distribution to that of the former active male population. Therefore Chapter 2 was designed to answer two research questions: 1) Are the chronotype and *PER3* VNTR genotype distributions and relationships similar in South African physically active compared to low physical activity individuals? and 2) Are the chronotype and *PER3* VNTR genotype distributions and relationships similar in South African male and female runners?

The majority of the data concerning diurnal variation and performance has analysed various components of performance and shown that performances peaks in the evening concurring with the peak in core body temperature. Furthermore, the data regarding diurnal variation and time-trial performance has proven to be somewhat controversial. In line with this, is the fact that chronotype, or a preference for mornings or evenings, may have an impact on performance and more recently, so may habitual training time-of-day. Since there is a known association between chronotype and *PER3* VNTR genotype, we speculated that this too would contribute to or help explain diurnal variation in performance. Therefore the aim of Chapter 3 was to analyse diurnal variation in swimming performance and other variables by assessing time-trial performance within a swimming group according to chronotype, *PER3* VNTR genotype and habitual training time-of-day.

4.2 NOVEL FINDINGS OF THIS THESIS

In conlusion, in the South African runners that we studied we found that more of them were morning-types compared to the inactive males. This together with the data from Kunorozva *et al.* (2012) strongly suggests that (i) level of physical activity is related to chronotype and (ii) South Africans in general are more morning-type in nature. Secondly, and in line with the latter observation, it was apparent that *PER3* VNTR genotype distribution was also associated with activity level. In particular, the low physical activity individuals had similar numbers of people genotyped as $PER3^{44}$ and $PER3^{45}$, and interestingly only one individual was genotyped as $PER3^{55}$. On the other hand, the active individuals (male runners) had a higher frequency of the $PER3^{55}$ genotype than the inactive group (Figure 10A), and a statistically significant lower $PER3^{4}$ allele frequency (Figure 10B). The $PER3^{4}$ allele frequency in the runners analysed here (58%) was higher than the 42% observed in the South African runners in a previous study (Kunorozva *et al.,* 2012), and likely related to the fact that there was no chronotype-genotype association within these male runners. On further investigation it was revealed that these runners were largely recreational. Therefore, conditioning to early mornings, due to the nature of the sport of running, most probably "shifted" the chronotype of the runners towards morningness, thus resulting in a mis-match between chronotype and *PER3* VNTR genotype within this population. These findings were re-iterated when the runners were analysed with regard to their respective race disciplines (Fig 15), with the *PER3* VNTR genotype distribution of the more committed runners mimicking what was previously reported in an active male running population (Kunorozva *et al.,* 2012). The majority of the amateur or less committed runners were the "outliers"; carrying the $PER3^{4}$ or "eveningness" allele, despite being classified as morning-types.

Interestingly, the female runners had an even greater preference for mornings compared to the male runners. However, there was no hint of a relationship between chronotype and *PER3* VNTR genotype, within this active population of female runners, even when race discipline was considered in the analysis. This finding suggests that *PER3* VNTR genotype does not appear contribute to chronotype in females. Therefore, there must be other factors influencing the greater preference for mornings observed in females, one of which may be conditioning or a needs-driven habituation to morning-timed activities.

There was an absence of diurnal variation in swimming performance when this group of masters male and female swimmers was analysed (Chapter 3). However, further investigation revealed that a diurnal variation in swimming time-trial performance was largely dependent on one's chronotype as well as habitual training time-of-day. This was in line with the findings of Chapter 2, that chronotype of athletes,

and thus essentially conditioning which relates to habitual training time-of-day, appears to have a profound influence on individuals in comparison to other factors such as genotype. Furthermore, the fact that RPE and mood state as well as stroke count also showed a diurnal variation with regard to chronotype and habitual training time-of-day reiterates the latter finding. These findings demonstrate that individual differences in circadian rhythmicity help in explaining individual differences in performance at different times of the day. This could be imperative for coaches and athletes, even more so regarding swimming performance since heats are generally scheduled in the mornings and finals in the evening. Thus it is essential that swimmers adapt to performing in both mornings and evenings in order to ensure that outcomes are not negatively affected. On the other hand, due to the small sample size of the three genotype groups, no sound conclusion regarding the role of *PER3* VNTR genotype could be determined. However, the fact that there was a weak trend for diurnal variation to be related to genotype highlights the fact that this needs to be investigated further in the future. Nevertheless, in response to the question that Chapter 3 set out to answer, it is apparent that it is important to take individual differences in circadian rhythmicity in account when analysing performance of athletes at different times of the day.

