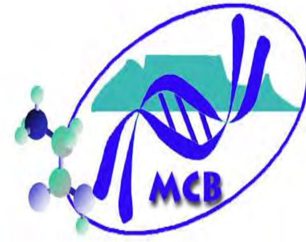

Chronotype in the South African population: the influence of longitudinal location

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Abstract

Most human beings experience the pull of three different daily timers, the solar clock, their endogenous circadian clock and the societal clock. Solar time is generated by the Earth's revolution on its axis, resulting in its surface being alternately exposed to and shielded from the sun every 24 hours. The endogenous clock, or circadian oscillator, is driven by a network of transcriptional-translational feedback loops, and has a period of close to 24 hours. The circadian oscillator is synchronised to the 24 hour light-dark cycle of the solar clock. The third timer is the standardised societal clock that organises and schedules work, school, transport, appointments and free time in a 24 hour period. The way an individual's endogenous clock synchronises to the solar clock, through advances or delays relative to sunrise and sunset, results in a phenomenon known as diurnal preference or chronotype. A person may have a morning-chronotype, where they enjoy rising and being active early in the day, an evening-chronotype where they prefer to be active later in the day into the late night, retiring in the early morning hours, or have no strong preference for early or late rising. This renders it easy for some to cope with the demands of the societal clock and others to struggle. Chronotype has both genetic and environmental influences. As society's schedule is governed by the standardised clock, it was hypothesised that chronotype may be influenced by one's longitudinal location within a time zone. South Africa presents an interesting case because although it uses just one time zone, in the most Easterly regions of the country, the sun rises and sets up to an hour earlier than in the most Westerly regions throughout the year. Sunrise times have an impact on the way the endogenous clock synchronises to the solar clock. It was hypothesised firstly, that South Africans living in the East of the country may have a greater preference for mornings (more morning-chronotypes) than those living in the West; and secondly, that this difference would not be due to genetic differences in the populations, particularly two gene polymorphisms previously shown to influence chronotype. Therefore the aims of this study were to describe and compare the distribution of chronotype in Eastern (n=222) and Western (n=205) sample populations with the use of a validated tool, the Horne–Östberg Morningness, Eveningness Questionnaire. Secondly to describe the genotype and allelic frequency distributions of the *PER2* single nucleotide polymorphism (SNP) G3853A (rs934945) in the Eastern (n= 184) and Western (n=186) populations, and the *PER3* variable number tandem repeat (VNTR) polymorphism in the Eastern (n=143) and Western (n=176) populations from buccal cell samples. There was a significantly higher proportion of morning-types in the Eastern population (60.6%) than in the Western population (40.5%) ($p<0.001$). Whereas there were higher proportions of neither-types and evening-types in the Western population (50.8% and 8.7% respectively) than in the Eastern population (35.1% and 4.3% respectively) ($p<0.001$). There were no significant differences in distribution of the *PER2* genotype (p=0.121) and allele frequencies (p=0.051) between the Eastern and Western populations nor in the *PER3* genotype (p=0.879) and allele (p=0.075) frequencies. Although previous studies have shown associations between chronotype and *PER2* G3853A and *PER3* VNTR genotypes, no significant associations were observed in either the Eastern (*PER2* p=0.769; *PER3* p=0.221) or the Western (*PER2* p=0.584; *PER3* p=0.733) populations. These findings indicate that, in South African populations, longitude influences chronotype independently of genotype. Factors that may contribute to this may be the difference in the rising times of the sun, which is exacerbated to some extent by the study areas being at dissimilar latitudes and thus experiencing slight differences in climate. The impact of the differences in chronotype but the maintenance of the same societal temporal organisation in the Eastern and Western regions were not assessed. However, they may be revealed by investigating certain general health indicators in such as quality of sleep and prevalence of depressive symptoms which are affected when there is incongruence between societal time and endogenous time.

List of Abbreviations

AMPK…………………	Adenosine monophosphate-activated protein kinase AMPK
ANOVA …………….....	Analysis of Variance
BA……………………..	Black ancestry
β-TRCP..…....……...	*Beta*-transducin repeats-containing proteins
BMAL1………………..	Brain and muscle aryl hydrocarbon receptor nuclear transporter-like 1 (NB :the convention used in this thesis is italics for nucleic acids)
bp……………………...	Base pairs
CBT…………………...	Core body temperature
CK1……………………	Casein kinase 1
CK1ε…………………..	Casein kinase 1 epsilon
CK1δ ………………....	Casein kinase 1 delta
CK2……………………	Casein kinase 2
CLOCK………………..	Circadian Locomotor Output Cycles Kaput
CRY…………………...	Cryptochrome
CSM…………………...	Composite scale of morningness
DNA…………………...	Deoxyribonucleic acid
DSPD………………....	Delayed sleep phase disorder
E.coli………………….	*Escherichia coli*
E………………………	East
EDTA………………....	Ethylendiaminetetraacetic acid
EEG. ………………….	Electroencephalographic/electroencephalogram
ET……………………..	Evening –types
FASPS………………...	Familial advanced sleep phase syndrome
GCP...………………….	Good Clinical Practice
gDNA………………....	Genomic Deoxyribonucleic acid
GSK-3…………........	Glycogen synthase kinase-3
h………………………	Hours
H-Ö MEQ…………..….	Horne-Östberg morningness-eveningness questionnaire
H-Ö score……………..	Horne-Östberg morningness-eveningness questionnaire score
IA ……………………..	Indian ancestry
ipRGCs………………..	intrinsically photosentsitive retinal ganglion cells
MA…………………….	Mixed ancestry
MAPK………………....	Mitogen-activated protein kinases
min…………………….	Minutes
MSFsc…………………	Midpoint of sleep on free days
MT…………………….	Morning-type
NLS……………………	Nuclear localisation signal
NPAS2………………...	Neural PAS domain-containing protein 2
NREM…………………	Non Rapid Eye Movement
NT……………………..	Neither-type
OA…………………….	Other ancestry
PAS……………………	Period ARNT Single-minded

PCR……………………	Polymerase chain reaction
PER……………………	Period
PP1……………………	Protein phosphatase 1
REM…………………...	Rapid Eye Movement
RNA……………………	Ribonucleic acid
RORE…………………	retinoic acid-related orphan receptor response elements
S………………………	South
SCF^{Fbxl3}……………..….	Skp1/cullin/F-box protein and leucine rich repeat protein 3
SCN…………………...	Suprachiasmatic Nuclei
SD…………………….	Standard deviation
SDS……………………	Sodium dodecyl sulphate
Sec…………………….	Seconds
SEM..…………………	Standard Error of the Mean
SIRT1……………….	Sirtuin 1
SNP……………………	Single nucleotide polymorphism
SUMO…………………	Small ubiquitin-related modifier protein
TAE…………………...	Tris acetate EDTA
β-TRCP..…....……...	Beta-transducin repeats-containing proteins
UTC…………………...	Universal coordinated time
v/v……………………..	volume per volume
VNTR…………………	Variable number tandem repeat
w/v…………………….	Weight per volume
WA……………………	White ancestry
yr ……………………...	Years

Table of Contents

Plagiarism Declaration……………………………………………………………………i

Acknowledgements……………………………………………………………………...ii

Abstract…………………………………………………………………………….......iii

List of Abbreviations…………………………………………………………….……..iv

List of Figures………………………………………………………………………....viii

List of Tables……………………………..……………………………………………..…x

Chapter 1………………………………………………………………………………...1

1.0 INTRODUCTION…………………………………….……………….…………….....1

1.1 The Circadian Oscillator……….……………………………………………………..3

1.2 Molecular Basis of the Circadian Oscillator…….……………………………………4

1.2.1 Posttranslational modifications…...……….……….……………………………5

1.3 Circadian rhythm, Sleep and the Sleep Homeostat….……………………………………8

1.3.1 Sleep disorders with a Circadian basis……………...…………………………11

1.3.1.1 Shift-work Sleep Disorder and Jet-Lag Disorder………...…………11

1.3.1.2 Familial Advanced Sleep Phase Syndrome……………...………….12

1.3.1.3 Delayed Sleep Phase Disorder…..…………………………………..13

1.3.14 Non-24h Sleep-Wake Syndrome………...……………………………..14

1.4 Chronotypes………...…………………………………………………………………15

1.4.1 Polymorphisms present in core clock gene associated with chronotype……...17

1.4.2 Factors that influence chronotype……………………………………...…22

1.5 Research Question and Study Aims…………………………………..……………….…24

Chapter 2………………………………………………………………………………….28

2.0 MATERIALS AND METHODS…………………………………………………………28

2.1 Participants………………………………………...…………………………………...28

2.2 Study design……………………………………………………………………………28

2.3 Detailed testing procedure……………………………………………………………..29

2.3.1 Horne- Östberg morningness-eveningness personality questionnaire………...29

2.3.2 Buccal Cell Samples…………………………………………………………...29

2.3.3 Genomic DNA extraction……………………………………………………...30

2.3.4 *PER2* Single nucleotide polymorphism genotyping…….……………………30

2.3.5 DNA Cloning and Transformation…………………………………………….32

2.3.6 *PER3* Variable number tandem repeat polymorphism genotyping……………33

2.4. Statistical Analyses……………………….…………………………………………...36

Chapter 3…………………………………………………………………………………37

3.0 RESULTS….……………………………………………………………………………..37

3.1 General Characteristics…………………………………………………………..37

3.2 Chronotype Distribution……………………...………………………………….39

3.3 *PER2* and *PER3* Genotype Frequency…………………………………………...42
3.4 *PER2* and *PER3* allele frequency distribution…………………………………...48
3.5 Relationships between *PER2* and *PER3* genotypes and chronotype…………….52

Chapter 4……………………………………………………………………………….58
4.0 DISCUSSION……………..…….……………………………………………………..58
4.1 Chronotype and the environment…….…………………………………………..58
4.2 Effect of age, sex and ethnicity on chronotype.……………………………….…61
4.3 Clock genes and ethnicity………………………………………………………..62
4.4 Clock genes and region…………………………………………………………..64
4.5 Genotype–phenotype relations…………………………………………………..65
4.6 Conclusion……………………………………………………………………….68
4.7 Limitations……………………………………………………………………….71

5.0 REFERENCES………………………………………….………………………..73

Appendix A
Appendix B
Appendix C
Appendix D

List of Figures

Figure 1.1. The molecular mechanism of the mammalian circadian oscillator……..…………..**8**

Figure 1.2. A schematic diagram of the interaction of the endogenous circadian oscillator “clock”, the sleep homeostat “homeostat” and the sleep-wake cycle…………………..……………………..…**11**

Figure 1.3. The mean rectal temperature in M-types (morning-types, n=7, bold line) and E-types (evening-types, n=7, thin line) was measured and recorded during constant routines in degrees Celsius.………………………………………………………………………………………...**17**

Figure 1.4. Map of Southern Africa showing longitudes and time zones from http://mapsof.net/map/africa-time-zones...……………………**24**

Figure 2.1. Amplification products of the *PER2* 137bp region containing the G3853A SNP:…..**31**

Figure 2.2. Restriction digestion of amplification products of the *PER2 137*bp region:..……….**32**

Figure 2.3. Amplification products of the *PER3* VNTR polymorphic region:………………..….**34**

Figure 2.4. Restriction digestion of the amplification products of the *PER3* VNTR polymorphic region:……………………….………………………………..…………………………**35**

Figure 3.1. Chronotype frequency distribution in the Eastern region and the Western Cape:…..…………………………………………………………………………………...**39**

Figure 3.2. The effect of sex on Chronotype:………..……………...…………………………...**40**

Figure 3.3. Relationship between age and Chronotype:.…………………….…...…………………..**41**

Figure 3.4. Mean chronotype in the individual ancestry groups:...............……..…………….…**42**

Figure 3.5. Genotype frequency distribution for *PER2* (A) and *PER3* (B) in the individual ancestry groups: ....………...………………………………………………………………..…**43**

Figure 3.6. Genotype frequency distribution for *PER2* (A) and *PER3* (B):…………………..….**45**

Figure 3.7. Genotype frequency distribution for *PER2* (A) and *PER3* (B) within the white ancestry group:.…………………….…………………………………………………….……..**46**

Figure 3.8. *PER2PER3* genotype frequency distribution:…………………………..………..…**47**

Figure 3.9. Allele frequency distribution for *PER2* (A) and *PER3* (B) in the individual ancestry groups:…………………………………………………………..……...……………….……**48**

Figure 3.10. Allele frequency distributions for *PER2* (A) and *PER3* (B):…….……….………..**50**

Figure 3.11. Allele frequency distribution for *PER2* (A) and *PER3* (B) within the white ancestry group:.……………………….………………………………………………………………..**51**

Figure 3.12. Chronotype frequency distribution in *PER2* (A) and *PER3* (B) genotypes:………………...…………………………...……………………………..…..………**53**

Figure 3.13. Relationship between *PER2* genotype and chronotype in the Eastern (A) and Western (B) populations:……………………..……...…………………………………………..……**54**

Figure 3.14. **Relationship between *PER3* genotype and chronotype in the Eastern (A) and Western (B) populations:**……………….……………..………………………………………………**55**

Figure 3.15. Relationship between *PER2* genotype and chronotype within the White ancestry group in the Eastern (A) and Western (B) populations:…....…………………………………..…**56**

Figure 3.16. Relationship between *PER3* genotype and chronotype within the White ancestry group in the Eastern (A) and Western (B) populations:……………….…………………………**57**

List of Tables

Table 2.1: Expected fragment sizes of the.*PER2* 137bp region after restriction digestion with *Bam*HI ……………………………………………………………………………………………….…...**32**

Table 2.2: Expected fragment sizes of the *PER3* VNTR polymorphic region containing the M1037T SNP after restriction digestion with *Nco1*………………………...………………...........................**35**

Table 3.1: General Characteristics of participants from the Eastern region and the Western Cape….**38**

Table 4.1: Sunrise and sunset times for Cape Town, Johannesburg and Pietermaritzburg at the summer and winter solstices………………………………………………………………………..**59**

Chapter 1

1.0 INTRODUCTION

As much as are we creatures of our own will, our lives are organised by three timers: the solar clock, a biological clock and a social clock (Roenneberg *et al*., 2003). The solar clock produces a twenty four hour light/dark cycle as a result of the earth's surface being periodically shielded from and exposed to the sun as it rotates on its axis (Roenneberg *et al*., 2007b).The biological clock is an autonomous endogenous oscillator that has a period of close to twenty-four hours (24h). It controls central aspects of biology and physiology from gene expression to complex behaviour and can be synchronised to the external environment by environmental stimuli (Wittmann *et al*., 2006; Roenneberg *et al*., 2003). One of the most important stimuli is light which entrains the biological clock to the 24h light/dark cycle i.e. the solar clock (Wittmann *et al*., 2006; Roenneberg *et al*., 2003). These two clocks when properly synchronised result in a harmonious internal environment in which there is optimal timing of physiological functions and the ability to adapt to and anticipate changes in the external environment (Wright *et al*., 2013). There is inter-individual variance in this synchronisation which gives rise to differences in preferred wake and sleep times (Horne and Östberg, 1976; Wittmann *et al*., 2006). The social clock is the temporal way in which society is organised this includes work and school schedules as well as social activities (Wittmann *et al*., 2006), although social activities are scheduled for the most part around school and work. The social clock can either complement the harmonious relationship between the biological and solar clocks or result in its alteration or interruption.

In the past, our daily exposure to light was determined by the rising and setting of the sun. However, all that changed in the 1930s, beginning with North American and European populations when electricity was used to supply them with bright indoor electric lighting (Wright *et al*., 2013). This enabled them to spend more time awake and be active indoors in an 'artificially' created light environment (Wright *et al*., 2013). This has since spread to the rest of the world, creating a common discrepancy between our social time and sun rise and sun set times (Roenneberg *et al*., 2007b). The social clock in many societies runs in such a way that school and/or work are scheduled early in the day (Wittman *et al*., 2006). Depending on how early in the day, an individual's endogenous clock may be unable to timeously anticipate and prepare for the change from rest to activity, particularly in individuals who prefer to sleep late and wake late, as a result of their endogenous clock. In this group of

people it would result in early awakening as demanded by the social clock and late sleep times as enforced by their biological clocks (Wittman *et al*., 2006). The cumulative effect of this over a period of a week results in sleep-debt which is generally compensated for over the weekend or other free days. This time of day difference in rest and activity between work days and free days is called 'social jetlag' (Wittman *et al*., 2006). In a similar way, individuals who prefer early sleep and wake times as a result of their biological clock experience this phenomenon when they stay up late for social or work reasons and are unable to wake late due to their internal predisposition (Wittman *et al*., 2006). Shift work schedules that result in late night exposure to unnatural light interrupt the timing of the endogenous clock because light is being received at a time when dark is anticipated which can have a more severe consequence than social jetlag. Despite not performing direct statistical analysis, Wittman *et al*. (2006), suggest that the correlation they described between lower sleep quality, higher stimulant and alcohol consumption, and preferred late sleep and wake times was as a result of social jetlag experienced by the group with these preferences. According to Steinhausen and Metzke, (1998) high stimulant and alcohol consumption are behavioural indicators of an inability to cope with social demands.

Before the 1930s, children went to school/work and adults went to work and may have engaged in shift work (e.g. working as watch men) but their level of light exposure after sunset was minimal as it was supplied by moon light, candles, kerosene lamps and campfires which do not produce as much light as electric bulbs. Wright *et al*. (2013) conducted a study where participants were exposed to two sets of environments for a period of a week. The first was typical of the dictates of the social clock in which they were exposed to a combination of natural and artificial light which surpassed the timing of the availability of natural light. This was followed by camping which involved exposure to only the natural light of the sun during the light period of the day and the light supplied by a camp fire and moonlight at night. They reported that in the former case the biological clock was delayed with regard to the solar clock, whereas under natural light conditions these two clocks were tightly synchronised. Czeisler *et al*. (1994) reported that the physiological markers of sleepiness such as low cognitive performance occur approximately 2 hours after habitual wake time. However, Wright *et al*. (2013) reported that when participants lived under natural light conditions only, the physiological markers of sleepiness (such as low cognitive performance) occurred a minimum of 50 min prior to wake up time. This would indicate that our social clocks which expose us to light past the setting of the sun result in low cognitive performance levels

occurring after the time of sleep offset. This is undesirable as this is typically the time at which people are at work or at school. This is just one of the outcomes that results in adverse effects when these three clocks are incongruent.

1.1 The circadian oscillator

The biological clock is also referred to as the circadian oscillator, and as earlier stated is synchronised to the solar clock by the major time-giver (*zeitgeber*) light (Roenneberg *et al*., 2003). There is a master oscillator housed in the Suprachiasmatic Nuclei (SCN) of the anterior hypothalamus (Gekakis *et al*., 1998; Jin *et al*., 1999; Reppert and Weaver, 2002) which is responsible for synchronising oscillators in all cells to the light environment (Reppert and Weaver, 2002; Duguay and Cermakian, 2009). The nuclei are organised in such a way that the entire clockwork machinery resides in each cell (Jin *et al*., 1999). They are self-sustaining and although these individual 'clocks' are separate, their interaction orders them so as to generate coordinated circadian outputs which govern the physiological and behavioural 24h rhythms observed (Jin *et al*., 1999). Photic signals enter the eye and reach the retinal rod and cone photoreceptors, as well as intrinsically photosensitive retinal ganglion cells (ipRGCs) which express melanopsin, a photopigment which makes them photo receptive (Altimus *et al*., 2010). Together they facilitate the transduction of photic signals via the retinohypothalamic tract to the SCN (Macchi and Bruce, 2004). This pathway is the main route through which synchronising information reaches the SCN (Reppert and Weaver, 2002). From the SCN the GABAergic input reaches the paraventricular nucleus. Efferents from the paraventricular nucleus go through the forebrain bundle and reticular formation and project to the intermediolateral column of the cervical spinal cord where preganglionic adrenergic fibres then transmit the input to the superior cervical ganglion from which postganglion adrenergic fibres reach the pineal gland (Macchi and Bruce, 2004). This gland produces melatonin which is a circadian fluctuating hormone, its production is limited to night time and, its synthesis is inhibited by light (Lucas *et al*., 1999). It therefore 'informs' the rest of the body which portion of the day it is i.e. day or night by its presence or absence.

1.2 Molecular basis of the circadian clock

The circadian system is responsible for ensuring all physiological and metabolic processes occur in an efficient and timely fashion. A hierarchy of oscillators manages and maintains the circadian systems in mammals. The SCN are at the top of this hierarchy (Ko and Takahashi, 2006). There are approximately 20,000 individual neurons that contain independent circadian oscillators and yet couple to form a robust oscillatory network (Mohawk *et al*., 2012). It has been reported that when timed individually each neuron is capable of exhibiting a circadian period ranging from 22h to as high as 30h (Mohawk *et al*., 2012). However, coupling among the individual nuclei results in the synchrony of the complete body of cells leading to a narrower range of circadian period and thus precision. When isolated and cultured the SCN are able to sustain daily rhythms for weeks (Berson, 2003). Other clocks have been reported in other parts of the brain such as the paraventric nucleus, arcuate nucleus, pineal gland, olfactory bulb and forebrain (Dardente and Cermakian, 2007). The peripheral or slave oscillators, despite their ability to generate oscillations independently, receive cues from the SCN which results in a harmonized rhythm throughout the organism (Ko and Takahashi, 2006; Dardente and Cermakian, 2007). They are present in peripheral organs/tissues such as the liver, heart, kidneys and muscle as well as almost every cell in the body (Dardente and Cermakian, 2007; Mohawk *et al*., 2012). Each oscillator has the ability to function autonomously without any external influences, however, environmental cues such as dark/light cycles, temperature rhythms, to mention a few are able to reset or entrain them (Dardente and Cermakian, 2007).

The oscillators present in the SCN and the peripheral organs are similar at molecular level and consist of a system of transcriptional-translational feedback loops. This system together creates a close to 24h expression pattern of core clock components (Ko and Takahashi, 2006). The genes responsible for the production of proteins that are necessary for generation and management of circadian rhythms within individual cells throughout the organism are referred to as the core clock genes (Ko and Takahashi, 2006).

The system has a primary feedback loop with positive and negative elements. The positive elements are transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK; which has a paralogue, NPAS2 in the SCN) (Mohawk *et al*., 2012) and brain and muscle aryl hydrocarbon receptor nuclear transporter-like 1 (BMAL1). CLOCK and BMAL1 are part of the basic helix-loop-helix-Period-ARNT Single-minded (PAS) transcription factor family,

and their DNA binding and dimerization is mediated through their PAS domains (Ko and Takahashi, 2006; Dardente and Cermakian, 2007; Huang *et al*., 2012). They form a heterodimer and initiate transcription of target genes containing E-box *cis* regulatory enhancer sequences (Gekakis *et al*., 1998; Hogenesch *et al*., 1998). These include *PERIOD* (*PER*) genes *PER1*, and *PER2*, as well as *CRYPTOCHROME* (*CRY*) genes *CRY1* and *CRY2*, which are core clock components (Mohawk *et al*., 2012). Their protein products PER and CRY heterodimerize within the cytoplasm and repress their own transcription by their action on the CLOCK:BMAL1 complex resulting in negative feedback (Ko and Takahashi, 2006; Dardente and Cermakian, 2007).

CLOCK:BMAL1 are part of a secondary regulatory loop in which they initiate transcription of retinoic acid-related orphan nuclear receptors, REVERBα and RORα. Their protein products competitively bind to the *BMAL1* promoter. Specifically to retinoic acid-related orphan receptor response elements (*RORE*), with *ROR* (α, β and γ) activating transcription of *BMAL1* and *REV-ERB* (α and β) repressing it (Ko and Takahashi, 2006). Thus *BMAL1* is highly regulated by both the retinoic acid orphan nuclear receptors and the PER:CRY complex. Approximately 24h are required to complete these self-regulating feedback loops which make up the circadian molecular clock. These processes however do not take place in isolation but are facilitated and governed by post-translational modifications (Ko and Takahashi, 2006). These modifications are responsible for the precision of the mammalian clock and play significant roles in the stability and nuclear translocation of the core clock proteins (Ko and Takahashi, 2006).

1.2.1 Posttranslational Modifications

Posttranslational modifications facilitate the delay between transcription, activation and repression of core clock proteins (Eide *et al*., 2002). Several of the clock proteins have a short half-life which is tightly controlled and enables their levels to fluctuate over a 24 hour period (Eide *et al*., 2005). Therefore, a considerable delay within the negative feedback loop is required to generate a circadian rhythm (Reischl and Kramer, 2011). They are able do this in different ways including through regulation of subcellular localization, interaction with other proteins, enzymatic activity or stability (Duguay and Cermakian, 2009).

Casein kinase 1 delta (CK1δ) and epsilon (CK1ε) both phosphorylate PER1 and PER2 proteins (Eide *et al*., 2002). CK1δ and ε are able to regulate PER proteins at different levels.

PER transcription takes places in the nucleus after which the PER proteins proceed to the cytoplasm (Mohawk *et al*., 2012). CK1δ/ε initiate sub-cellular relocalisation of PER proteins by regulating the phosphorylation status of the region which houses the nuclear localisation signal (NLS). Phosphorylation of PER1 by CK1ε in one instance masks the NLS thus trapping it in the cytoplasm (Vielhaber *et al*., 2000). In another instance phosphorylation of PER1 and 3 by CK1ε promotes nuclear entry (Takano *et al*., 2004). Glycogen synthase kinase-3 (GSK-3) phosphorylates PER2 in the cytoplasm which leads to nuclear entry (Litaka *et al*., 2005) or retention or both (Dardente and Cermakian, 2007). Nuclear entry of PER allows for repression of CLOCK/BMAL1 (Mohawk *et al*., 2012).

The phosphorylation of PER proteins by CK1 in the cytoplasm also results in the recruitment of the F-box protein β-TRCP which, binds a region just amino-terminal to the CK1ε binding domain (Eide *et al.,* 2005), and ultimately leads to the degradation of PER via the 26S proteasome (a large multi-protein complex involved in the regulated degradation of ubiquitinated proteins in the cell) after ubiquitylation has been stimulated (Bedford *et al*, 2010; Lowery and Takahashi, 2011; Shanware *et al*., 2011). Phosphorylation is a reversible process and dephosporylation of PER2 by protein phosphatase 1 (PP1) results in the stabilization of PER2 protein (Reischl and Kramer, 2011) in the cytoplasm. SIRT1 binds to CLOCK-BMAL1 and PER2 in a circadian manner and supports the deacetylation and degradation of PER2 (Asher *et al*., 2008).

Translated CRY proteins translocate to the cytoplasm where there is a co-dependency between PER and CRY proteins for effective nuclear translocation (Lee *et al*., 2001). CRY proteins are phosphorylated by CK1δ and CK1ε (Eide *et al*., 2002). CK1ε plays a role in PER-dependent phosphorylation of CRY (Eide *et al*., 2002). As this phosphorylation only occurs after the formation of the CRY/PER complex, CK1ε and CRY proteins are brought into close proximity by PER proteins which act as scaffolds (Eide *et al*., 2002). This complex is then translocated to the nucleus. CRY2 is also a substrate of GSK3β. GSK3β phosphorylates CRY2 after another protein has phosphorylated it, this promotes its nuclear localization, targets it for ubiquitination and finally for degradation via the proteasome (Harada *et al*., 2005; Reischl and Kramer, 2011). Another kinase, adenosine monophosphate-activated protein kinase (AMPK) has been reported to play a role in the degradation of CRY by Lamia *et al*., (2009). The Skp1/cullin/F-box protein and leucine rich repeat protein 3 (SCF^{Fbxl3}) ubiquitin ligase plays a salient role in the degradation of CRY1 and CRY2 in the

cytoplasm (Busino *et al*, 2007). After phosphorylation, CRY undergoes ubiquitination by SCF^{Fbxl3} which directs it for degradation (Busino *et al*, 2007).

BMAL1 is phosphorylated by CK1ε and mitogen-activated protein kinases (MAPK) which both modulate BMAL1-CLOCK dependent transcription (Asher *et al*. 2008; Eide *et al*., 2002). Phosphorylation of BMAL1 is also carried out by GSK3β which destabilises it and targets it for proteasomal degradation (Reischl and Kramer, 2011). On the other hand CK2 phosphorylation of BMAL1 governs its nuclear entry (Tamaru *et al*., 2009). Another posttranslational modification that aids in tight and robust regulation of the oscillator is SUMOylation. This refers to the covalent linking of small ubiquitin-related modifier protein (SUMO) to lysine residues (Cardone *et al*., 2005). It is reversible and controlled by an enzymatic pathway similar to the ubiquitin pathway. The findings of Cardone *et al*. (2005) indicate that BMAL1 undergoes rhythmic sumoylation *in vivo*, with a timing that parallels BMAL1 circadian activation. Therefore sumo-modification is required for BMAL1 rhythmicity, however a functional CLOCK protein is also essential for SUMO-modification (Cardone *et al*., 2005) their findings also indicate that the absence of the SUMO modification process may affect BMAL1 protein turnover. Lee *et al*. (2008) further reported that SUMOylation is a prerequisite for ubiquitination and occurs before nuclear localisations and subsequent proteasomal degradation. Figure 1.1 below is a simplified diagram of the molecular mechanism of the circadian oscillator.

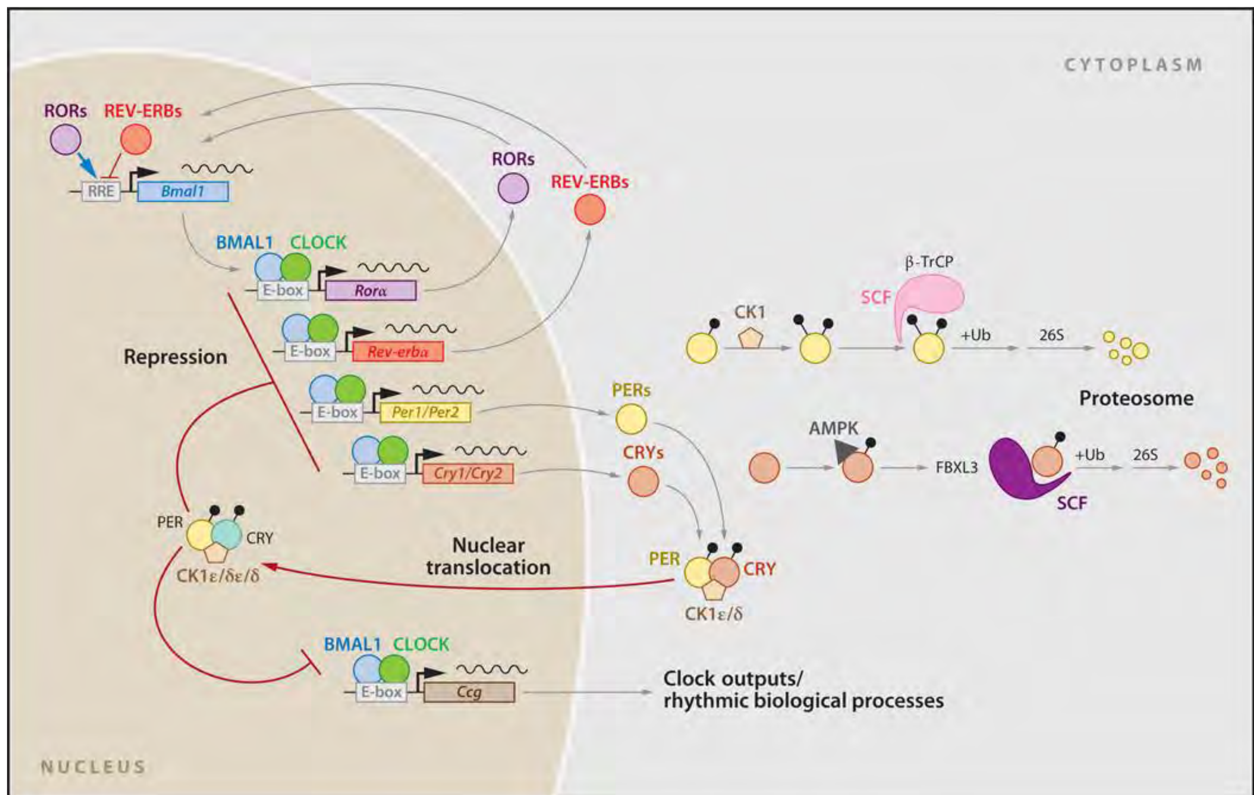


Figure 1.1. The molecular mechanism of the mammalian circadian oscillator: The core oscillator is an auto-regulatory transcriptional-translational feedback loop. CLOCK and BMAL1 are part of the positive regulators and activate the *PER1*, *PER2*, *CRY1* and CRY2 genes. Their gene products (proteins) in turn form a negative-feedback repressor complex. There are other auxiliary feedback loops also driven by CLOCK and BMAL1. One involves *REV-ERBα* and *RORα* that repress *BMAL1* transcription resulting in an antiphase oscillation in its expression. Other target genes referred to as clock controlled genes are regulated by CLOCK and BMAL1. The stability of the PER and CRY proteins is regulated by SCF (Skp1-Cullin-F-box protein) E3ubiquitin ligase complexes involving β-TrCP and FBXL3 respectively, after transcription. Casein Kinase 1 ε/δ (CK1ε/δ) and AMPK, phosphorylate the PER and CRY proteins to facilitate polyubiquitination by their respective E3 ubiquitin ligase complexes this process targets them for degradation by the 26S proteasome complex. Phosphorylation by CK1ε/δ also results in stabilization of the PER and CRY protein complex to facilitate nuclear entry, adapted from Mohawk *et al*. (2012).

1.3 Circadian Rhythm, Sleep and the Sleep homeostat

Circadian is derived from Latin words which mean "about a day" (*circa*: about and *diem* or *dies*: a day).Circadian rhythms have the characteristics of spanning a close to 24h period and possessing a phase because they have defined ranges of magnitude at particular clock hours (e.g. concentration, temperature) (Korczak *et al*., 2008; Czeisler *et al*., 1999). They have been

favoured by natural selection such that they are present in plants, cyanobacteria, mice and humans (Dunlap, 1999) and in the absence of environmental stimuli, continue to oscillate with a period close to that of the 24h light-dark cycle (Czeisler *et al*., 1999).

In order to confirm the presence of circadian rhythms in humans Czeisler *et al*. (1999) assessed the intrinsic period of the circadian oscillator in young and older subjects in a number of different protocols. These protocols created environments devoid of time cues with controlled exposure to a light-dark cycle and imposed sleep-wake schedules. This was done in order to separate internally generated time and external time as generated by the 24h light-dark cycle. The conditions created by Czeisler *et al*., (1999) resulted in each individual's endogenous rhythms, driven by the circadian oscillator, being desynchronised from their sleep-wake cycle. This enabled them to measure the patterns in the fluctuations of potential circadian markers. The markers measured were core body temperature (CBT), plasma melatonin and plasma cortisol and their estimated intrinsic periods were reported to have a high correlation when analysed within individual participants (Czeisler *et al*., 1999). The younger subjects had a mean estimated intrinsic period of 24.18h ± 0.04(SEM) while that of the older subjects was 24.18h ± 0.04 (SEM). These data give evidence for both the stability and precision of the circadian oscillator and its generation of approximately 24h rhythms (Czeisler *et al*., 1999).

The process of synchronisation or entrainment results in a predictable phase relationship between the *zeitgeber* and the oscillator (Roenneberg *et al*., 2003). Commonly used reference points for the phase of the oscillator are dawn and/or dusk (Roenneberg *et al*., 2003). This is because at these times there are drastic changes in light availability and light, according to current knowledge, is the most potent *zeitgeber*. The phase of entrainment of the oscillator to the *zeitgeber* is flexible. It is dependent on the period of the endogenous oscillator, the period of the *zeitgeber* as well as its strength or amplitude (Roenneberg *et al*., 2003).

An individual's sleep-wake pattern is a behavioural circadian rhythm regulated by the circadian system and the sleep homeostat (Dijk and Czeisler, 1995). It was reported that lesions in or near an area homologous to the SCN in humans results in perturbations of the sleep-wake cycle (Cohen and Albers, 1991). This has also been shown in animals with lesions of the SCN (Dijk and Lockley, 2002). Furthermore, Duffy *et al*. (2001) found a significant correlation between intrinsic circadian period and timing of habitual awakening. This gives evidence of the involvement of the circadian oscillator in sleep. The circadian

oscillator is thought to generate a rhythm that influences the time at which falling asleep is easiest, by playing a bidirectional role where sleep is promoted at certain phases and alertness at others. Homeostatic regulation on the other hand infers that sleep is dependent on duration of wakefulness i.e., it regulates the average level of sleep debt (Marano *et al*., 2011; Mistlberger, 2005; Dijk and Lockley, 2002). Therefore during sleep, sleep debt dissipates and during wakefulness it accumulates (Dijk and Lockley, 2002). In humans the SCN is thought to generate a wake or arousal signal that increases in strength throughout the biological day (i.e., during the habitual wake episode) peaking in later in the day at approximately 22h00. The strength of this signal declines during the biological night (i.e., during the habitual sleep episode) to reach a minimum at approximately 06h00, which coincides with the temperature nadir (Dijk and Lockley, 2002). The fluctuations of the SCN generated arousal signal work together with the sleep homeostasis processes and generate pressure for sleep to occur in one episode. However, when this arousal signal is absent the sleep homeostat would presumably work alone and this would result in several episodes of sleep during the 24h period because sleep consolidation has been lost (Dijk and Lockley, 2002). Both the sleep homeostat and the circadian processes contribute equally to waking performance as well as sleep consolidation (Dijk and Lockley, 2002). To strengthen this point, a consolidated 8h sleep episode can only be achieved at a particular phase relationship between the sleep-wake cycle and the endogenous circadian rhythm. Sleep should be initiated shortly after the crest of the wake propensity rhythm, roughly 6h before the temperature nadir if an uninterrupted 8h of sleep is desired (Dijk and Lockley, 2002).

Additionally, certain stages of sleep (aspects of sleep structure) are linked to endogenous CBT rhythm that is regulated in a circadian manner. At the nadir, or shortly thereafter, of CBT rhythm, sleep propensity and Rapid eye movement (REM) sleep peak (Dijk and Czeisler, 1995). Dijk and Czeisler, (1995) estimated that the circadian rhythm of sleep propensity has its peak located at the minimum of CBT rhythm, while its nadir is estimated to be close to habitual bedtime. Dijk and Czeisler, (1995) also estimated that the peak of the circadian rhythm of sleep propensity coincides with the minimum of the CBT rhythm which is close to habitual wake time. Furthermore Dijk and Czeisler, (1995) estimated the nadir of the circadian rhythm of sleep propensity to be located close to habitual bed time which is after the peak of CBT and corresponds to the evening wake-maintenance zone which is 2-3h before bedtime (Strogatz *et al*., 1987). This is because after the peak of the circadian rhythm of sleep propensity the circadian drive for wakefulness rises gradually and only begins to rise

steeply 4-8h after the minimum of CBT rhythm (Dijk and Czeisler, 1995). Therefore as core body temperature is rising sleep propensity is diminishing so that when CBT is at its peak, sleep propensity is at its minimum. After this CBT starts its decrease while sleep propensity starts to increase during the wakefulness maintenance zone after which is bed time. While sleep propensity and REM are linked to the circadian oscillator stage 3 of non-REM (NREM) as well as electroencephalographic (EEG) power density in NREM sleep are for the most part governed by the duration of prior sleep and wakefulness and are virtually uninfluenced by the endogenous circadian phase (Dijk and Czeisler, 1995). Figure 1.2 below depicts the interaction of the circadian oscillator, the sleep homeostat, the light-dark cycle and the sleep-wake cycle.

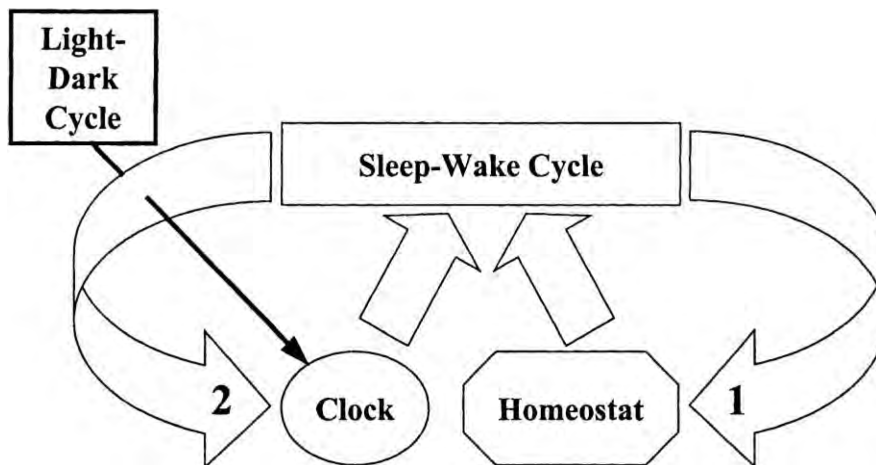


Figure 1.2. A schematic diagram of the interaction of the endogenous circadian oscillator "clock", the sleep homeostat "homeostat" and the sleep-wake cycle. Arrow 1 depicts that the oscillation of the sleep homeostat is principally determined by the sleep-wake cycle. Arrow 2 depicts that the sleep-wake cycle determines the light input that is received by the endogenous oscillator. Finally the oscillator and the sleep homeostat regulate the sleep-wake-cycle and sleep structure, adapted from Dijk and Lockley, (2002).