4.3 FINAL REMARKS

In conclusion, both chronotype and *PER3* VNTR genotype appear to be related to the level of physical activity that individuals choose to undertake. This thesis also showed that there are sex differences in *PER3* VNTR genotype distribution and that *PER3* VNTR genotype does not contribute to morning preference in females, specifically active females. It is useful to examine inter-individual differences in circadian rhythmicity in order to explain and predict diurnal variation in performance, as these differences may have consequent effects on performance of individuals at different times of the day.

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Period3 Structural Variation : A Circadian Biomarker Associated with Breast Cancer in Young Women. *Cancer Epidemiology, Biomarkers and Prevention*. **14**(1), 268–270, 2005.

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APPENDICES

APPENDIX 1

CIRCADIAN RHYTHM, PHYSICAL ACTIVITY AND GENDER

PARTICIPANT INFORMATION SHEET

Dear Volunteer,

Thank you for agreeing to participate in the University of Cape Town's study entitled **Circadian Rhythm, Training Habits and Sports Performance (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, low physically active population of South African males
   (b) well-trained male runners and
   (c) well-trained female runners.
2. To genotype the same five populations for the variable number tandem repeat (VNTR) polymorphism within the *Per3* gene and other circadian clock associated genes.

Procedures

You will be asked to complete a General Questionnaire (personal details, medication / supplement use, physical activity history 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. Alternatively, a trained phlebotomist will take a 5 ml sample of blood from the ante-cubital vein in your forearm using the venipuncture technique. DNA will be extracted from your buccal or blood sample to establish your genotype for circadian clock associated genes. 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. The potential risks to you during blood collection are minimal and are related to the blood sample collection technique from the veins on your arm (ante-cubital veins). The risks are: infection, delayed healing,

blood clot (haematoma), physical pain, mental discomfort and injury to a nerve or a vessel. These risks are small and will be minimized by the use of staff that are trained to take blood samples (trained phlebotomists), use of sterile techniques and the use of disposable, single-use materials.

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 circadian clock associated genes, 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 2

CIRCADIAN RHYTHM, PHYSICAL ACTIVITY AND GENDER

INFORMED CONSENT
ALL participants to read and sign

I, the undersigned, have been fully informed about the University of Cape Town's study entitled **Circadian Rhythm, Training Habits and Sports Performance (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.
- Either a buccal sample will be obtained from the insides of my cheeks using sterile swabs or a blood sample will be obtained from an ante-cubital vein in my forearm by venipuncture. DNA will be extracted from these samples to establish my genotype for the VNTR polymorphism within the *Per3* gene, and other circadian clock associated genes.

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 circadian clock associated genes, 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 3

HORNE-OSTBERG Morning-Evening Personality QUESTIONNAIRE

Name:_____

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 .....☐

Based on: Horne JA and Ostberg O (1976) A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* **4**:97-110

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APPENDIX 3

HORNE-OSTBERG Morning-Evening Personality QUESTIONNAIRE

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 ☐

Based on: Horne JA and Ostberg O (1976) A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* **4**:97-110

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APPENDIX 3

HORNE-OSTBERG Morning-Evening Personality QUESTIONNAIRE

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 .....☐

Based on: Horne JA and Ostberg O (1976) A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* **4**:97-110

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APPENDIX 3

HORNE-OSTBERG Morning-Evening Personality QUESTIONNAIRE

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 .....☐

Based on: Horne JA and Ostberg O (1976) A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* **4**:97-110