1.3.1 Sleep disorders with a circadian basis

1.3.1.1 Shift-work sleep disorder and Jetlag disorder

Given the role of the circadian oscillator in the anticipation and regulation of the sleep-wake rhythm, school or work schedules that result in the misalignment of their timing can have significant negative health consequences. When the external environment (24 h light-dark cycle) is altered relative to the circadian oscillator as is usually the case in night shift work or

trans meridian travel, circadian rhythm disorders such as shift-work sleep disorder or jetlag disorder may result. Shift-work sleep disorder is characterized by excessive sleepiness during work hours and/or insomnia during the desired period of sleep. This insomnia frequently results in the reduction of the sleep episode by 1 to 4 hours (Zee *et al*., 2013). The postulated reasons for this are the problems in the alignment of the circadian oscillator and the enforced sleep-wake cycle due to work schedules. The circadian oscillator is tightly entrained to the 24 h light-dark cycle. This is accomplished by light-facilitated advances in the earlier part of the day and delays in the later part of the day. However, due to work schedules shift workers are exposed to light at atypical times of the day (Drake *et al*., 2004). The result is an inability to fully adapt to the sleep-wake schedule enforced by their work. Additionally particularly in night shift work one is forced to stay awake during the period that the circadian alertness signal may be at its lowest (Zee *at al*., 2013). In the case of rapid travel across several time zones is made, the endogenous clock requires time to entrain to the new light dark cycle. During this period insomnia and excessive daytime sleepiness occur as the sleep-wake cycle is realigning itself with the new time zone. It is usually a temporary condition and is called jetlag disorder (Morgenthaler *et al*., 2007). Another way disorders occur is when there is an alteration in the circadian oscillator itself which impacts the way it entrains to the 24 h light dark cycle, e.g. in delayed sleep phase disorder, and familial advanced sleep phase syndrome (Okawa and Uchiyama, 2007), which are discussed below.

1.3.1.2 Familial Advanced Sleep Phase Syndrome

It has been observed in temperate countries, during the shorter days of winter and autumn, that from the ages of about 20 to 50, sleep onset tends to follow nightfall by 4 to 5 hours with awakening spontaneously occurring in the 1 to 2 hours after sunrise (Young and Kay, 2001). In familial advanced sleep phase syndrome (FASPS) sufferers experience evening sleepiness and have relatively early sleep onset, usually between 18h00 and 21h00, as well as early morning awakening, between 01h00 and 04h00 (Cermakian and Boivin, 2003). Reid *et al.* (2001) investigated this syndrome in 32 family members and reported that dim light melatonin onset was earlier in family members suffering from FASPS than in those not affected. Despite the advance in melatonin phase and sleep-wake cycle, sleep duration and architecture were not affected (Reid *et al*., 2001).

FASPS is an autosomal dominant transmitted behavioural trait (Xu *et al*., 2005) in which the *PER2* gene has a SNP resulting in an amino acid substitution, S662G (Toh *et al*., 2001). Toh

et al. (2001) reported that not all carriers of the S662G mutation were FASPS sufferers, some individuals who were relatives of the sufferers were also reported to have the SNP and a high H-Ö score. In a study of 15 family members spanning three generations, a different SNP was identified in FASPS sufferers, an A to G change in the DNA sequence of *CK1δ*, which causes a threonine to alanine alteration at amino acid 44 in the protein (CK1δ-T44A) (Xu *et al*., 2005). In the study, the mutation was reported to co-segregate with the FASPS phenotype only, and controls did not have this change (Xu *et al*., 2005).

Interestingly, these two polymorphisms identified in FASPS sufferers are mechanistically linked as CK1δ and CK1ε phosphorylate PER1 and PER2 proteins (Eide *et al*., 2002). The *PER2* SNP at position 2106(A→G) which leads to serine being changed to glycine in the protein is located in the CK1ε binding region of PER2, a modification that leads to hypo-phosphorylation of PER2 by CK1ε in vitro (Toh *et al*., 2001; Vanselow *et al*., 2006). It is postulated that hypo-phosphorylation of PER2 S662G may attenuate its CK1ε mediated degradation and thus accelerate its auto-repression resulting in phase advanced molecular rhythms (Vanselow *et al*., 2006). In mammals, rhythmic phosphorylation of PER proteins has been shown to be essential for rhythm generation itself as well as for correct circadian period and phase (Toh *et al*., 2001; Vanselow *et al*., 2006) and a short endogenous period, or *tau* is characteristic of FASPS (Xu *et al*., 2005).

1.3.1.3 Delayed Sleep Phase Disorder (DSPD)

DSPD was first identified in 1979 and described in 1981 (Weitzman *et al*., 1981) and was defined as a delayed sleep-phase syndrome characterized by a delay of the usual sleep period by as much as 2 to 6h. It is now called a disorder (Lack *et al*., 2009) as a disorder is a minor ailment or disease, while a syndrome is group of concurrent symptoms of a disease. Affected individuals have difficulty or the inability to fall asleep at a socially acceptable or desired time and an inability to wake spontaneously at the desired time in the morning (Lack *et al*., 2009). DSPD displays a range in severity from less severe cases sleeping from 02h00 to 10h00 to extreme delayed cases for example 05h00 to 14h00 (Lack *et al*., 2009). These unconventional sleep-wake patterns usually result in the inability to cope with normal work schedules. This disorder is associated with delayed endogenous circadian rhythms including delayed sleep timing parameters, melatonin circadian rhythms and CBT rhythms. Uchiyama *et al*. (2000) reported that sleep length and the time period between the nadir of CBT and

sleep offset were significantly longer in DSPD sufferers than in healthy controls. In another study conducted by Shibui *et al*. (1999) it was reported that the time from dim light melatonin onset to sleep offset was longer in DSPD sufferers than in healthy controls. Uchiyama *et al*. (2000) postulated that as a result of the lengthened sleep period after CBT nadir the phase advance that would have taken place due to exposure to light when one is awake may be masked due to sleep.

A genetic basis for this disorder has been investigated. A haplotype with the following polymorphisms in the *PER3* gene: G647, P864, $PER3^{4}$, T1037 and R1158 was reported by Ebisawa *et al*. (2001) and was found to be significantly higher in DSPD sufferers than in a control population of healthy people with no sleep disorders. Archer *et al*. (2003) investigated the association between the *PER3* VNTR polymorphism and DSPD and reported a higher frequency of the $PER3^{4}$ allele in DSPD sufferers in comparison to the morning, intermediate and evening preference groups combined. In contrast DSPD sufferers were reported by Pereira *et al*. (2005) to have a significantly higher frequency of the $PER3^{5}$ than the $PER3^{4}$ allele when compared to both a general population and a group with evening preference. This was posited to be due to the effect of different latitudes on the phenotype-genotype association.

1.3.1.4 Non-24-hour sleep-wake syndrome

As previously stated, the circadian oscillator is synchronised to the external light-dark cycle which results in a close to 24 h sleep-wake cycle. However, when individuals are in isolation and not exposed to a normal 24 h light-dark cycle their sleep-wake cycle is longer than 24 h resulting in progressively later sleep and wake times (Uchiyama *et al*., 2002). This is because their endogenous oscillator is being allowed to free-run and the period of the oscillator is usually slightly longer than 24 h (Czeisler *et al*., 1999). There is a disease condition, non-24-h sleep-wake syndrome, in which an individual’s sleep-wake cycle is longer than 24 h. It is less common among sighted individuals than it is among blind people and people who spend more time exposed to artificial light such as astronauts and sub-mariners (Cermakian and Boivin, 2003). Blind people are more prone to this syndrome because transmission of light stimuli through the pupil to the retina is hampered or sometimes impossible depending on the reasons for blindness (Uchiyama *et al*., 2000). This means that the circadian oscillator is unable to receive photic cues and therefore unable to synchronise to the to the environmental

24 h light–dark cycle. In sighted individuals despite exposure to the 24 h light-dark cycle the circadian oscillator is unable to entrain.

This condition affects the sleep-wake patterns of an individual such that they have a chronic one to two hour delay in sleep onset and offset which follows a steady pattern resulting in a sleep-wake cycle that is consistently longer than 24 hours. These longer than 24 h rest-activity cycles occur over several weeks and may be interspersed with periods of the rest-activity cycle aligning to the 24h day. Long periods of 24-40h without sleep may occur followed by long periods, 14-24h, of uninterrupted sleep (Cermakian and Boivin, 2003). In addition to problems with sleep initiation sufferers also experience episodes of disturbance where there are difficulties with sleep maintenance and awakening in the morning (Okawa and Uchiyama, 2007).

This syndrome severely affects one's ability to cope with school or work schedules. Similar to DSPD sufferers, non-24-h sleep-wake syndrome sufferers were reported by Uchiyama *et al.* (2000) to have a longer period of sleep between the nadir of CBT and sleep offset than healthy controls. However, in contrast to DSPD a shorter interval between sleep onset and CBT nadir was experienced in non-24-h sleep-wake syndrome sufferers (Uchiyama *et al*., 2000). The human melatonin 1a receptor has variants R54W and A157V which have shown a higher frequency in patients with non-24-h sleep-wake syndrome; however, this difference was not significant and there are no known effects of these variants (Cermakian and Boivin, 2003).

1.4 Chronotypes

Behavioural traits such time of day preference for activity-rest and habitual sleep-wake time have been observed and recorded to differ among people. Morning-type people are those who prefer to wake early, be active earlier in the day and retire to bed relatively early, whereas evening-types prefer to wake late and be active much later in the day. Neither or intermediate types are a separate group having no strong preference for a particular time of day (Kerkhof and Van Dongen, 1996; Taillard *et al*, 1999; Carney *et al*., 2006).

An individual's diurnal preference is referred to as a chronotype. Chronotypes have been assessed mainly by questionnaires designed to associate individuals to tendencies coined "morningness" or "eveningness" (Duffy *et al*., 1999; Roennenberg *et al*., 2003; Mongrain *et al*., 2004). One questionnaire in particular, the Horne–Östberg morningness-eveningness questionnaire, (H-Ö MEQ) used to subjectively determine diurnal preference was validated against daily oral temperature variation (Horne and Östberg, 1976). A correlation was found between a preference for relatively later activity and rest times and later peaks and nadirs of temperature. A similar relationship was observed in individuals with preference for earlier activity and rest times and earlier peaks and nadirs of temperature (Horne and Östberg, 1976). However, as it was not clear whether behavioural traits resulted in these internal differences or vice-versa further investigations have been carried out to this end.

Kerkhof and Van Dongen (1996) reported that morning-types reached a peak in rectal temperature approximately four hours before evening-types, this was measured while participants were ambulatory. Other measurements including alertness, midpoint of estimated period of sleep (mid-sleep), and sleep-wake behaviour which each have a 24 h periodicity, were also taken during this time. Peaks and nadirs were significantly earlier in morning-types than in evening-types (Kerkhof and Van Dongen, 1996). Rectal temperature and alertness were again measured under a 24 h constant routine and similarly the nadir of CBT was significantly earlier in morning-types and the peak of alertness was significantly later in evening-types (Kerkhof and Van Dongen, 1996).

Phases of plasma melatonin and CBT were measured and reported by Duffy *et al*. (1999) to differ between morning and evening-types with the evening-types having a later phase. Furthermore, the interval between circadian phase and usual wake time was longer in morning-types than in evening-types. This reveals that while evening-types may wake up at a later clock hour than morning-types, they wake up at a relatively earlier circadian phase. Duffy *et al*. (2001) went further in their investigation using CBT as a circadian marker and made measurements of both period and phase under constant routine and forced desynchrony protocols. They found that earlier habitual wake times, shorter periods and an earlier phase of CBT corresponded to morningness. They also reported longer periods, later phase of core body temperature and later habitual wake time corresponded to eveningness. Therefore, individuals with shorter intrinsic periods will have circadian phases (e.g. of CBT, plasma melatonin) relatively early within their sleep-wake cycle whereas those with longer periods

will have circadian phases relatively later within their sleep-wake cycles. (Duffy *et al*., 1999; Duffy *et al*., 2001).These reports taken together provide evidence for the contribution of a circadian mechanism to the observed behavioural traits of morning and evening-types. Figure 1.3 illustrates the differences in core body temperature variation between morning and evening types.

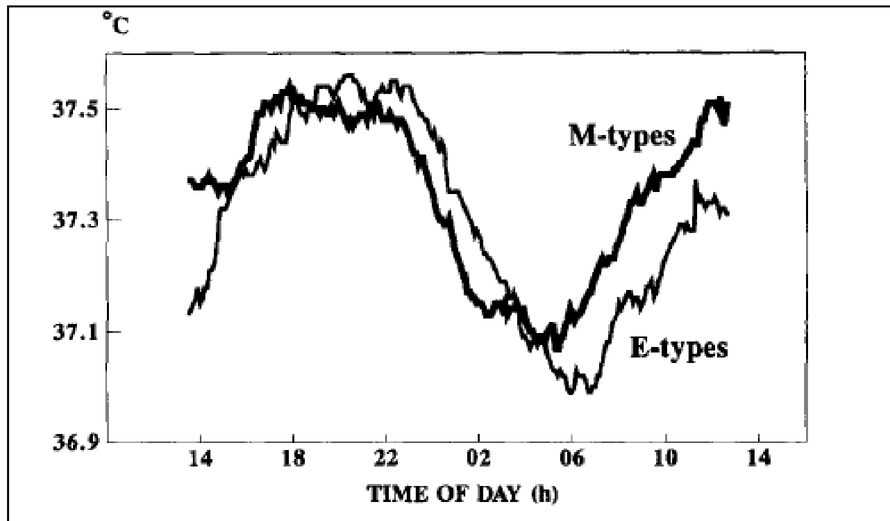


Figure 1.3. The mean rectal temperature in M-types (morning-types, n=7, bold line) and E-types (evening-types, n=7, thin line) was measured and recorded during constant routines in degrees Celsius. The calculated mean times of minimum temperature were 4h38 for the M types and 6h45 for the E types, adapted from Kerkhof and Van Dongen (1996).

1.4.1 Polymorphisms of core clock genes associated with chronotype

The first clock gene with a polymorphism observed to associate with diurnal preference was *CLOCK*. The polymorphism is located in the immediate 3' untranslated region at position 3111 and is a C to T nucleotide substitution (Katzenberg *et al*., 1998). Katzenberg *et al.* (1998) investigated this polymorphism in a population of 410 participants 57.1 % of whom were male and 95% of the total population were Caucasians, the mean age was 50.0 ± 7.9 (SD). They reported a correlation between higher H-Ö scores and the homozygous T allele carriers and lower H-Ö scores and the C allele carriers whether heterozygous or homozygous. They concluded that the C allele associates with evening preference. Mishima *et al.* (2005) also investigated this SNP in a Japanese population of 421 participants, with a mean age of 35.5 years ± 0.5(SEM), 27.1% male. They reported that subjects with *3111C/C CLOCK* genotype had significantly lower scores than those with either the 3111T/C or 3111T/T genotype. Thus, they concluded, like Katzenberg *et al.* (1998), that CC is associated with

evening preference. However, unlike the previously mentioned group they found no difference between the H-Ö scores of the TT and TC genotypes. Mishima *et al*. (2005) found the presence of the T allele (whether heterozygous or homozygous) correlated with higher scores, whereas Katzenberg *et al*. (1998) found that the TC heterozygotes had significantly lower H-Ö scores than the TT homozygotes.

Choub *et al*. (2011) carried out their investigation in 156 people of Italian origin aged between 21 and 56 years (33.1 ± 12.0, mean ± SD). They found no significant difference in T or C allele frequency among the different diurnal preference groups (measured using the H-Ö MEQ) nor was a difference in genotype frequency observed. Robilliard *et al*. (2002) investigating a relationship between H-Ö score and the 3111C/T polymorphisms observed no significant difference in the frequency of the C allele among the different groups of diurnal preference. This was the original population later used and described by Carpen *et al*. (2006). Barclay *et al*. (2011), also observed no association between the *CLOCK* 3111 genotype and diurnal preference. Likewise a study carried out in a Brazilian population of 161 people showed that there was no association between either T3111C or T257G polymorphism in the *CLOCK* gene with diurnal preference measured by H-Ö MEQ (Pedrazzoli *et al*., 2007). This population of 161 consisted of 68 morning-types, 50 neither-types and 43 evening-types selected on the basis of their H-Ö scores from an initial population of 1245 volunteers. The mean age was 28.02± 7.21 years (mean ± SD), 64% were females and 77.6% of the total population were Caucasians. Chang *et al*. (2011) also observed no significant difference in the *CLOCK 3111T/C* genotype between those with morning or evening preference nor did they find a significant difference in allele frequency between the groups. They had 179 participants, 74% of whom were white and the H-Ö MEQ was used to assess diurnal preference.

A synonymous SNP on exon 18 in the *PER1* gene was reported by Carpen *et al*. (2006) to associate with diurnal preference. Carpen *et al*. (2006) selected 263 participants, aged between 18 and 81 years, from a group of 1,590 volunteers recruited in London, England, and the H-Ö MEQ was used to assess diurnal preference. No information on their ethnic background was provided. The 263 participants were made up of 5% of the highest and lowest scoring participants (n=160), an intermediate group of the 80 participants closest to the linear regression line (Carpen *et al*., 2006) and finally 23 Delayed Sleep Phase Disorder sufferers. For this SNP, T2434C, a significant difference in the frequency of the C allele was

found, with the individuals with extreme morning preference having a higher frequency of the allele than in those with extreme evening preference. The frequency in the DSPD sufferers did not differ significantly from the general population.

A number of polymorphisms in *PER2* have been reported to associate with diurnal preference (Toh *et al*., 2001; Carpen *et al*., 2005; Lee *et al*., 2011; Matsuo *et al*., 2007). Carpen *et al*., (2005) used the H-Ö MEQ to determine diurnal preference in a population of 484 participants recruited in London (this population was different from one investigated by Carpen *et al*., 2006). From this original number, 105 participants were selected, 35 within the 7 percentile of extreme morning preference, 35 within the 7 percentile of extreme evening preference and 35 with intermediate preference. These 105 participants were genotyped for the C111G SNP which is located in the 5' untranslated region of exon 2 of the *PER2* gene and the G allele was reported to have a significantly higher frequency in subjects with extreme morning preference in comparison to those with extreme evening preference. They also genotyped these 105 participants for the G3853A and C-1228T SNPs within the same gene and found no significant difference between the frequencies of the alleles in the extreme and intermediate diurnal preference groups. Lee *et al*. (2011) were able to find, within a Korean population, using the Composite scale of morningness, (CSM) that individuals carrying the 3853G allele of the *PER2* polymorphism had significantly higher scores, which denote morningness, than the carriers of 3853A. They also genotyped their participants for the C111G and found no association with diurnal preference. Similarly, Choub *et al*. (2011) did not observe any significant difference in the frequency of the C and G alleles among the different diurnal preference groups. Neither did they observe a significant difference in genotype frequencies. *PER2* G3853A showed a significant association with 'activity planning' and 'morning alertness', both subscales of the CSM (Randler and Diaz-Morales, 2007) as reported by Ojeda *et al*. (2013). Activity planning is related to the self-perception of morningness and the time of retiring; and morning alertness is a reflection of the degree of alertness in the first half hour of wakefulness in the morning. However, no association was found between the polymorphism and diurnal preference which would be indicated by the total score. Ojeda *et al*. (2013) also reported the *PER3* M1037T polymorphism associated with the 'morningness' subscale of the CSM which correlates with the effort required to get up in the first half hour of wakefulness. Matsuo *et al*. (2007) investigated a small healthy population of 71 Japanese, 50% female and reported an association between the A allele of the G2114A SNP of the *PER2* gene and eveningness. Diurnal preference was assessed using the H-Ö MEQ.

PER3 has a variable number tandem repeat polymorphism (VNTR), within exon 18, with five or four 54 base pair repeats (Ebisawa *et al*., 2001). The 5-repeat allele ($PER3^5$) was reported to associate with morning preference and the 4-repeat allele ($PER3^4$) was reported to associate with evening preference by Archer *et al*., (2003) in the same population described by Carpen *et al*. (2005). Pereira *et al*. (2005) also observed a significantly higher frequency of the $PER3^5$ in the group with morning preference than in those with evening preference. Pereira *et al*. (2005), had 110 participants, mean age 48.91 ± 17.35 (SD); 60% of them were women and 76.36% of all the participants were Caucasian. Kunorozva *et al*. (2012) also reported a significant correlation between the *PER3* VNTR and diurnal preference. Their population comprised 625 active males of European descent aged 25-50 years. They used the H-Ö MEQ to assess chronotype in 374 participants and found the $PER3^{5/5}$ to associate with morningness and the $PER3^{4/4}$ with eveningness. Conversely Barclay *et al*. (2011) found no association between the *PER3* VNTR genotype and diurnal preference, using the H-Ö MEQ, in a population of 947 males and females from the United Kingdom aged 18-27 years. Voinescu and Coogan (2012) used the CSM to assess diurnal preference in a Romanian population (n=154) with self-reported sleep problems. Of the original number, 72 were genotyped for the *PER3* VNTR. They reported no association between this polymorphism and diurnal preference, which may have been due to small sample size or the presence of sleep problems within their population.

Another polymorphic region exists within the promoter region of the *PER3* gene. Three novel single nucleotide polymorphisms (G-320T, C-319A and G-294A) as well a 21-bp one or two variable number tandem repeat were reported by Archer *et al*. (2010). They also reported the-319A allele was at a significantly higher frequency in the intermediate group than in either the morning or evening-types. The population investigated was the same one described by Carpen *et al*. (2006).

Choub *et al*. (2011) found no association between genotype and phenotype for the *CLOCK T3111C* polymorphism which may be due to the low number of participants in comparison to that of Mishima *et al*. (2005) or Katzenberg *et al.* (1998). Out of the studies investigating the *CLOCK T3111C* polymorphism (Katzenberg *et al*., 1998; Robilliard *et al*., 2002; Mishima *et al*., 2005; Carpen *et al*., 2006; Pedrazzoli *et al*., 2007; Chang *et al*., 2011), Mishima *et al*. (2005) and Katzenberg *et al*. (1998) analysed, the highest number of participants although Katzenberg *et al*. (1998) had a greater proportion of male participants. Both the Japanese

(Mishima *et al*., 2005) and Caucasian (Katzenberg *et al*., 1998) populations were not prone to differences in genotype frequency due to ethnicity (Carleglio *et al*, 2008). These two factors may have contributed to their ability to detect associations. In the case of Robilliard *et al*. (2002) and Carpen *et al*. (2006) no information on ethnic background of participants was provided therefore, ethnicity may have contributed to their findings.

The CSM (Smith *et al*., 1989) and H-Ö MEQ (Horne and Östberg, 1976) are validated tools for assessing diurnal preference. However, it would appear that despite Greenwood (1994) reporting the CSM is psychometrically as sound as the H-Ö MEQ there may be a few differences in what aspects of diurnal preference are assessed by the questionnaires. For instance, the *PER2 C111G* polymorphism was found to associate with diurnal preference when the H-Ö MEQ was used by Carpen *et al*. (2005). However, when Lee *et al*. (2011) investigated a similar relationship using the CSM to assess diurnal preference no association was found. Furthermore, Lee *et al*. (2011) and Ojeda *et al*. (2013) found an association between diurnal preference using CSM score and CSM subscale scores and the *PER2 G3853A* polymorphism. Whereas Carpen *et al*. (2005) did not find an association between the G3853A polymorphism and diurnal preference as assessed by the H-Ö MEQ. Similarly the *PER3* VNTR polymorphism has been reported to associate with diurnal preference when the H-Ö MEQ was used (Archer *et al*., 2003; Pereira *et al*., 2005; Kunorozva *et al*., 2012) but not with CSM (Voinescu and Coogan, 2012). However, as indicated earlier, the population studied by Voinescu and Coogan (2012) was not a general population and were specifically selected due to suffering from sleep problems. However, the study by Barclay *et al*. (2011) used the H-Ö MEQ for diurnal preference, had a large study population and did not see an association between *PER3* VNTR genotype and diurnal preference, an indication that there are other factors that may contribute to the conflicting findings. Other important considerations in these clock polymorphism-chronotype association studies are the effects of environment such as latitude (Pereira *et al*., 2005), and longitude, (Roenneberg *et al*., 2007a). This is cannot be overlooked in studies for example Pedrazolli *et al*. (2007) apart from investigating a population with approximately 25% mixed ethnicity had participants residing at different latitude/longitude locations to those studied by either Mishima *et al*. (2005) or Katenberg *et al*. (1998). This may have contributed to their findings.

1.4.2 Factors that influence Chronotype

Chronotype studies have been carried out in different parts of the world highlighting a few of the factors that affect chronotype or have an effect on it. In one of the earlier large scale chronotype studies that was conducted by Chelminski *et al*. (1997) the H-Ö MEQ was used to determine chronotype in a population of 1617 students (1041 females, 576 males) enrolled at the University of North Dakota; their ages ranged from 18 to 53 years. They reported that the majority of the students, 62.4%, were neither-types; evening-types accounted for 29.3% of the population and 8.3% were morning-types. The students with the morning-type scores were significantly older than the students in the other two categories. Despite there being no significant difference between mean H-Ö scores of males and females, females tended more towards morningness. This is similar to what was reported by BaHammam *et al*. (2011) who used a reduced validated Arabic version of the H-Ö MEQ to assess chronotype in a population of 759 young Saudi college students aged 18-32 years. 540 of who were male and 219 female. Chronotype scores followed a normal distribution with the majority of participants scoring as neither-types and no gender differences were found in mean scores or chronotype frequencies. However, Randler (2007) conducted a meta-analysis of studies which had reported on sex and chronotype and found in contrast to the above mentioned studies that females were significantly more morning-type than males.

A study conducted by Mishima *et al*. (2005) recruited 421 healthy adults, 305 female, 116 male, aged 20-58 (mean age 35.5 years) who were employees of several medical facilities in the Akita Prefecture of Japan and completed the H-Ö MEQ. Although approximately two thirds of the participants were shift workers, no significant difference was found in the H-Ö scores between the shift workers and non-shift workers. This was an interesting finding considering what has been stated about shift work and the circadian oscillator (see section 1.3.2). The H-Ö scores showed a normal distribution. They found a significant positive correlation between age and H-Ö score.

Merikanto *et al*. (2012) assessed chronotype in 3696 women and 3162 men aged 25-74 years in six areas in Finland using six questions taken from the original 19-question H-Ö MEQ. The range of scores of the six-question questionnaire was from 5 (extreme eveningness) to 27 (extreme morningness). The cut-off points were modified in order for the ranges to correspond with the H-Ö MEQ scaling, with Morning-types scoring: 19-27, intermediate types: 13-18 and evening-types: 5-12. They reported 11.8% were evening-types, 40.9% were

intermediate types and 47.3% were morning-types. They also found that there were significantly more reports of insomnia symptoms and sleep medication use among evening-types than among morning-types. This indicated an influence of chronotype on sleep. One of the other findings of this study was eveningness was more common among women than among men. This was in contrast to the findings of Roennneberg *et al*. (2007b) who found men more prone to eveningness than women until the average age of menopause.

Randler (2008a) used the CSM and midpoint of sleep on free days (MSFsc) to assess chronotype in adolescents residing in three different climate zones in both the Northern and Southern Hemispheres (tropical, subtropical or temperate zones). Adolescents living in the subtropics had the greatest preference for evenings using both measurements. Those in the temperate zone were earliest in terms of MSFsc while adolescents in the tropics were the most morning-chronotypes in terms of CSM score. A significant association was reported between longitude and latitude and both CSM score and MSFsc as well as an inclination towards morningness in Adolescents living more towards the North and the East. Borisenkov, (2010) conducted a study in the Republic of Komi (Russia) from February to March, where 772 men and women completed the Munich Chronotype Questionnaire (Roenneberg *et al*., 2003) for chronotype assessment. Participants were aged 10 to 77 years, and mid-sleep time was used to measure chronotype. An assessment of the effect of latitude on chronotype was carried out by measuring chronotype in school-going children, aged 13 to 17, in two cities which are 4.5° apart in terms of latitude. Borisenkov (2010) reported a significant increase in the frequency of evening-chronotypes in the more northern latitude. A significant increase in the frequency of evening-chronotypes in the residents of Komi republic in comparison to residents of Central Europe was also reported. These two studies confirm the effect of latitude on chronotype.

Roenneberg *et al*. (2007a) conducted a study in a German population of 21,600, 51% women, where chronotype was assessed by midpoint of sleep on free days (MSFsc). Participants were grouped according to the size of the population where they lived; 1) ≤300,000 inhabitants, 2) 300,001-500,000 inhabitants, or 3) >500, 000 inhabitants. Roenneberg *et al*. (2007a) reported that chronotype, which was averaged in longitudinal bins, was tightly coupled to sun time within the first group, it was observed that MSFsc decreased from West to East. Within the other two groups a progressively weaker coupling to sun time was observed as well as a generally later chronotype than in the first group. These results suggest that the human

circadian oscillator is principally entrained by sun time as opposed to social time. They also indicate that this entrainment was independent of latitude. Roenneberg *et al*. (2007a) further suggested that the observations in the inhabitants of larger cities (>300,000) may be due to less exposure to outdoor light which results in the attenuation of the natural light-dark cycle as a *zeitgeber*. This study reveals the influence longitude exerts on chronotype.

1.5 Research Question and Study Aims

Chronotype and clock genes have previously been studied in a South African population (Kunorozva *et al*., 2012). Chronotype distribution has also been compared between different regions (Randler, 2008a). However, a study exploring chronotype, region and clock genes, as far as the author is aware, has not yet been conducted. South Africa represents a unique opportunity to conduct such a study. It has a large land mass which stretches from the Atlantic Ocean in the West to the Indian Ocean in the East. The entire country observes the same astronomical time which is based on the longitude 30' east of the Greenwich meridian (0'). This longitude does not pass through the centre of South Africa's landmass; it passes through the eastern side of the country close to the Indian Ocean (Figure 1.4). The longitude 15'east passes through the Atlantic ocean close to the Western coast of the country. The result is that while most of the land mass is in the universal coordinated time (UTC) +02:00 time zone, some of the landmass lies in the UTC +01:00 time zone (http://mapsof.net/map/africa-time-zones).

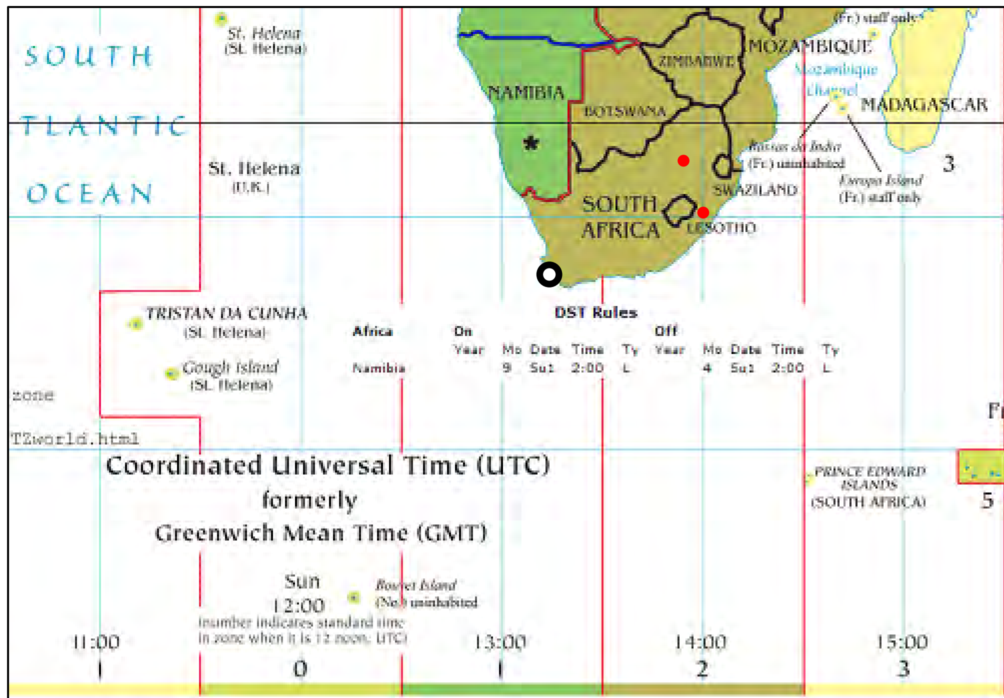


Figure 1.4 Map of Southern Africa showing longitudes and time zones from http://mapsof.net/map/africa-time-zones. The approximate locations of Cape Town (open circle), Johannesburg and Pietermaritzburg (red filled-circles) are indicated on the map.

The clock gene *PER2 G3853A* SNP has been investigated in three populations: those reported by Carpen *et al*. (2005), Lee *et al*. (2011) and Ojeda *et al*. (2013). Only Carpen *et al*. (2005) used the H-Ö MEQ, the other two used the CSM. *PER3* VNTR has been studied widely and several reports of an association between chronotype (H-Ö MEQ score) and the VNTR polymorphism have been made including by Kunorozva *et al*. (2012).

The roles of the *PER2* and *PER3* genes have for the most part been elucidated by mouse-model mutation studies. Zheng *et al*. (1999) reported that a mutation in the *PER2* gene leading to the deletion of 87 residues of the PAS domain of the mPER2 protein resulted in circadian instability in the mice. Another observation of mPER2 protein disruption was a reduction in levels of m*CLOCK* gene expression within the SCN (Shearman *et al*., 2000b). Zheng *et al*. (1999) also reported that m*PER2* mutant mice displayed a short circadian period followed by a loss of circadian rhythmicity when placed in constant darkness. Ultimately the disruption of *mPER2* destabilizes the circadian system and in the absence of the priming stimulus of the daily light-dark cycle, leads to severely disrupted rhythmicity in constant conditions in mice (Bae *et al*., 2001). Toh *et al*. (2001) were able to give insight into the essential role played by PER2 in humans when they reported a naturally occurring *PER2*

missense mutation that results in FASPS. This mutation leads to an altered free-running period brought about through changes in protein stability. These results demonstrate the prominent and crucial role played by *PER2* in the circadian oscillator (Zheng *et al*., 1999).

Shearman *et al*. (2000a) reported that only a very subtle change in circadian rhythmicity occurred when the *mPER3* gene was disrupted in mice. The free running rhythm period was moderately shorter in constant conditions by approximately 0.5h in comparison to wild type mice (Shearman *et al*., 2000a; Bae *et al*., 2001). Shearman *et al*, 2000a postulated that the mPER3 protein most likely participated in protein-protein interactions among essential clock proteins within the SCN neurons. Bae *et al*. (2001) also suggested it may be involved in regulating circadian outputs other than locomotor activity. Therefore PER3, unlike PER2, is not essential for maintaining circadian rhythmicity (Bae *et al*., 2001). However, the *PER3* gene has been reported to play a non-circadian role. Viola *et al*. (2007) reported that it affects sleep latency, slow wave sleep, theta activity in the waking electroencephalogram, and the decrement in waking performance in response to sleep loss, each of which are key markers of sleep homeostasis.

The role of *PER2* in the circadian system and the paucity of investigations of the G3853A polymorphism and its correlation with diurnal preference made it a good choice for study in a novel population. The well-studied *PER3* VNTR polymorphism widely reported to associate with diurnal preference, including in a population of South African Caucasian, male athletes (Kunorozva *et al*., 2012) was identified as another suitable polymorphism for investigation in this study. The tool used to determine diurnal preference was the Horne-Östberg morningness-eveningness questionnaire which was validated in 1976 against oral body temperature and wake and sleep time logs (Horne and Östberg, 1976). Duffy *et al*. (1999) demonstrated that H-Ö score correlates with the relationship between the sleep-wake cycle and circadian phase.

The position of South Africa within the two time zones creates a continuum of sunrise time from East to West. As sunrise is a powerful *zeitgeber* (Roenneberg *et al*., 2007a), it was hypothesized firstly that South Africans living in the East of the country would have a greater preference for mornings (more morning-chronotypes) than those living in the West. Secondly, that this difference would not be due to genetic differences in the populations, particularly the two clock gene polymorphisms, *PER2 G3853A* and the *PER3* VNTR. The aims of this study were to 1) describe and compare the distribution of chronotype in Eastern

and Western sample populations with the use of the H-Ö MEQ; and 2) describe the genotype and allelic frequency distributions of the *PER2 G3853A* (rs934945) and the *PER3* VNTR polymorphisms in the same two populations.

Chapter 2

2.0 MATERIALS AND METHODS

2.1 Participants

Two hundred and ninteen people were recruited from the Western Cape province (Cape Town: 34°5.3'S/18°35.3'E), and two hundred and forty one people from KwaZulu Natal (Pietermaritzburg: 29°36.0'S/30°22.8'E) and Gauteng (Johannesburg: 26°12.2'S/28°2.8'E) provinces which are in the Western and the Eastern parts of the country respectively. The approximate locations of these towns are indicated in Figure 1.4. Participants were aged 25 to 55 inclusive from different ancestral backgrounds including, African, European, Asian, Indian, Mixed and Other ancestry. The inclusion criteria for participants were that they: 1) were not taking any medication or substances chronically or sporadically that would have an effect on their sleep/wake cycle or interfere with their circadian rhythm, 2) had not done any night-time shift work in the three months prior to participating in the study; and finally 3) they had been living in their respective provinces for 3 years consecutively. Night time shift work was defined as 'working for pay, profit or income for at least 3h between midnight and 05h00 on a regular (a minimum of once a week) basis' (adapted from International Labour Organisation, 1990; Council of the European Union, 1993).

2.2 Study design

Advertisements to raise awareness about the study were done through radio media. Recruitment of participants was carried out in places open to the general public such as a library, shopping centres, work places and the Universities of Cape town and KwaZulu Natal. The Faculty of Science research ethics committee gave approval for the study **SFREC 017_2012**. A description of the purpose and requirements of the study and was given to participants verbally and in writing (Appendix A) on request, and written, informed consent was obtained from all participants (Appendix B). Participants provided a buccal swab and filled out a general questionnaire (Appendix C) as well as the Horne-Östberg Morningness-Eveningness personality questionnaire (Appendix D).

In brief, the general questionnaire captured personal information, the length of time without interruption that an individual had stayed in the particular province, as well as information regarding involvement in night-time shift work and medication. The buccal swabs were obtained in order to perform genotyping of participants for 1) a Single Nucleotide Polymorphism G3853A present in the coding region of the *PER2* gene as performed by Lee *et al*. (2011) and 2) for the variable number tandem repeat polymorphism in the *PER3* gene reported by Ebisawa *et al*. (2001).The study adhered to the principles described in the Declaration of Helsinki (October 2008, Seoul), ICH and South African Good Clinical Practice (GCP) guidelines.

2.3 Detailed testing Procedure

2.3.1 Horne-Östberg Morningness-Eveningness personality questionnaire

All participants completed the H-Ö MEQ. This is a self- assessment questionnaire that was developed in 1976 by Horne and Östberg and has been widely used and validated, including in a South African population (Kunorozva *et al*., 2012). The questionnaire consists of 19 questions, 14 of which are multiple-choice regarding a person's preferred waking, bed and activity times. The responses are assigned a numerical score and summed. A score of 16-30 classifies someone as a definite evening-type, 31-41 as a moderate evening-type, 42-58 as a neither-type, 59-69 as moderate morning-type and 70-86 as a definite morning-type. Horne and Östberg (1976) were able to observe a consistent association between the timing of peaks and nadirs in fluctuations of body temperature and the scores for "morningness", "neither-type" or "eveningness" generated by the questionnaire. The morning-types with higher scores had earlier phased peak body temperatures than the evening-types with the lower scores.

2.3.2 Buccal Cell samples

Buccal cells were obtained from participants using Epicentre Catch-All™ Sample Swabs (Epicentre Biotechnologies, Madison, USA) or Isohelix DNA swabs (Cell Projects Ltd., Kent, UK). Swabs were introduced into the mouths of participants and rubbed thoroughly on the insides of both cheeks for 10-30 seconds. The swabs were then stored at room temperature for a maximum of 30 days before further processing.

2.3.3 Genomic DNA extraction

gDNA was extracted from buccal swabs using a protocol based on the one described by Aljanabi and Martinez, (1997). Briefly, swabs were placed in 50 μl Ethylenediaminetetraacetic acid (EDTA) buffer pH 8, with 10 % (w/v) sodium dodecyl sulphate (SDS) for 1h at room temperature, followed by incubation for 3h at 56°C in the presence of 10 mg ml^{-1} proteinase K (Thermo Scientific, Vilnius, Lithuania). This was followed by addition of 6 M NaCl, centrifugation at 12,000 x*g* for 10min, pelleting using isopropanol and finally washing with 70 % (v/v) ethanol. Isohelix DNA Isolation kits DDK-50 (Cell Projects Ltd., Kent, UK) were also used for DNA extraction according to the manufacturer's protocol. The gDNA was stored at -20 °C. Quantification of DNA was carried out on Nanodrop ND-1000 spectrophotometer (Nanodrop ® Technologies, USA). Quality was assessed by visualisation of 100-500 ng of gDNA on a 1 % (w/v) agarose gel in 1 X Tris Acetate EDTA (TAE; 40 mM Tris, 1 mM EDTA and 0.11 % (v/v) glacial acetic acid) buffer stained with ethidium bromide.