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APPENDIX 4

CIRCADIAN RHYTHM, PHYSICAL ACTIVITY AND GENDER

GENERAL QUESTIONNAIRE

PERSONAL DETAILS

Name: _____

Surname: _____

Postal address: _____

_____ Code: _____

Email address: _____

Phone number: _____ Cell phone: _____

Date of birth: _____ Age: _____

Gender: _____

Ethnic group (only required and used for research purposes):

Black/African ☐ White ☐ Mixed Ancestry (coloured) ☐

Indian ☐ Asian ☐ Other ☐

Ancestry (Tribal or national background - E.g.: Xhosa, Dutch, Italian): _____

Dominant hand: Left ☐ Right ☐

Dominant leg: Left ☐ Right ☐

Height (cm): _____

Weight (kg): _____

MEDICATION AND SUPPLEMENT USE

What medication, if any, are you currently using?

Name of medication	Years taken

What dietary supplements / vitamins, if any, are you currently using?

Type of supplement	Name	Years taken

GENERAL SPORT PARTICIPATION

Do you **currently** participate in a sport/physical activity on 2 or more days per week?

Yes **No**

Since leaving school, have you ever participated in any type of sport or physical activity on 2 or more days per week? **Yes** **No**

If yes, type of activity/sport: _____ Number of years: _____

type of activity/sport: _____ Number of years: _____

APPENDIX 5

CIRCADIAN RHYTHM, PHYSICAL ACTIVITY AND GENDER

RUNNING TRAINING AND RACING HISTORY

RUNNING TRAINING HISTORY

For how many years have you been running? _____ Years

In the last **three months**:

How many days do you train per week? _____ Days/wk

How many hours do you train per week? _____ Hours/wk

What is your weekly training distance? _____ Km/wk

What time of the day do you train? _____

Is this your preferred / chosen time of the day to train? **Yes** **No**

If no: What time of the day would you choose to train? _____

Why are you unable to train at your chosen time? _____

RUNNING RACING HISTORY

For how many years have you been competing in running races? _____ Years

At what level of competition have you participated? _____

In the last **two years**:

How many races have you competed in? _____

What was your average race distance? _____ Km

Race times:

	Current time	Year	PB time	Year
Comrades				
Two Oceans				
Marathon				
Half-marathon				

APPENDIX 6

CIRCADIAN RHYTHM, TRAINING HABITS AND SWIMMING PERFORMANCE

PARTICIPANT INFORMATION SHEET

Dear Volunteer,

Thank you for agreeing to participate in the University of Cape Town's study entitled **Circadian Rhythm, Training Habits and Swimming Performance (Part 2)** 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

Circadian rhythms are the 24-hour cycles that almost all aspects of our beings display. Perhaps the most obvious is our sleep wake cycle. Other examples include core body temperature, release of hormones, concentration, reaction time, flexibility. Each one of these has a time of day when it is highest/fastest/best and a time of day when it is lowest/slowest/worse. For example body temperature is at its lowest in the early hours of the morning (just before you wake up), increases throughout the day to a peak in the later afternoon early evening, where after it decreases again. While these rhythms are driven by a group of genes known as our "clock" genes, they are also reset each day by external factors such as daylight, temperature and social cues. It is now understood that there are inter-individual differences in our rhythm lengths – some of us have rhythms just shorter than 24-hours, while others are closer to the 24-hour mark. This manifests as us being "larks" – morning people, or "owls" – evening people.

While it has been well established that athletic performance varies in a circadian rhythmic manner (we tend to perform best in the afternoon/evening when our body temperatures are highest), it seems that the actual peak in performance time may be slightly different for morning and evening types. Furthermore, competition and training times can be at any time of the day, and the extent to which circadian rhythm or morning- and evening-types impacts our ability to adapt to training or perform is not well understood. Questions remain such as "If I am to compete in the morning, but am an evening-type person – should I train in the morning or evening?"