2.3.4 *PER2 G3853A* Single Nucleotide polymorphism genotyping

A 137 bp fragment of the *PER2* gene was amplified using KAPATaq DNA polymerase (Kapabiosystems, Boston, USA) under the following conditions 95 °C for 5min, 36 cycles of 95 °C for 30 sec, 61.5 °C for 30 sec, 72 °C for 30 sec then 72 °C for 3 min using primers described by Malison *et al*. (2006). Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Vilnius, Lithuania) was also used for amplification under the following conditions denaturation at 98 °C for 30 sec, 40 cycles of 98 °C for 10 sec, 63 °C for 30 sec and 72°C for 30 sec and 7 min elongation at 72 °C. This region contains a SNP (rs934945) at position 3853 (G→A) that causes a Glycine to Glutamine change in the *PER2* protein. Confirmation of amplification was performed by electrophoresing the PCR product on a 2 % (w/v) agarose gel in 1 X TAE buffer stained with ethidium bromide. Examples of the amplification products are shown in Figure 2.1.

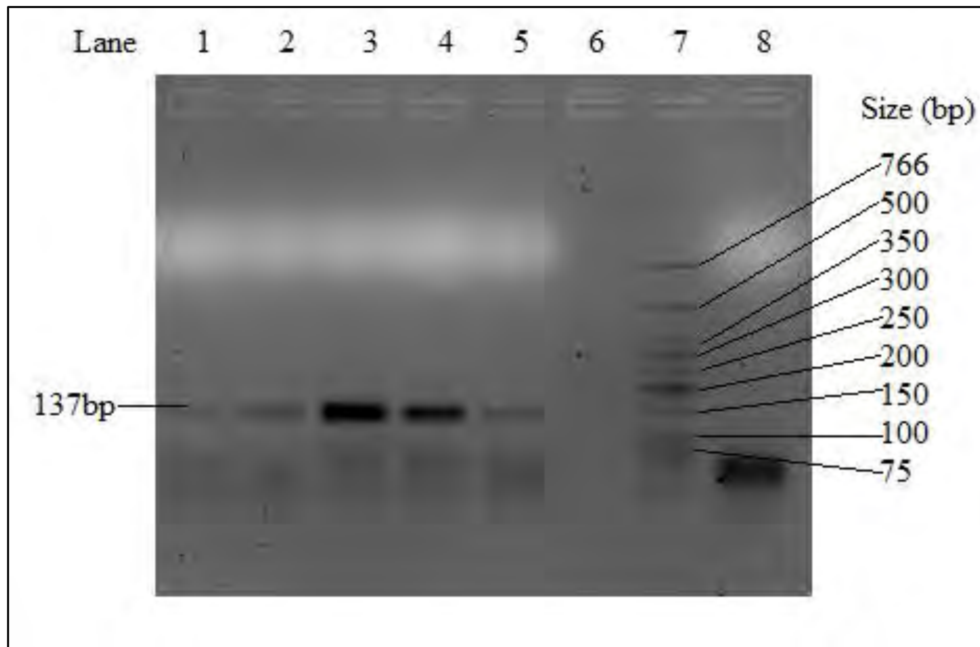


Figure 2.1. Amplification products of the *PER2* 137bp region containing the G3853A SNP: Polymerase chain reaction was performed on genomic DNA using primers described by Malison *et al*. (2006). The products were then resolved on a 2 % (w/v) agarose gel, stained with ethidium bromide and visualised under ultra violet light. Lanes 1-5 are participant samples each containing the 137bp amplification product. Lane 6 is empty, lane 7 contains the Low Molecular Weight ladder, New England BioLabs (New England BioLabs, Massachusetts, USA) and lane 8 contains the negative control.

Restriction digestion using *Bam*H1 (Thermo scientific, Vilnius, Lithuania) at 37 °C for 7-16 h was carried out on the resulting amplicon. *Bam*H1 binds and cleaves the following sequence 5'-GGATCC-3' on each DNA strand just after the 5'- guanine. Digestion confirmed the presence of the SNP and this was resolved and visually confirmed on 2 % (w/v) agarose gel in 1 X TAE buffer stained with Novel Juice (GeneDirex®, Taoyuan County, Taiwan) or ethidium bromide. An example of the banding pattern observed after restriction digestion is shown in Figure 2.2 below.

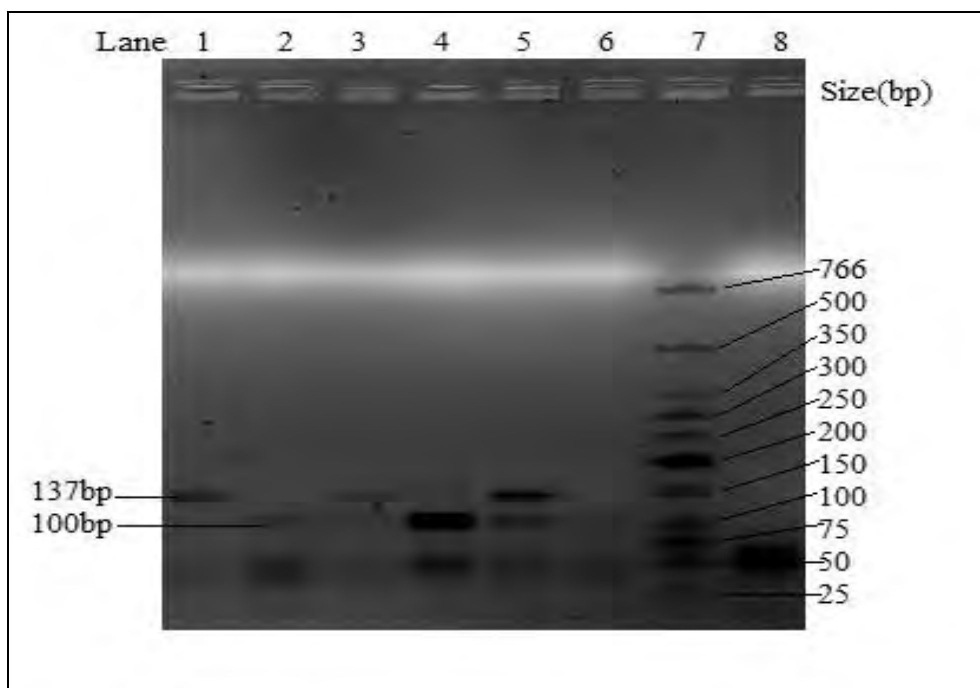


Figure 2.2. Restriction digestion of amplification products of the *PER2 137*bp region: After amplification the *PER2* 137bp region was digested with *Bam*H1 (Thermo scientific, Vilnius, Lithuania) the resulting products were resolved on a 2 % (w/v) agarose gel stained with ethidium bromide and visualised under ultra violet light. Lanes 1- 6 are participant samples. Lane 1contains the undigested 137bp product representing the AA genotype. Lanes 2, 4 and 6 each containing the digested 100bp product representing the GG genotype and lanes 3 and 5 contain both the undigested 137bp band and the digested 100bp band which represent the GA genotype. Lane 7 contains the Low Molecular Weight ladder, New England BioLabs (New England BioLabs, Massachusetts, USA) and lane 8 contains the negative control.

Table 2.1: Expected fragment sizes of the *PER2* 137bp region after restriction digestion with *Bam*HI

Genotype	Band 1(bp)	Band 2(bp)	Band 3(bp)
AA	137	-	-
GA	137	100	37
GG	-	100	37

2.3.5. DNA cloning and transformation

In order to confirm that it was indeed the targeted region of the *PER2* gene which was being amplified and digested for the purposes of genotyping, the 137 bp region was amplified according to the protocol described above (2.3.4) and purified using a Biospin purification kit (Gerham Pharmaceuticals, USA) according to the manufacturer’s instructions. This amplicon

was ligated into the Clone JET vector (CloneJET® kit, Thermo scientific, Vilnius, Lithuania) specifically employing the blunting protocol. The Sambrook and Russell (2006) calcium chloride protocol was employed to generate *Escherichia coli* (*E. coli*) DH5α competent cells. These cells were then transformed and plated onto Luria Bertani broth Agar containing 60 µg ml^{-1}ampicillin and placed at 37 °C for 12-16 h (Sambrook and Russell, 2006). The resulting colonies were individually cultured in Luria Bertani broth containing 60 µg ml^{-1} ampicillin at 37 °C for a further 12-16 h. Plasmid DNA was extracted from these cultures using an alkaline lysis protocol (Sambrook and Russell, 2006). PCR was performed to confirm the presence of the region of interest and the resulting product resolved and visually confirmed on 2 % (w/v) agarose gel in 1 X TAE buffer stained with ethidium bromide. Macrogen (Macrogen, Seoul, South Korea) sequenced the plasmid DNA using the pJET1_2F primer. Vector sequences were removed using Vecscreen (www.ncbi.nlm.nih.gov/tools/vecscreen) and homology searches using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed the identity of the amplified sequence.

2.3.6 *PER3* Variable Number Tandem Repeat (VNTR) polymorphism genotyping

Genotyping of individuals for this polymorphism was carried out by amplification of exon 18 and the adjacent introns on the *PER3* gene with the use of primers described in Ebisawa *et al*. (2001) and Archer *et al*. (2003). The polymerases used were supplied by Bioline, (MyTaq Polymerase, Bioline, London, UK) and Thermo Scientific (Phusion High-Fidelity DNA Polymerase, Thermo Scientific, Vilnius, Lithuania). Amplification conditions for Bioline MyTaq were: denaturation at 94 °C for 5 min, 36 cycles of 94 ° C for 45 sec, 55 °C for 45 sec, 72 °C for 1 min and 5 min elongation at 72 °C and those for Phusion High-Fidelity DNA Polymerase were denaturation at 98°C for 30 sec, 40 cycles of 98 °C for 10 sec, 60 °C for 30 sec and 72 °C for 30 sec and 7 min elongation at 72 °C. These amplification processes both resulted in a 650bp or 600 bp amplicon, for the $PER3^5$ allele or the $PER3^4$ allele respectively. Amplification was visually confirmed by electrophoresing the PCR product on a 2 % (w/v) agarose gel in 1 X TAE buffer stained with ethidium bromide. Examples of the amplification products are shown in Figure 2.3. In order to differentiate between the $PER3^4$ and $PER3^5$ alleles which differ by only 54 base pairs (Archer *et al*., 2003) restriction digestion using *Nco1* (Thermo Scientific, Vilnius, Lithuania), which recognises and cleaves the sequence 5'-CCATGG-3’ just after the 5’ cytosine on each strand, was performed. Restriction digestion was carried out at 37 °C for 7-16 h. This digestion facilitates resolution not only between the

$PER3^4$ and $PER3^5$ alleles but also enables a differentiation between $PER3^4$ alleles which may or may not harbour the thymine (T) to cytosine (C) SNP at position 3110 (Ebisawa *et al*., 2001) and one without. This T to C substitution results in an amino acid substitution from Methionine to Threonine at position 1037 in the PERIOD3 protein (Ebisawa *et al*., 2001). The digestion products were resolved and visually confirmed on 2 % (w/v) agarose gel in 1 X TAE buffer stained with Novel Juice (GeneDirex®, Taoyuan County, Taiwan) or ethidium bromide. Examples of the restriction digestion products are shown in Figure 2.4, and the expected fragment sizes after *Nco1* restriction digestion are show in Table 2.2 from Kunorozva (2011).

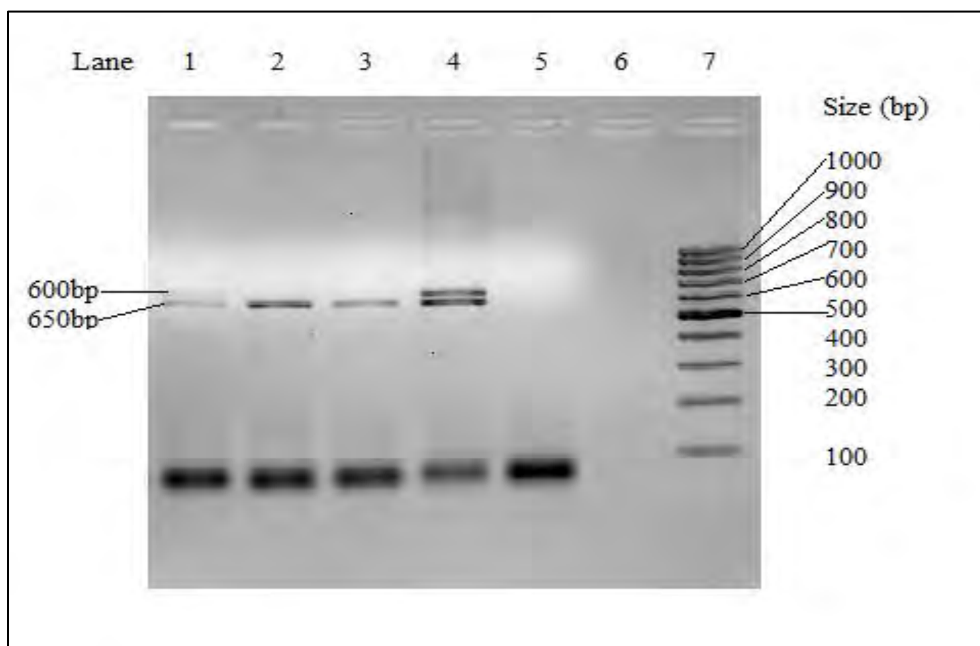


Figure 2.3. Amplification products of the *PER3* VNTR polymorphic region: Polymerase chain reaction was performed on genomic DNA using primers described by Ebisawa *et al*. (2001). The products were then resolved on a 2% (w/v) agarose gel, stained with ethidium bromide and visualised under ultra violet light. Lanes 1-4 are participant samples. Lanes 1 and 4 have both the $PER3^5$ and $PER3^4$ alleles, while lane 2 and 3 only have the $PER3^4$ allele, lane 5 is the negative control, lane 6 is empty and lane 7 contains the 100bp O'Gene Ruler (Thermo Scientific, Vilnius, Lithuania).

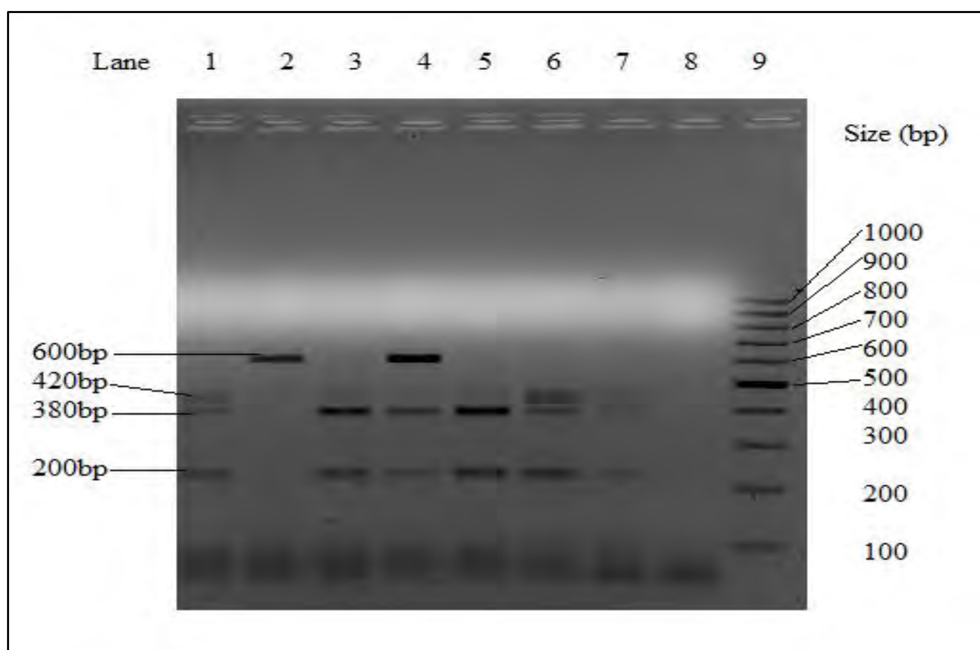


Figure 2.4. Restriction digestion of the amplification products of the *PER3* VNTR polymorphic region: After amplification, the *PER3* VNTR polymorphic region was digested with *Nco1* the resulting products were resolved on a 2% (w/v) agarose gel stained with ethidium bromide. Lanes 1-6 contain participant samples. Lanes 1, 3, 5 and 6 contain both the $PER3^4$ and $PER3^5$ allele, lane 2 contains only the $PER3^5$ allele, lane 4 contains the $PER3^4$ allele, lane 7 contains the 100bp O'Gene Ruler (Thermo Scientific, Vilnius, Lithuania) and lane 8 contains the negative control.

Table 2.2: Expected fragment sizes of the *PER3* VNTR polymorphic region containing the M1037T SNP after restriction digestion with *Nco1*

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

2.4. Statistical Analyses

All quantitative data collected were reported as the mean ± standard deviation. In order to determine whether quantitative data were normally distributed the Shapiro-Wilks test was used. The general characteristics of the participants were compared using the Pearson's Chi-Squared test or the two-tailed Fisher's exact test (http//in-silico.net/tools/statistics/fischer_exact_test) for the categorical data, and the Mann-Whitney U test for quantitative data that was not normally distributed. Chronotype, genotype and allele frequency distributions were compared using the Pearson's Chi-Squared test and Bonferroni's correction was used for post hoc analysis. Chronotype frequency distribution for each genotype was compared using the two-tailed Fisher's exact test. Correlation analysis for data that was normally distributed was performed using ANOVA and for data that was not normally distributed Kruskal-Wallis ANOVA by ranks was used. Hardy-Weinberg exact tests (GenePop, web version 4.2, http://genepop.curtin.edu.au/genepop_op1.html) were used to determine whether allele frequencies were in Hardy Weinberg equilibrium. Statistical significance was accepted at $p<0.05$. Statistica versions 10 and 12 (Statsoft Inc, Tulsa Oklahoma, USA) were used for all the data analyses that were not performed *in silico*.

Chapter 3

3.0 RESULTS

A total of 460 people took part in the study, 241 from the Eastern region and 219 from the Western Cape. Females comprised 59.3% of the population and the sex of two participants was unknown. The Eastern region was comprised of participants from Johannesburg in Gauteng (26°12.2'S/28°2.8'E) and Pietermaritzburg in KwaZulu Natal (29°36.0'S/30°22.8'E). The people who participated in the study had lived in their particular region for a minimum of three years prior to the date of participation in the study (refer to materials and methods for inclusion criteria). The Horne-Östberg morningness eveningness data (H-Ö scores) and genotype data for either *PER2*, *PER3* or both loci were available for use in analysis from a total of 367 participants. For 60 participants only H-Ö scores were available as either the participant did not submit a swab (n=17), or genotyping attempts failed due to low yield or poor quality of DNA obtained. Genotypic data only was available for analysis from 33 participants due to shift work or medication (refer to materials and methods for exclusion criteria), and they were excluded from the H-Ö MEQ score-genotype association analyses. All the questionnaire data were entered on a database and each entry was verified twice, first by the person entering the data and then by another individual who did not participate in data entry. Forms with atypical responses were assessed individually according to an agreed set of criteria.

3.1 General Characteristics

The general characteristics of the two populations are presented in Table (3.1) below. The sex distribution in the Eastern region was similar to that observed in the Western Cape, a Pearson's Chi-Squared test therefore showed no significant difference, p=0.981, between the populations. There was a greater percentage of females in both populations. A significant age difference between the populations ($p<0.0001$) was found with the participants from the Eastern region being older than the Western Cape participants. The H-Ö score was significantly higher ($p<0.0001$) in the Eastern region than in the Western Cape. Ancestry was used instead of ethnicity because each of the major populations groups Black Ancestry (BA), Indian Ancestry (IA), Mixed Ancestry (MA) and White Ancestry (WA) were made up of

different ethnicities i.e. either tribes or nationalities. A two tailed Fisher's exact test revealed a significant difference in representation of ancestry groups between the two regions (p<0.0001). The groups which had n<5 where combined to form the Other Ancestry (OA) group. The differences were particularly evident in the IA group which made up 16.2% of the population in the East and <3.2% in the West. Similarly the MA group made up 28.4% of the Western Cape participants and accounted for <2.9% in the East. The BA group was not well represented in either region and the WA group was well represented in both regions. This is as a result of limitations during the recruitment process. The Hardy-Weinberg test performed on the *PER2* and *PER3* alleles revealed that the allele frequencies did not deviate significantly from Hardy-Weinberg equilibrium; the p-values were p=0.475 for the *PER2* alleles and p=0.805 for the *PER3* alleles.

Table 3.1: General Characteristics of participants from the Eastern region and the Western Cape

	Eastern region	**Western Cape**	**p-value**
Sex			0.981 (Pearson's Chi-squared test)
Female	59.3% (n=143)	59.5% (n=129)	
Male	40.7% (n=98)	40.6% (n=88)	
Age (yrs)	39.1± 8.8 (n=241)	35.6±8.2 (n=218)	0.000005* (Mann-Whitney U)
H-Ö Score	60.56 ± 10.2 (n=231)	55.07 ± 10.6 (n=196)	0.000000* (Mann-Whitney U)
Ancestry			<0.0001* (Two tailed Fisher's exact test)
Black Ancestry (BA)	12.9% (n=31)	19.7% (n=43)	
Indian Ancestry (IA)	16.2% (n=39)	<3.2% (n=4)	
Mixed Ancestry (MA)	<2.9% (n=4)	28.4% (n=62)	
Other Ancestry (OA)	2.9% (n=7)	3.2% (n=7)	
White Ancestry (WA)	68% (n=164)	48.6% (n=106)	

Mean values are presented as values ± standard deviation. (*) denotes a significant difference between the Eastern region and the Western Cape. Significance was set at p<0.05.

3.2 Chronotype Distribution

The H-Ö scores obtained for each person fell in to one of 5 categories namely: Definite Morning-type (score: 70-86), Moderate Morning-type (score: 59-69), Neither-type (score: 42-58), Moderate Evening-type (score: 31-41) and Definite Evening-type (score: 16-30). For purposes of analysis these categories were combined to form three broad groups, morning-type (MT: 59-86) which includes the definite and moderate morning-types, evening-type (ET: 16-41) which includes the definite and moderate evening-types and the neither-type (NT: 42-58) which remained as it was.

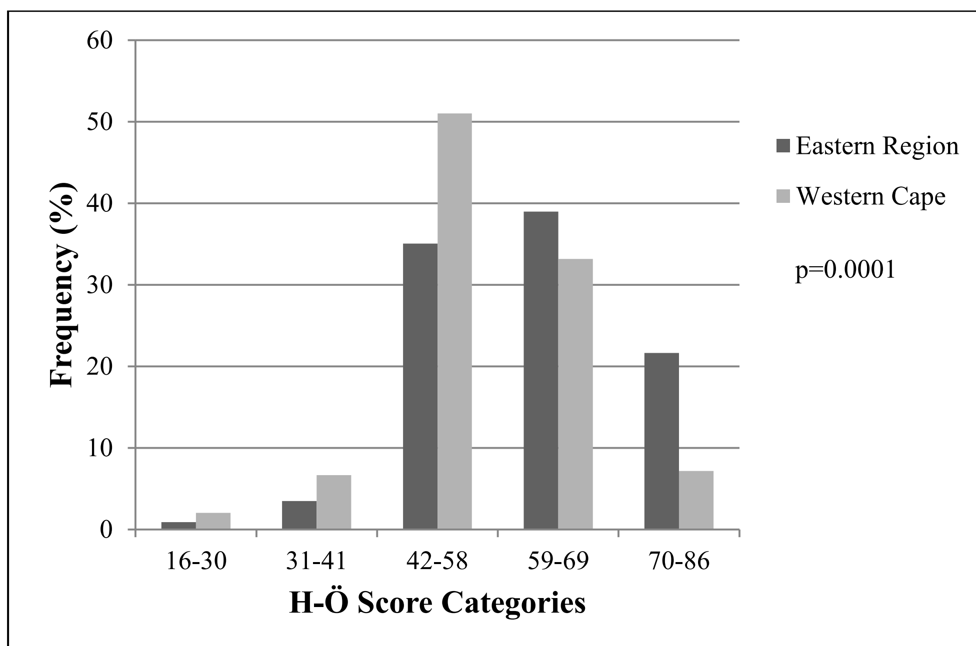


Figure 3.1. Chronotype frequency distribution in the Eastern region and the Western Cape: Chronotype frequency, based on the H-Ö Scores, was compared between the Eastern region (n= 231) and the Western Cape (n=196). H-Ö Scores from; 16-41 are evening-types, 42-58 are neither-types and 59-86 are evening-types. The Pearson's Chi-Squared test was used for comparison.

Chronotype frequency distribution was compared between Eastern and Western populations using a Pearson's Chi-Squared test which revealed a significant difference, Figure 3.1, p=0.0001. This finding remained significant even after Bonferroni's post hoc correction. Evening-types were the least represented chronotype in both regions, but occurred with twice the frequency in the West than in the East. Distinct differences were observed between the populations as the Eastern region showed a higher proportion of morning-types (MT: 60.6%, NT: 35.1% and ET: 4.3%) while the Western Cape showed a higher proportion of neither-

types (MT: 40.5%, NT: 50.8% and ET: 8.7%). The Shapiro Wilk's test was used to assess whether the Eastern and Western populations had normal distributions. For this test a p-value <0.05 suggests the data distribution differs significantly from a normal distribution. The Western Cape H-Ö score data followed a normal distribution (p=0.449; skew -0.25) whereas the Eastern region's did not, (p=0.00002) and was more skewed towards the higher scores (skew -0.637). When H-Ö score data for the Eastern and Western populations was combined The Shapiro Wilk's test gave a p-value of 0.00007 and the skew of this data was -0.436.

The effect of sex on chronotype was tested using combined data for Eastern and Western populations, Figure 3.2. No significant difference in H-Ö score between males and females was found when a Mann-Whitney U test was performed, p=0.704. The mean H-Ö score for female participants was 57.97±10.8 and for males 58.17±10.6, (mean ± SD). All further analyses include both male and female participants.

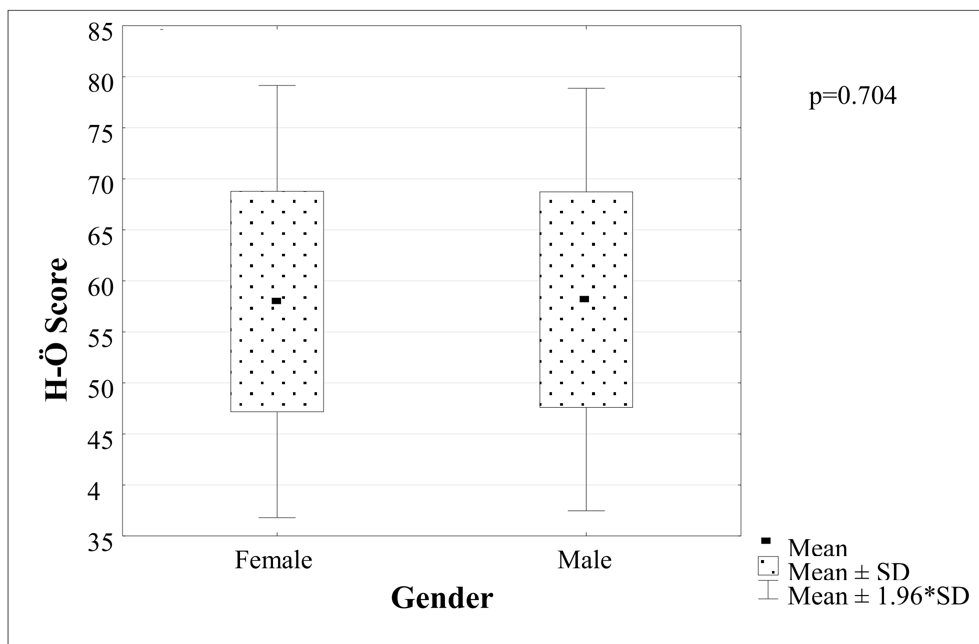


Figure 3.2. The effect of sex on Chronotype: The Eastern Region and Western Cape were combined and analysis was performed to determine if there was a difference in chronotype, as assessed by H-Ö Score, as a result of sex, female (n=272) and male (n=186). A Mann Whitney U test was used for comparison.

The Eastern and Western populations were combined and a significant correlation between age and H-Ö score was observed, Figure 3.3 (Spearman-R=0.216, p<0.0001). Spearman's rank correlation was used because both the age (Shapiro Wilk's test, p=0.0000, skew, 0.235) and H-Ö scores were not normally distributed.

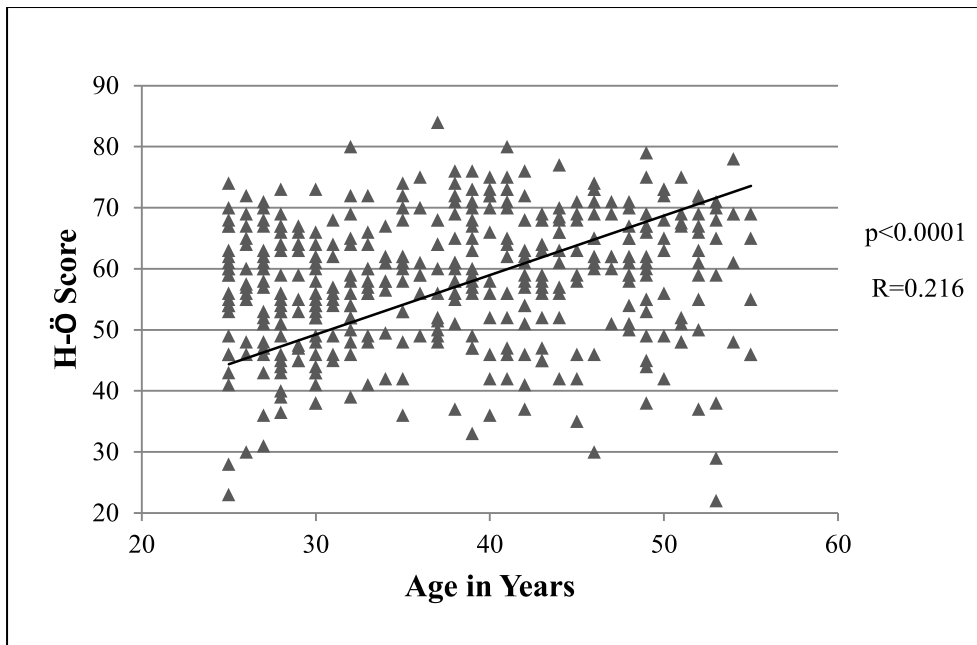


Figure 3.3. Relationship between age and Chronotype: In the combined population of the Eastern region and Western Cape (n= 459) the relationship between age and chronotype as assessed by H-Ö score was analysed using Spearman's correlation by ranks.

Mean chronotype was compared among the ancestry groups that accounted for a minimum of 12% of the population in either the Eastern or Western populations, that is, the Black, Indian, Mixed and White ancestry groups, Figure 3.4. There was no significant difference in chronotype among the different ancestry groups, p=0.299.

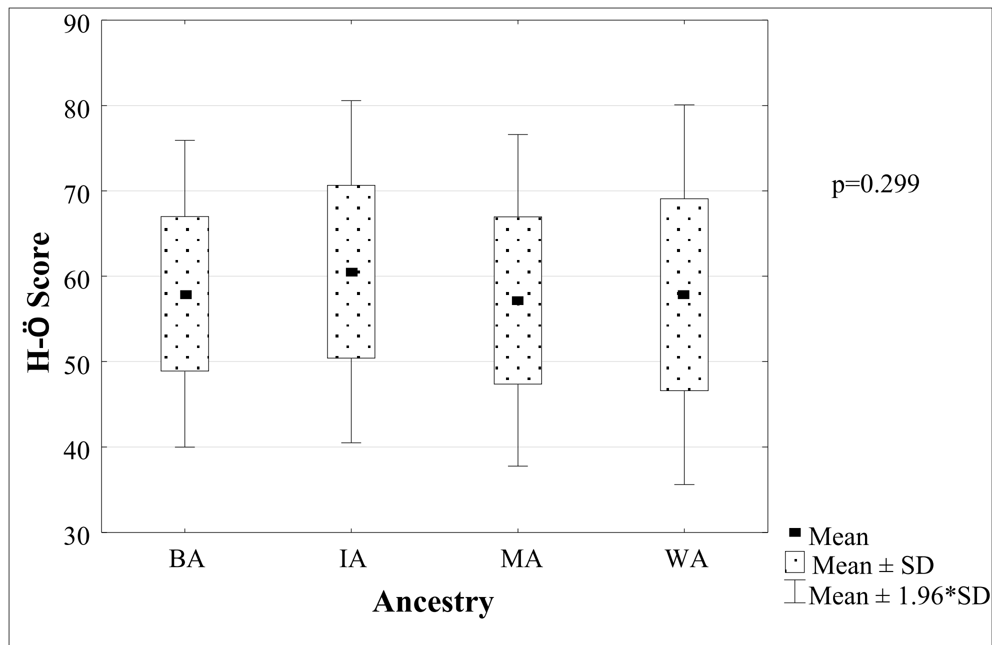
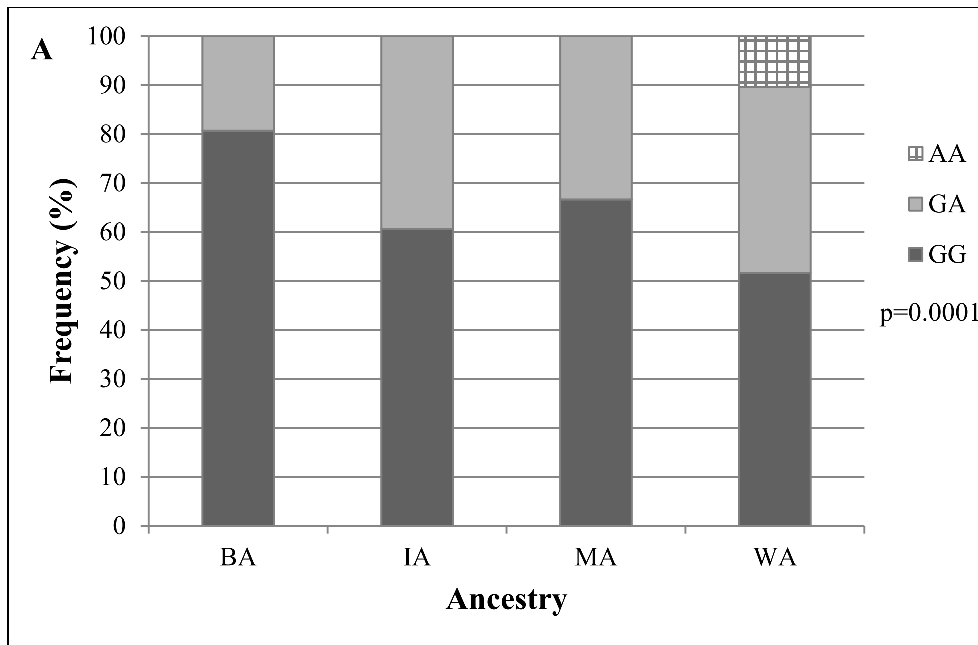


Figure 3.4. Mean chronotype in the individual ancestry groups: The chronotype based on H-Ö score was compared among the all the ancestry groups that accounted for a minimum of 12% of the population in either the Eastern or Western populations, BA (Black ancestry, n=66), IA (Indian ancestry n=42), MA (Mixed ancestry n=60) and WA (White ancestry n=253). Kruskal-Wallis ANOVA by ranks was used for this analysis.

3.3 *PER2* and *PER3* Genotype Frequency

A comparative analysis of *PER2* and *PER3* genotype frequency among ancestry groups BA, IA, MA and WA without regard to East or West was carried out using a Pearson's Chi-squared test, Figure 3.5.



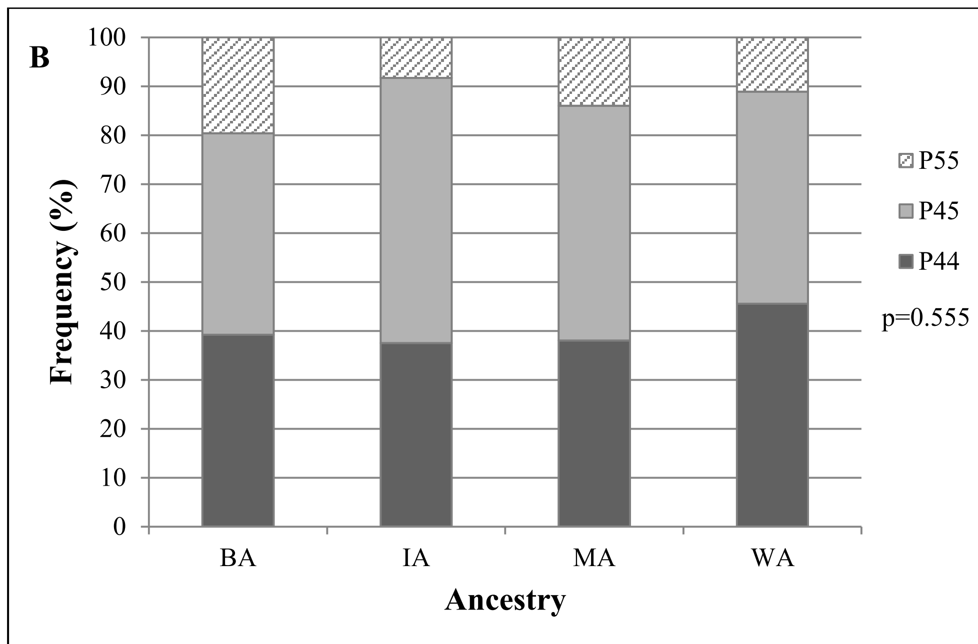
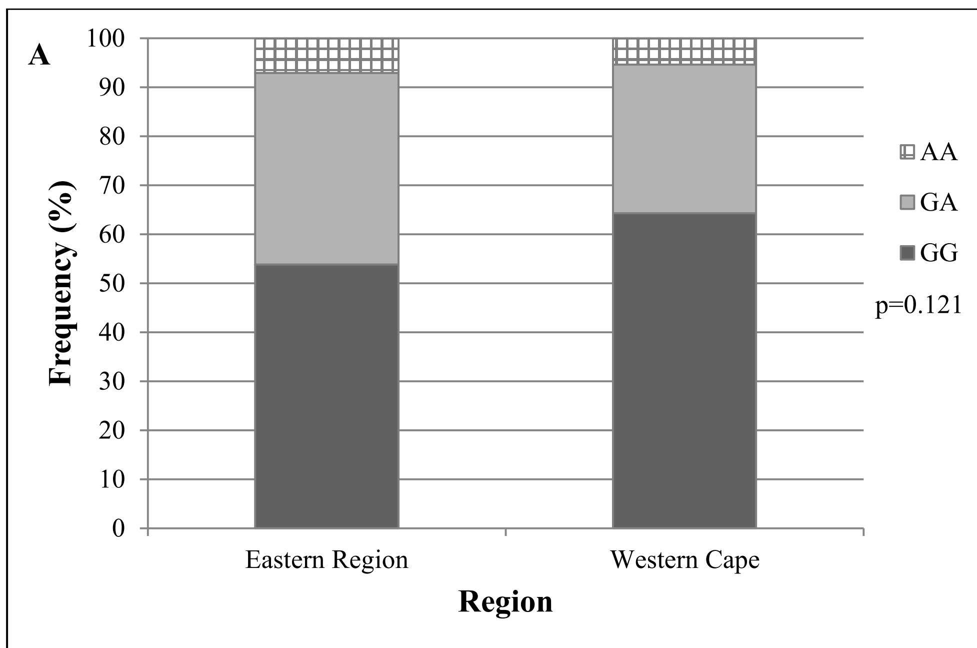


Figure 3.5. Genotype frequency distribution for *PER2* (A) and *PER3* (B) in the individual ancestry groups: *PER2* and *PER3* genotype frequencies were compared among the ancestry groups which made up at least 12% of either the Eastern or Western populations using the Pearson's Chi-Squared test. For *PER2* BA (n= 57), IA (n=33), MA (n=54) and WA (n=221). For *PER3* BA (n= 51), IA (n=24), MA (n=50) and WA (n=189). BA: black ancestry, IA: Indian ancestry, MA: mixed ancestry and WA: white ancestry, GG, GA and AA, are the *PER2* genotypes. P44, P45 and P55 represent the *PER3* genotypes $PER3^{4/4}$, $PER3^{4/5}$ and $PER3^{5/5}$ respectively.

This analysis revealed a significant difference in *PER2* genotypes among the different ancestries (p=0.0001), this remained significant after Bonferroni's post hoc correction. The

homozygous genotype of the rare A alleles is represented only in the WA group, possibly because of the greater sample size. The BA group has the highest frequency of the GG genotype (80%). A similar analysis was done of *PER3* genotype among the ancestry groups. No significant difference was found, p=0.555. All the *PER3* genotypes were represented in each ancestry group.