Based on previous work in our laboratory, we have observed that in individual sports such as running, cycling and Ironman triathlon (all of which tend to have early morning race times), there are more morning-types than in the general population in South Africa. We would like to see whether the same is true for swimmers. Next, we are interested in how habitual training time-of-day impacts performance in both morning- and evening-type swimmers. To address this, we are going to determine whether training habitually at a certain time of day is able to shift peak performance from the afternoon/evening to perhaps the morning.

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

- To determine the proportion of morning- and evening-types in trained swimmers
- To compare the performance of trained swimmers who train habitually either in the morning or evening in a 200m time trial at either 06h30 or 18h30.

We anticipate that these studies will lead to a better understanding of inter-individual athletic performance. This, together with future studies investigating the role of circadian rhythm on training

efficacy, could improve management of athletes' training and health based on our understanding of the role of the internal molecular clock and chronobiological principles.

Procedures

We ask that you please keep your training, work, eating and sleep patterns and habits as close to normal as possible for the duration of the study.

Session 1 – Baseline testing and familiarisation

- The researcher will explain the study in detail to you, including the risks and benefits associated with participating. You will be free to ask any questions you may have relating to the study. Should you agree to participate, you will sign a consent form and be asked to complete two questionnaires.
- The first is a general questionnaire requiring basic personal information, medication / supplement use, swimming training and racing history.
- The second is the Horne-Östberg Morningness/Eveningness personality questionnaire. This is a tool commonly used to assess our "chronotype" – i.e. personal preference for morning or evening activity.
- The investigator will also measure your height, weight and arm span.
- A trained phlebotomist will take a 5 ml (teaspoon) sample of blood from a vein in the crook of your arm. Alternatively, a buccal swab from the inside of your cheek will be taken using a sterile swab. This will be used to extract your DNA (genetic material) so that we can determine any variations you might have in the "clock" genes that control your body's 24-hour rhythms.
- You will then be taken through the testing procedures that will take place in the next two testing sessions.
- At the end of this first session the investigator will give you a digital oral thermometer to measure your core body temperature during the 24-hour period prior to sessions 2 and 3. This is important as the cycle of body temperature differs for morning- and evening-types and is thought to affect performance. In addition to allowing us to confirm these differences, it will also allow us to see where on your 24-hour temperature curve you are when you perform the time trial.
- Lastly, you will be given a diary, in which you will be asked to log your training, sleep, food/liquid intake and body temperature during the 24-hour period prior to sessions 2 and 3.

Prior to sessions 2 and 3

24 hours prior to these sessions:

- Record hourly oral body temperature, while you are awake in the diary provided.
- Log training, sleep and food/drink intake.

12 hours prior to these sessions:

- Refrain from training/racing.

8.5 hours prior to the 06h30 time trial:

- Fast – i.e. please don't consume food or liquid (other than water) after 22h00 the night before the time trial.

4 hours prior to the 18h30 time trial:

- Fast – i.e. please don't consume food or liquid (other than water) after 14h30 that afternoon.

Sessions 2 and 3 – 06h30 and 18h30 200m time trials

- These sessions will take place on two separate days beginning at either 06h00 or 18h00 and will be at least three days apart.

- While the order in which you perform these trials will be randomised, the procedures will remain identical.
- At the start of this session you will be weighed and asked to eat a standardised snack 30 minutes prior to the time trial.
- You will then complete the Profile of Mood States (POMS) questionnaire, which is used simply to assess your mental and physical state prior to each time trial.
- Your oral body temperature will be recorded and you will then perform a standardised warm-up – a 1000m swim at recovery pace with four short sprints. At the end of the warm-up you will be asked to rate your perception of effort of the swim (RPE).
- After 5 minutes of rest you will perform a 200m time trial, which will be maximal effort. During the time trial your lap time, breathing rate and stroke rates will be recorded. At the finish you will be asked to give your RPE of the time trial.

Potential Risks
The completion of a questionnaire is not associated with any risk. The potential risks to participants of blood collection are minimal and are related to 1) blood sample collection technique, and 2) the volume of blood collected prior to racing and the potential risk of a decreased performance in a subsequent time trial. The potential risks associated with blood collection technique from the vein in the crook of your arm are: infection, delayed healing, haematoma, physical pain, mental discomfort and injury to a nerve or a vessel. These risks are small and will be minimized by the use of trained phlebotomists, use of sterile techniques and the use of disposable, single-use materials. The risk of decreased performance as a result of blood collection will be reduced by not subjecting any participant to the collection of a blood volume exceeding 5 ml prior to the first time trial.

Benefits
On completion of the study you will receive feedback relating to your personal preference for either mornings or evenings as established by the Horne-Östberg questionnaire, body composition, 24-hour variation in body temperature, time trial performance as well as the general results of the study.

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.

You will not be included unless you 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.

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. Your identity 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.

Contact
Please do not hesitate to contact us should you require any additional information. Our contact information is listed below.

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Fax: (021) 406 6441
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Investigators
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072 141 3143

APPENDIX 7

CIRCADIAN RHYTHM, TRAINING HABITS AND SWIMMING PERFORMANCE

CONSENT FORM

I, the undersigned, have been fully informed about the University of Cape Town's study entitled **Circadian Rhythm, Training Habits and Swimming Performance (Part 2)** 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 as well as a questionnaire to determine my preference for mornings or evenings. I understand that testing for this study will take place on three occassions, each separated by approximately 3 days, at the SSISA or OM pool. I understand that the following measurements / tests **may** be conducted on me during this study, as described in detail in the accompanying Participant Information document:

Session 1

- Body composition assessment – height, weight, arm span
- A blood sample will be obtained from a vein in the crook of my arm using sterile needles and holders or a buccal sample from the inside of my cheek.

24-hour period prior to sessions 2 and 3

- Log training, sleep, diet and hourly oral body temperature
- Fast from 22h00 the night before the 06h30 session and from 14h30 on the day of the 18h30 session
- Ingest a standardised snack 30 minutes prior to each time trial

Sessions 2 and 3 (06h30 and 18h30)

- Profile of mood states (POMS) questionnaire
- Standardised warm-up
- 200m time trial

I have been fully informed about the risks inherent in participation in this trial. I understand that my DNA will be extracted from my blood sample, that it will only be used to analyse variations within the genes known to be involved with the circadian clock, and that it will be destroyed on completion of this 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:	_____	_____	_____
	Full name	Signature	Date
Investigator:	_____	_____	_____
	Full name	Signature	Date
Witness:	_____	_____	_____
	Full name	Signature	Date

APPENDIX 8

CIRCADIAN RHYTHM, TRAINING HABITS AND SWIMMING PERFORMANCE

GENERAL QUESTIONNAIRE

PERSONAL DETAILS

Name: _____ Surname: _____

Email address: _____

Phone number: _____ Cell phone: _____

Date of birth: _____ Age: _____

Gender: _____ Occupation: _____

Ethnic group (only required and used for research purposes):

Black/African ☐ White ☐ Mixed Ancestry (coloured) ☐

Indian ☐ Asian ☐

Ancestry (Tribal or national background - E.g.: Xhosa, Dutch, Italian): _____

Height (cm): _____

Weight (kg): _____

Arm span (cm): _____

TRANSMERIDIAN TRAVEL

Have you travelled across more than two time-zones in the last 2 months? _____

SHIFT WORK

Have you engaged in any shift work in the past two months? If yes, please provide details:

_____

FEMALE SWIMMERS

For females:

Are you currently menstruating? _____

When was the first day of your last menstrual cycle? _____

MEDICATION AND SUPPLEMENT USE

What **medication**, if any, are you **currently** using?

Name of medication Years taken

_____

_____

What **medication,** have you used in the **past 6 months**, but are **no longer taking**?