The *PER2* and *PER3* genotype frequencies were compared between Eastern and Western populations in Figures 3.6 A and B respectively. Differences in *PER2* genotype frequencies were insignificant (p=0.121) between East and West.



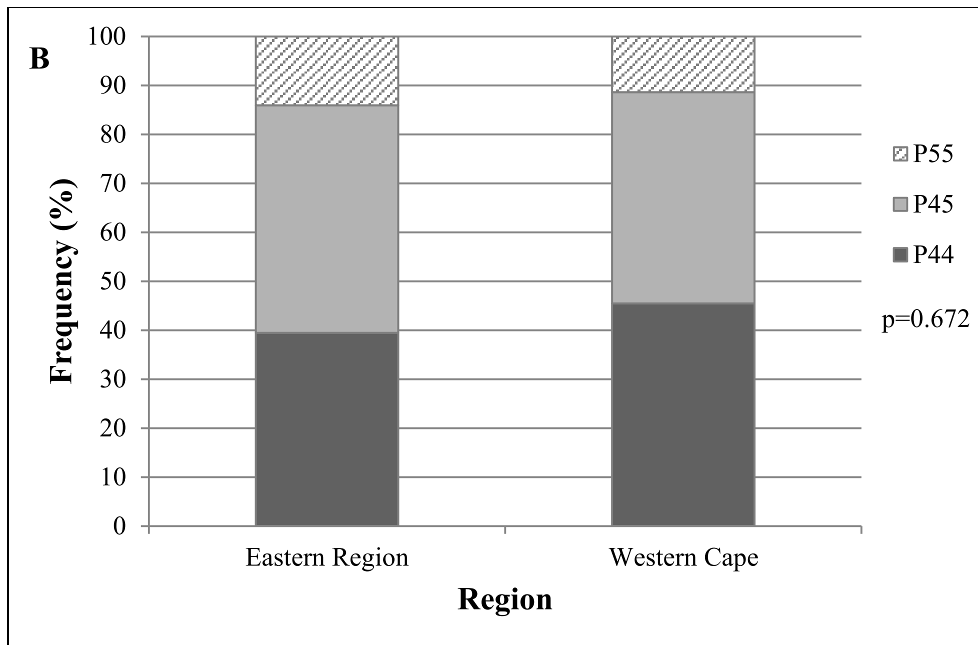
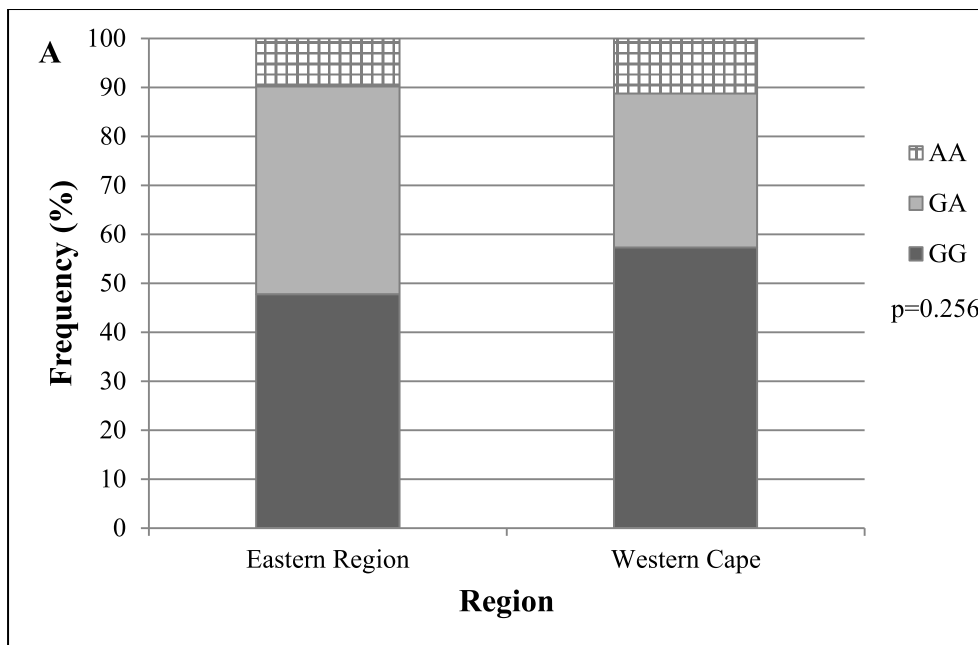


Figure 3.6. Genotype frequency distribution for *PER2* (A) and *PER3* (B): The frequency distribution of the *PER2* genotypes in the East (n=184) and West (=185) with all ancestry groups included was analysed.GG, GA and AA represent the three *PER2* genotypes. The same comparison was carried out for *PER3* in the East (n= 142) and West (n=176). P44, P45 and P55 represent the *PER3* genotypes $PER3^{4/4}$, $PER3^{4/5}$ and $PER3^{5/5}$ respectively. The Pearson's Chi-squared test was used for this analysis.

The *PER3* genotype also did not show a significant difference between the Eastern and Western populations (p=0.672). Despite the differences in *PER2* genotype frequencies being insignificant between Eastern and Western populations, there is at least a 9% difference in the GA and GG frequencies between the populations. This difference may be as a result of the difference in representation of the ancestry groups between these populations. Therefore a comparison of *PER2* and *PER3* genotype frequencies between Eastern and Western populations was again carried out however, in this analysis the only WA group was included, Figure 3.7.

PER2 genotype frequencies were not significantly different (p=0.256) within the WA group in the Eastern and Western populations. There was no significant difference in *PER3* genotype frequency within the WA group in the Eastern and Western populations (p=0.404) either.



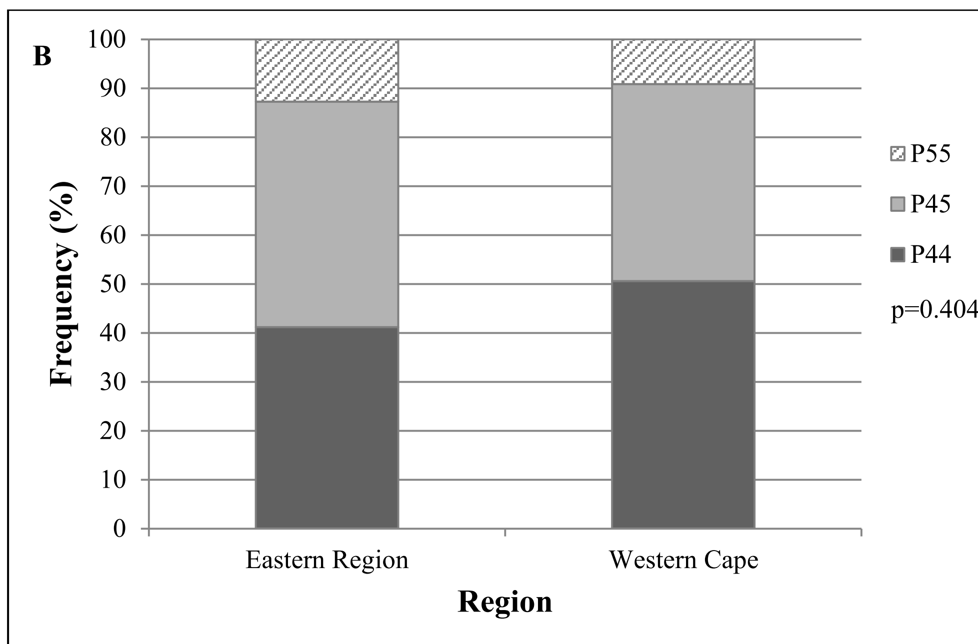


Figure 3.7. Genotype frequency distribution for *PER2* (A) and *PER3* (B) within the white ancestry group: The frequency distribution of the *PER2* genotypes was compared between the Eastern region (n=132) and the Western Cape (n=89) within the white ancestry group. GG, GA and AA represent the *PER2* genotypes. The frequency distribution of the *PER3* genotypes was also compared between the Eastern region (n=102) and the Western Cape (n=87) within the white ancestry group. P44, P45 and P55 represent the *PER3* genotypes $PER3^{4/4}$, $PER3^{4/5}$ and $PER3^{5/5}$ respectively The Pearson's Chi-squared test was used to perform the analyses.

A combination of the *PER2* and *PER3* genotypes results in 9 different genotypes. The distribution of these genotypes was compared between the Eastern and Western populations, Figure 3.8, excluding AAP55 because only one participant had this genotype.

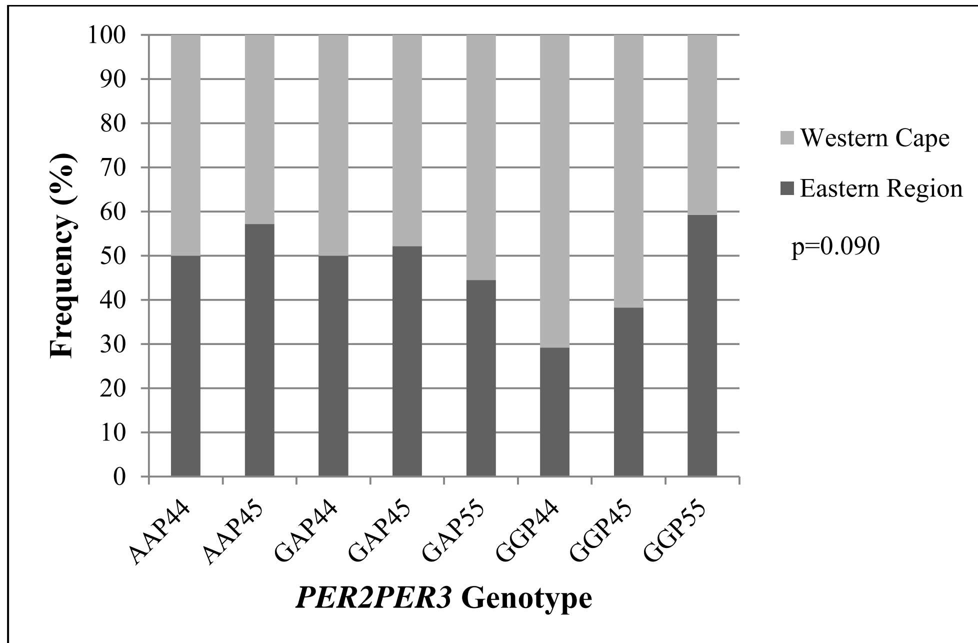


Figure 3.8. *PER2PER3* genotype frequency distribution: The frequency distribution of eight genotypes resulting from the combination of the *PER2* and *PER3* genotypes were compared between the Eastern (n=122) and Western populations (n=164). The Pearson's Chi-Squared test was used for this analysis.

The differences observed in the distribution of the *PER2PER3* genotypes between East and West are not significant based on Pearson's Chi-squared test which gave a p-value of 0.090.

3.4 *PER2* and *PER3* allele frequency distribution

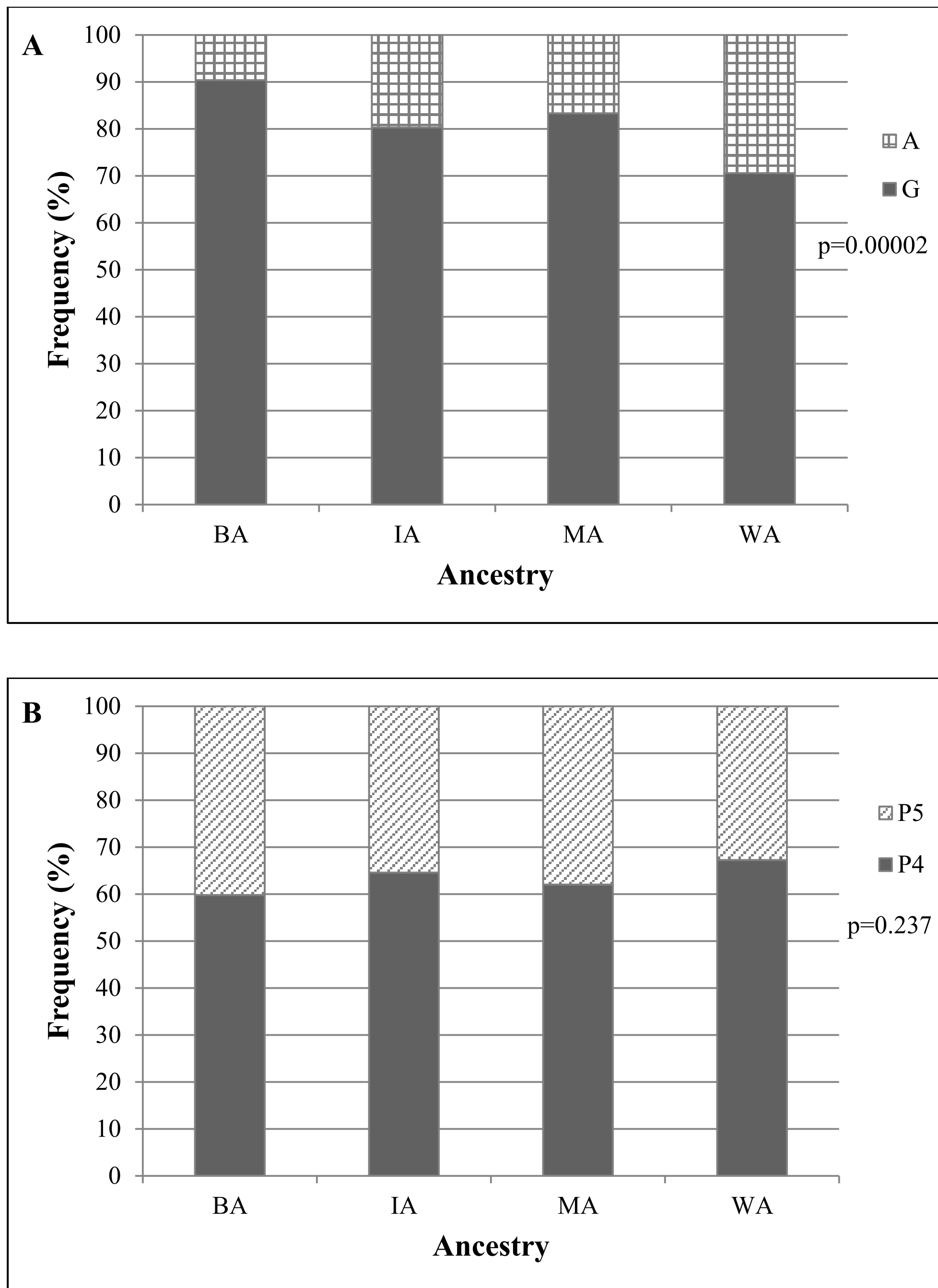
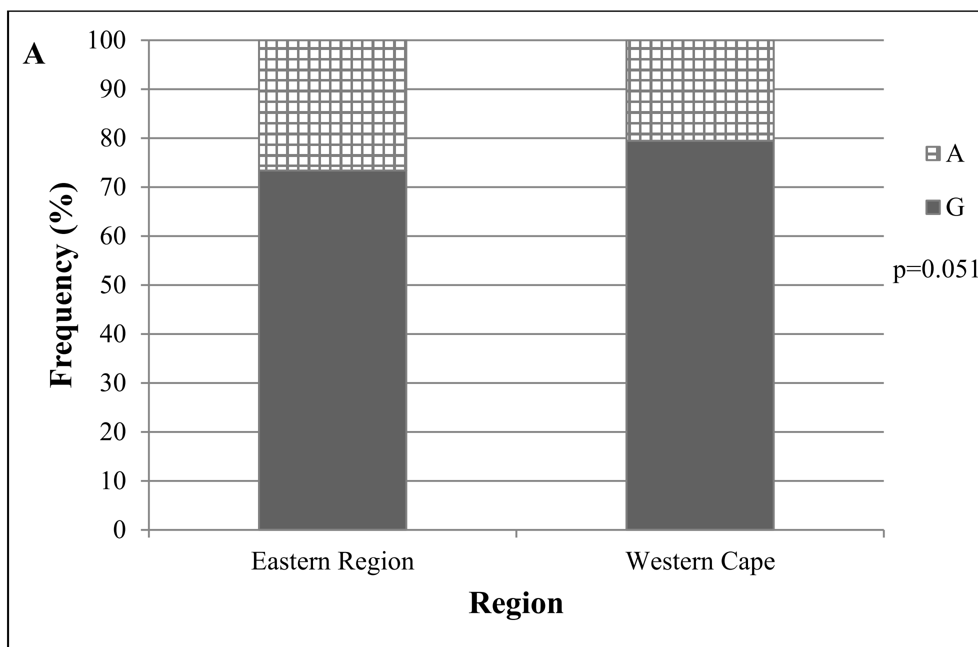


Figure 3.9. Allele frequency distribution for *PER2* (A) and *PER3* (B) in the individual ancestry groups: *PER2* and *PER3* allele frequencies were compared among the ancestry groups which made up at least 12% of either the Eastern or Western populations. For *PER2* BA (n= 57), IA (n=33), MA (n=54) and WA (n=221). For *PER3* BA (n= 51), IA (n=24), MA (n=50) and WA (n=189). BA: black ancestry, IA: Indian ancestry, MA: mixed ancestry and WA: white ancestry, G and A, represent the *PER2* alleles, P4 and P5 represent the *PER3* alleles $PER3^4$ and $PER3^5$ respectively. The Pearson's Chi-Squared test was used to perform the analysis.

Not surprisingly there is a significant difference (p=0.00002) among the ancestry groups in the *PER2* allele frequency distribution, Figure 3.9A, which was anticipated based on the observed the genotype frequencies (Figure 3.5A). This finding remained significant even after Bonferroni's post hoc correction. The frequency of the G allele ranged from 90% in the BA to 68.8% in the WA. The *PER3* allele frequency distribution was not significantly different (p=0.237) between Eastern and Western populations. The *PER3*[4] has the higher frequency in all ancestry groups.



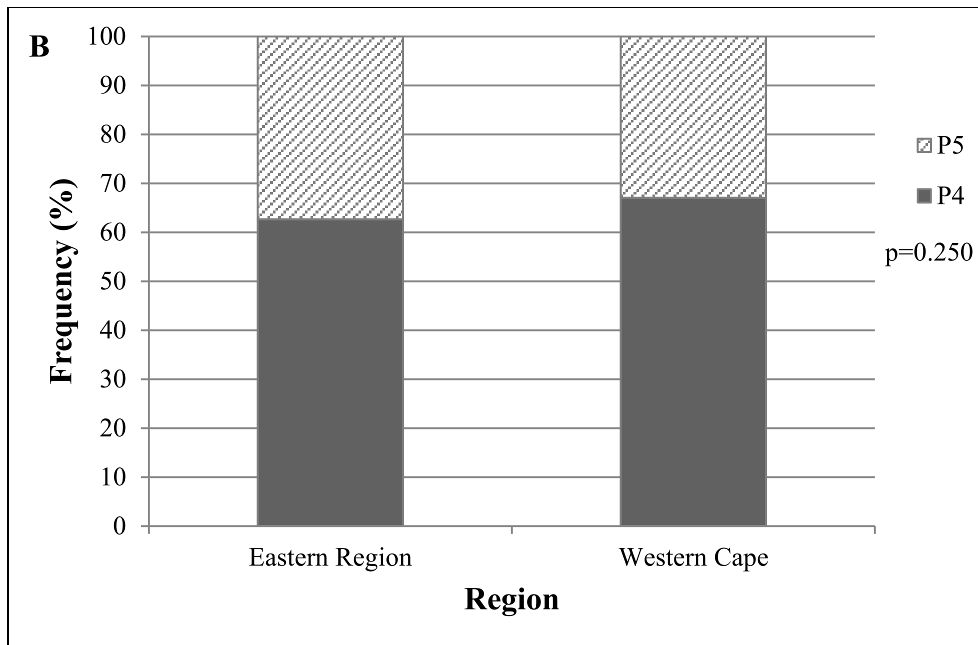
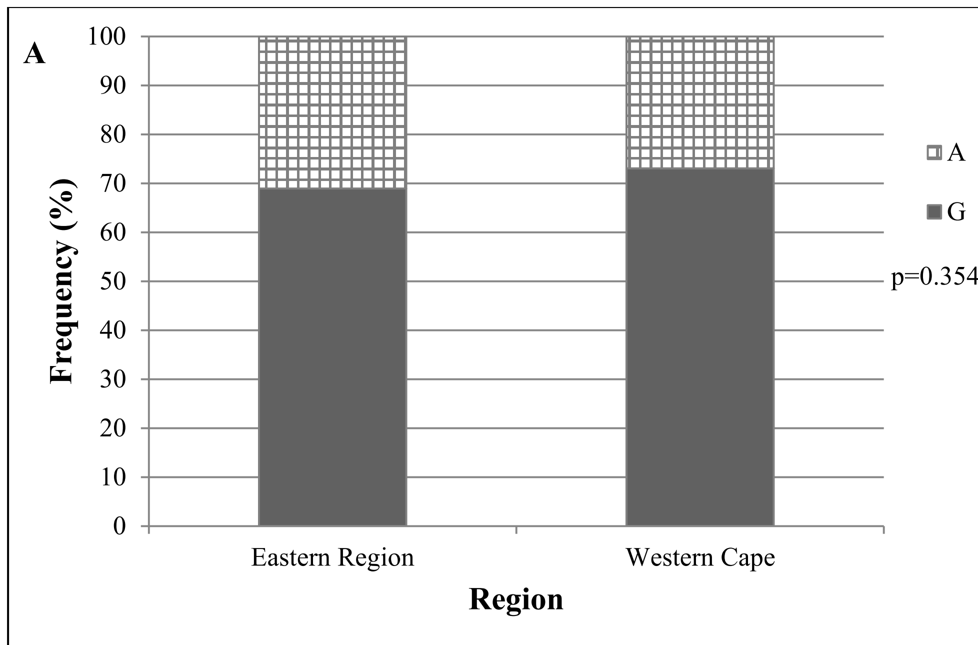


Figure 3.10. Allele frequency distributions for *PER2* (A) and *PER3* (B): The frequency distribution of *PER2* alleles in the East (n=184) and West (=185) with all ancestries included was analysed, A and G represent the *PER2* alleles. The same comparison was carried out for *PER3* in the East (n= 142) and West (n=176) P4 and P5 represent the *PER3* alleles $PER3^4$ and $PER3^5$ respectively. The Pearson's Chi-squared test was used for this analysis.

The p-value was just above the level set for significance (p=0.051) for the *PER2* allele distribution analysis, Figure 3.10A. This almost significant result can be attributed to the difference in genotype distribution among the ancestry groups which are part of the Eastern and Western populations. The fact that it is not significant may be attributed to the large representation of the WA group in both Eastern and Western populations which has a major influence on the allele frequency given it had a genotype (AA) that was absent in any of the other ancestry groups (refer to Figure 3.5A). There was no significant difference in the *PER3* allele frequency distribution between East ($PER3^4$=62.7% and $PER3^5$=37.3%) and West ($PER3^4$=67.1% and $PER3^5$=32.9%), Figure 3.10B.



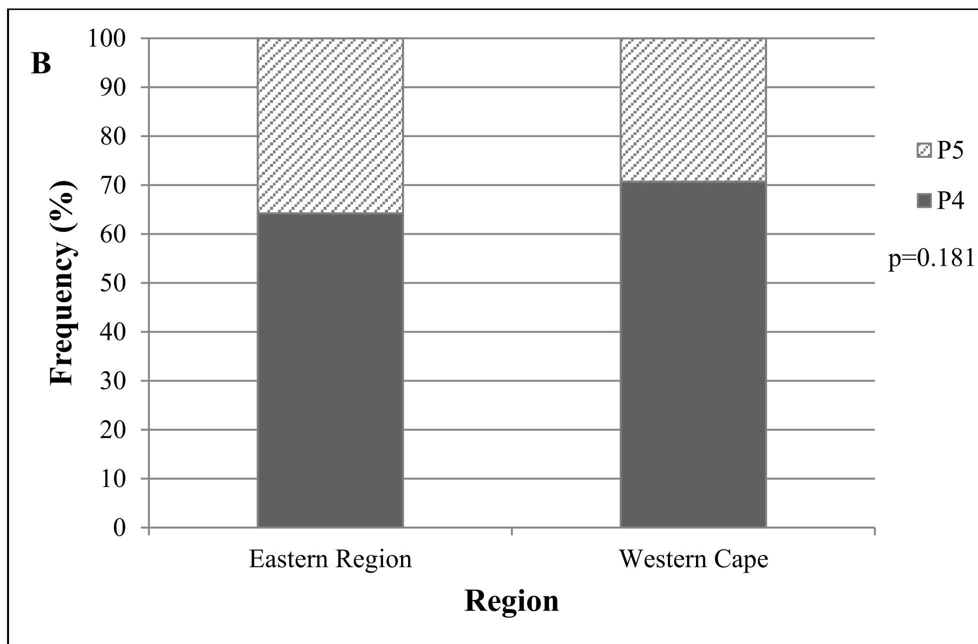
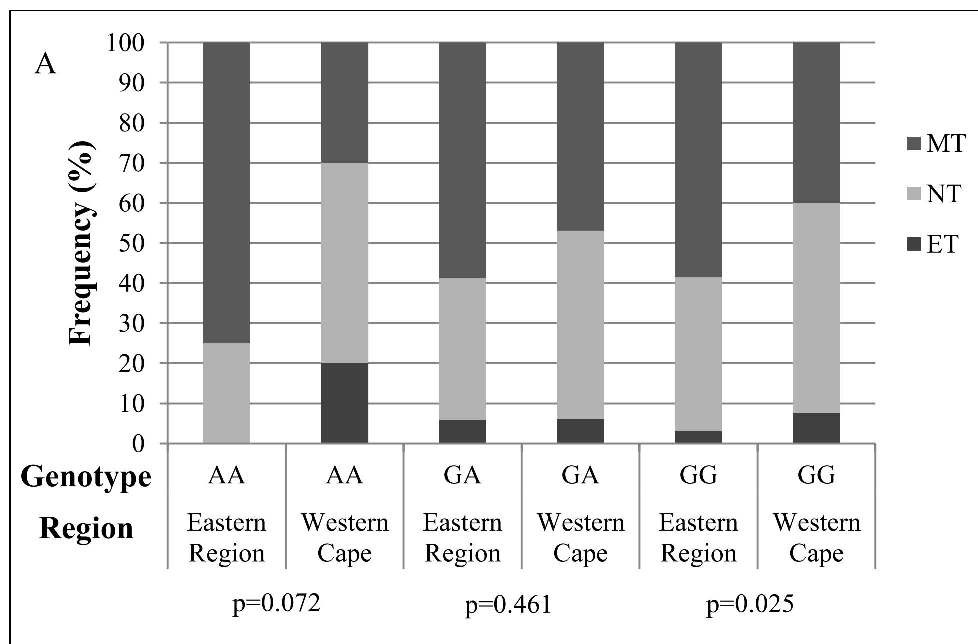


Figure 3.11. Allele frequency distribution for *PER2* (A) and *PER3* (B) within the white ancestry group: The frequency distribution of the *PER2* alleles was compared between the Eastern region (n=132) and the Western Cape (n=89) within the white ancestry group, G and A represent the *PER2* alleles. The frequency distribution of the *PER3* alleles was also compared between the Eastern region (n=102) and the Western Cape (n=87) within the white ancestry group. P4 and P5 represent the *PER3* alleles $PER3^4$ and $PER3^5$ respectively The Pearson's Chi-squared test was used to perform the analyses.

PER2 and *PER3* allele frequency distribution was examined within the white ancestry group, Figure 3.11. The *PER2* allele frequency distribution did not differ significantly in the white ancestry groups in the East and the West (p=0.354). The *PER3* allele frequency distribution showed no significant difference between the white ancestry groups in the East and the West either (p=0.181).

3.5 Relationships between *PER2* and *PER3* genotypes and chronotype

Since chronotype is influenced by both environment and genetics (Vink *et al*., 2001) the relationship between chronotype and the *PER2* and *PER3* genotypes was investigated in the Western Cape and Eastern region. These analyses can give insight into which environment results in a stronger genotype-phenotype association. An analysis was performed which compared how chronotype was distributed within each *PER2* (AA, GA and GG) and *PER3* ($PER3^{4/4}$, $PER3^{4/5}$ and $PER3^{5/5}$) genotype between the Eastern and Western populations using the two tailed Fisher's exact test for the analyses, Figure 3.12.



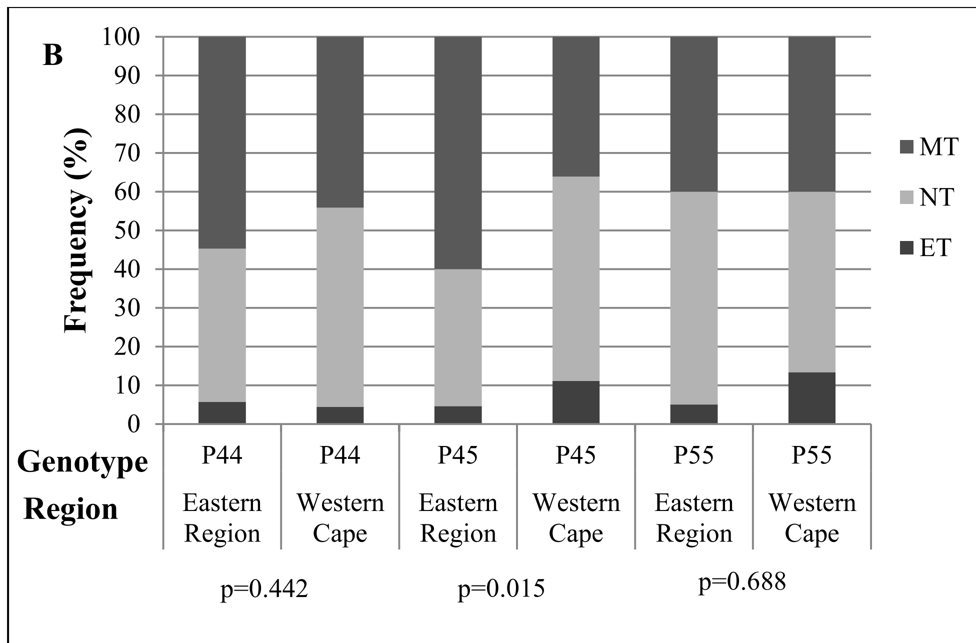


Figure 3.12. Chronotype frequency distribution in *PER2* (A) and *PER3* (B) genotypes: The chronotype frequency in each *PER2* genotype was compared between the Eastern (n= 174) and Western (n=164) populations, and the same comparison was made for the *PER3* genotypes in the Eastern (n=138) and Western populations (n=155). MT (morning-type), NT (neither-type) and ET (evening-type), GG, GA and AA represent the *PER2* genotypes and P44, P45 and P55 represent the *PER3* genotypes $PER3^{4/4}$, $PER3^{4/5}$ and $PER3^{5/5}$ respectively. The two tailed Fisher's exact test was used for these analyses.

The difference in chronotype distribution between East (MT: 75% and NT: 25%) and West (MT: 30%, NT: 50% and ET: 20%) for the *PER2* AA genotype was not significant (p=0.072). However, the evening-type chronotype was absent in the Eastern population. Despite these large differences in proportion in chronotype between the regions the differences are probably insignificant because of the low numbers of the AA genotype (n=12) in the East and (n=10) in the West. Likewise, there was no significant difference for the GA genotypes between the East and West. (p=0.462). Conversely the distribution of chronotype in the East and the West for the GG genotype was significantly different (p=0.025).

The $PER3^{4/4}$ and $PER3^{5/5}$genotypes showed no significant difference in chronotype distribution between the East and the West (p=0.442 and p=0.688 respectively). On the other hand there was a significant difference in chronotype distribution between East and West for the $PER3^{4/5}$ genotype (p=0.015).

The Kruskal-Wallis Analysis of Variance (ANOVA) by Ranks was used to test if significant relationships existed in the Eastern region between genotype and chronotype. To assess a

similar relationship in the West one-way ANOVA was used (this is because chronotype data was normally distributed in the West, refer to section.3.2) and a Chi-Squared test was used to assess genotype distribution.

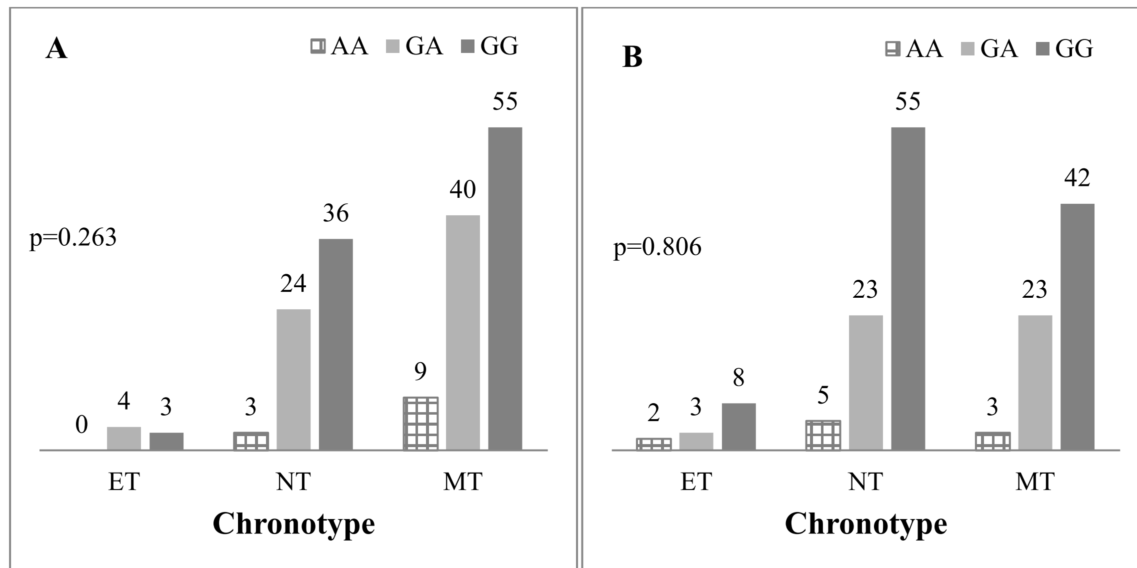


Figure 3.13. Relationship between *PER2* genotype and chronotype in the (A) Eastern and (B) Western populations: Correlation analyses were performed to investigate the relationship between *PER2* genotype and H-Ö score, in the Eastern region (n=174) and the Western Cape (n=164). MT (morning-type), NT (neither-type) and ET (evening-type), GG, GA and AA represent the *PER2* genotypes. The Kruskal-Wallis ANOVA by ranks was used for the analysis in the Eastern population and a one-way ANOVA was used for analysis in the Western population.

In Figure 3.13 the Eastern population showed no association between *PER2* genotype and chronotype (p=0.263). However, it is interesting to note that none of the participants in the Eastern population carrying the AA genotype were evening-types. The other two genotypes have all the chronotype categories represented in both populations, and the Chi-Squared test revealed that overall in the Eastern population there was no bias in genotype distribution (χ^2=3.8, p=0.146). In the Western Cape population, similarly, no relationship was found between *PER2* genotype and chronotype (F=0.215, p=0.806) and the three genotypes were again evenly distributed (χ^2=1.7, p=0.431). Each of the genotypes has all the chronotypes represented.

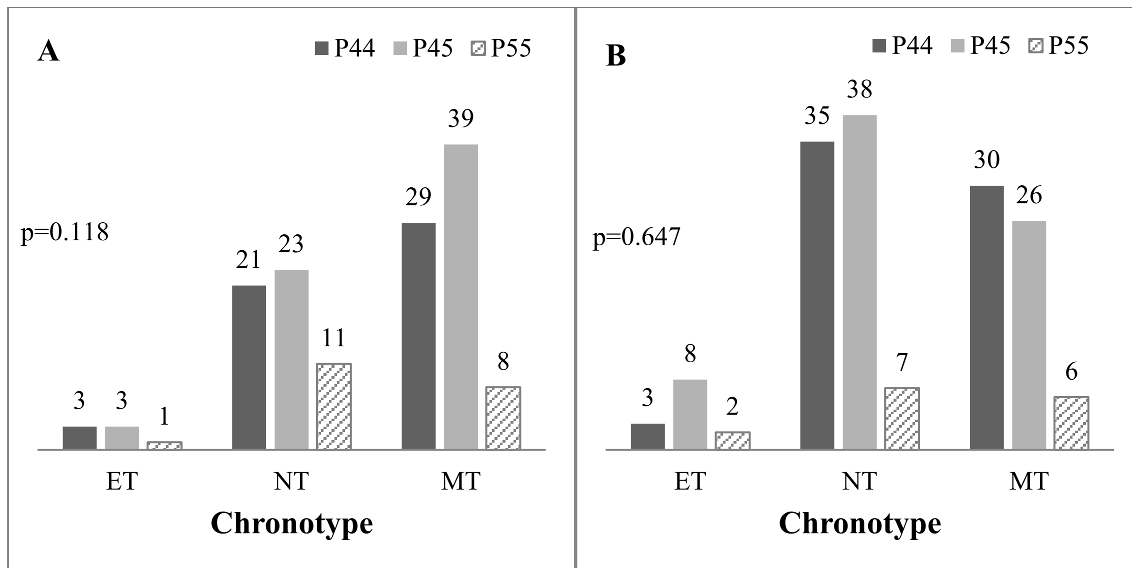


Figure 3.14. Relationship between *PER3* genotype and chronotype in the (A) Eastern and (B) Western populations: Correlation analyses were performed to investigate a relationship between *PER3* genotype and H-Ö score in the Eastern region (n= 138), and the Western Cape (n=155). MT (morning-type), NT (neither-type) and ET (evening-type), P44, P45, and P55 represent the *PER3* genotypes $PER3^{4/4}$, $PER3^{4/5}$ and $PER3^{5/5}$ respectively. Kruskal-Wallis ANOVA by ranks was used for the analysis in the Eastern population and a one way ANOVA was used for analysis in the Western population.

There was no association found between *PER3* genotype and chronotype, in both the Eastern (p=0.118) and in the Western populations (F=0.437, p=0.647), Figure 3.14. Each chronotype is represented within each genotype and the distribution of genotype shows no bias in the East (χ^2=3.8, p=0.149) or in the West (χ^2=1.3, p=0.510). In case the presence of more than one ancestry group was masking the relationship between genotype and phenotype this relationship was investigated in the white ancestry group only, Figure 3.15.

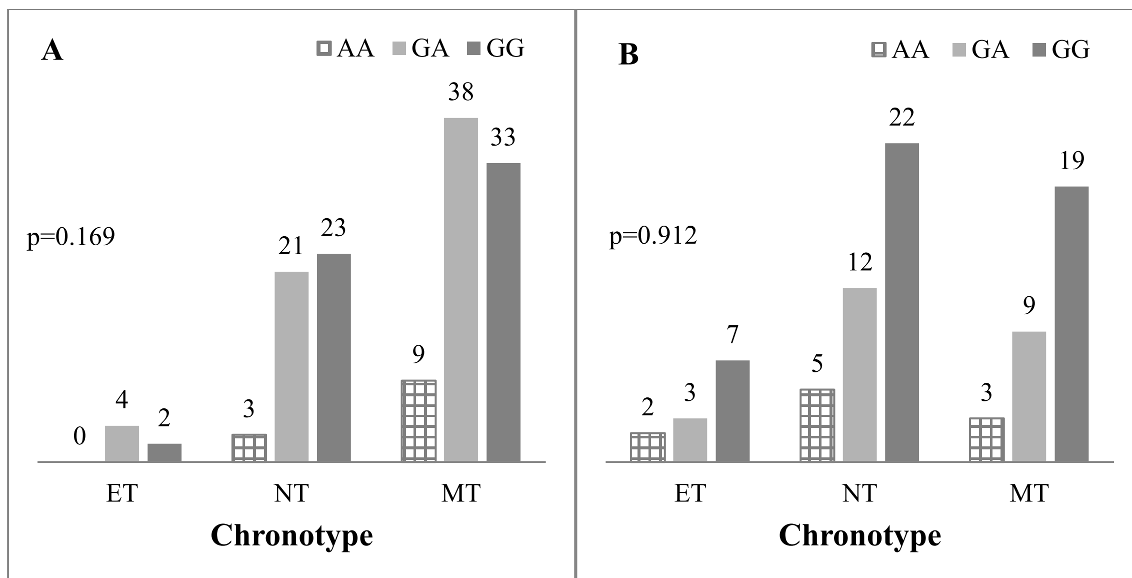


Figure 3.15. Relationship between *PER2* genotype and chronotype within the White ancestry group in the Eastern (A) and Western (B) populations: Correlation analyses were performed to investigate a relationship between *PER2* genotype and H-Ö score in the Eastern (n=133) and Western (n=82) populations within the white ancestry group. MT (morning-type), NT (neither-type) and ET (evening-type), AA, GG and GA represent the *PER2* genotypes. Kruskal-Wallis ANOVA by ranks was used for analysis in the Eastern population and a one way ANOVA was used for analysis in the Western population.

No correlation between *PER2* genotype and chronotype was observed both in the Eastern population, p=0.169 and in the Western population, F=0.082, p=0.921 within the white ancestry group. There was also no significant bias in chronotype distribution in the East (χ^2=4.2, p=0.125) or in