Name of medication Years taken

What **supplements**, if any, are you **currently** using?

Name of supplement Years taken

SWIMMING TRAINING HISTORY

For how many **years** have you been swimming? _____ Years

At which **phase** of your training cycle are you currently in? _____

Have you sustained an **injury within the last 6 months** that has prevented you from racing or training? Yes ☐ No ☐

If yes, please describe the date you sustained the injury, the nature of the injury, the treatment you underwent and the time that you were unable to train/race.

Training load

Describe your pool training during the **last 8 weeks**:

Days per week: _____

Sessions per week: _____

Hours per week: _____

Distance per week: _____

Describe your pool training during the **last season**:

Days per week: _____

Sessions per week: _____

Hours per week: _____

Distance per week: _____

Training time-of-day

Use the following table to describe the **normal times of your pool training sessions** during the last **8 weeks**. The first row serves as an example. There are two rows for you should you have more than one session per day.

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
05h30	05h30	05h30	18h30	05h30	07h00	

Use the following table to describe the normal times of your pool training sessions during the **last season**.

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday

If you could chose, at what **time** of the day would you train? _____

Have you participated in any other sport? Yes ☐ No ☐

If yes, which sport, when and for how long? _____

SWIMMING RACING HISTORY

At what level of competition do you **currently** participate? _____

What has been the highest level of competition that you have participated in? _____

Which events do you usually compete in? _____

_____

Describe your racing during the **last 8 weeks**:

Number of races: _____

Average race distance: _____

Current 400m freestyle race time: _____

Current 200m freestyle race time: _____

Describe your personal best times for the following:

400m freestyle race time: _____

200m freestyle race time: _____

APPENDIX 9

CIRCADIAN RHYTHM, TRAINING HABITS AND SWIMMING PERFORMANCE

TRAINING, SLEEP, TEMPERATURE AND FOOD DIARY

ALL participants to complete diary 24 hours prior to each swimming time trial

Name: _____ **Time-trial time:** 06h30 or 18h30

Diary start time and date: _____ (eg: 06h30, Tue 13 Mar)

Training

Session type: _____ Session type: _____

Time of day: _____ Time of day: _____

Duration: _____ (min) Duration: _____ (min)

Distance: _____ (km) Distance: _____ (km)

Intensity: _____ (1-10) Intensity: _____ (1-10)

(1 = very light; 10 = extremely hard)

Sleep

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

What time did you wake up? _____ (AM)

Body temperature (ºC)

05h00 _____	13h00 _____	21h00 _____
06h00 _____	14h00 _____	22h00 _____
07h00 _____	15h00 _____	23h00 _____
08h00 _____	16h00 _____	24h00 _____
09h00 _____	17h00 _____	01h00 _____
10h00 _____	18h00 _____	02h00 _____
11h00 _____	19h00 _____	03h00 _____
12h00 _____	20h00 _____	04h00 _____

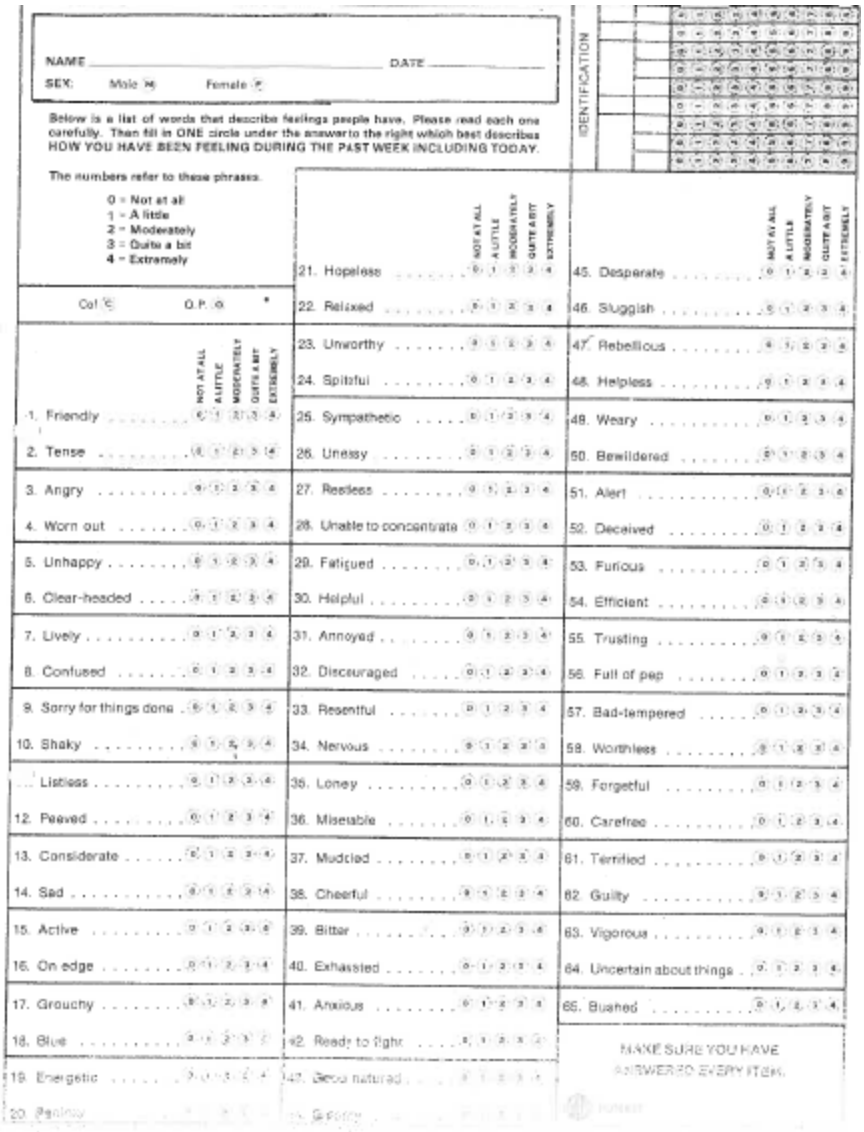
Diet

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 10

POMS QUESTIONNAIRE



NAME _____ DATE _____

SEX: Male (M) Female (F)

IDENTIFICATION

Below is a list of words that describe feelings people have. Please read each one carefully. Then fill in ONE circle under the answer to the right which best describes HOW YOU HAVE BEEN FEELING DURING THE PAST WEEK INCLUDING TODAY.

The numbers refer to these phrases:

0 = Not at all
1 = A little
2 = Moderately
3 = Quite a bit
4 = Extremely

Col (C) O.P. (O) *

	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
1. Friendly	0	1	2	3	4
2. Tense	0	1	2	3	4
3. Angry	0	1	2	3	4
4. Worn out	0	1	2	3	4
5. Unhappy	0	1	2	3	4
6. Clear-headed	0	1	2	3	4
7. Lively	0	1	2	3	4
8. Confused	0	1	2	3	4
9. Sorry for things done	0	1	2	3	4
10. Shaky	0	1	2	3	4
11. Listless	0	1	2	3	4
12. Peeved	0	1	2	3	4
13. Considerate	0	1	2	3	4
14. Sad	0	1	2	3	4
15. Active	0	1	2	3	4
16. On edge	0	1	2	3	4
17. Grouchy	0	1	2	3	4
18. Blue	0	1	2	3	4
19. Energetic	0	1	2	3	4
20. Panicky	0	1	2	3	4
21. Hopeless	0	1	2	3	4
22. Relaxed	0	1	2	3	4
23. Unworthy	0	1	2	3	4
24. Spiteful	0	1	2	3	4
25. Sympathetic	0	1	2	3	4
26. Uneasy	0	1	2	3	4
27. Restless	0	1	2	3	4
28. Unable to concentrate	0	1	2	3	4
29. Fatigued	0	1	2	3	4
30. Helpful	0	1	2	3	4
31. Annoyed	0	1	2	3	4
32. Discouraged	0	1	2	3	4
33. Resentful	0	1	2	3	4
34. Nervous	0	1	2	3	4
35. Lonely	0	1	2	3	4
36. Miserable	0	1	2	3	4
37. Muddled	0	1	2	3	4
38. Cheerful	0	1	2	3	4
39. Bitter	0	1	2	3	4
40. Exhausted	0	1	2	3	4
41. Anxious	0	1	2	3	4
42. Ready to fight	0	1	2	3	4
43. Good natured	0	1	2	3	4
44. Gloomy	0	1	2	3	4
45. Desperate	0	1	2	3	4
46. Sluggish	0	1	2	3	4
47. Rebellious	0	1	2	3	4
48. Helpless	0	1	2	3	4
49. Weary	0	1	2	3	4
50. Bewildered	0	1	2	3	4
51. Alert	0	1	2	3	4
52. Deceived	0	1	2	3	4
53. Furious	0	1	2	3	4
54. Efficient	0	1	2	3	4
55. Trusting	0	1	2	3	4
56. Full of pep	0	1	2	3	4
57. Bad-tempered	0	1	2	3	4
58. Worthless	0	1	2	3	4
59. Forgetful	0	1	2	3	4
60. Carefree	0	1	2	3	4
61. Terrified	0	1	2	3	4
62. Guilty	0	1	2	3	4
63. Vigorous	0	1	2	3	4
64. Uncertain about things	0	1	2	3	4
65. Bushed	0	1	2	3	4

MAKE SURE YOU HAVE ANSWERED EVERY ITEM.

APPENDIX 11

CIRCADIAN RHYTHM, TRAINING HABITS AND SWIMMING PERFORMANCE

DATA COLLECTION SHEET

DATE: _____ POOL: _____

Air Temp: _____ Humidity: _____ Pool Temp: _____

.....

NAME: _____ CODE: _____

SESSION (2 or 3): _____ AM: ☐ PM: ☐

24 hour diary: ☐

POMS: ☐

.....

BODY WEIGHT (kg): _____

BODY TEMP (°C): _____

PRE- TT Meal: ☐ Amount: _____ Time taken: _____

Warm-up: ☐ Distance: _____ Time: _____ RPE: _____

Rest: ☐ Time: _____

.....

TIME TRIAL:

Lane number: ☐

Start time: _____

Time 1: _____

Time 2: _____

Video analysis: _____

Splits

50m	100m	150m
.....	.....	.....

RPE POST- TT: _____

APPENDIX 12

CIRCADIAN RHYTHM, TRAINING HABITS AND SWIMMING PERFORMANCE

RPE SCALE

BORG'S RPE SCALE INSTRUCTIONS

While exercising we want you to rate your perception of exertion, i.e., how heavy and strenuous the exercise feels to you. The perception of exertion depends mainly on the strain and fatigue in your muscles and on your feeling of breathlessness or aches in the chest.

Look at this rating scale; we want you to use this scale from 6 to 20, where 6 means "no exertion at all" and 20 means "maximal exertion".

9	corresponds to "very light" exercise. For a normal, healthy person it is like walking slowly at his or her own pace for some minutes.
13	on the scale is "somewhat hard" exercise, but it still feels OK to continue.
17	"very hard" is very strenuous. A healthy person can still go on, but he or she really has to push him- or herself. It feels very heavy, and the person is very tired.
19	on the scale is an extremely strenuous exercise level. For most people this is the most strenuous exercise they have ever experienced.

Try to appraise your feeling of exertion as honestly as possible, without thinking about what the actual physical load is. Don't underestimate it, but don't overestimate it either. It's your own feeling of effort and exertion that's important, not how it compares to other people's. What other people think is not important either.

Look at the scale and the expressions and then give a number

BORG'S RPE SCALE

6	No exertion at all
7	
	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard (heavy)
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion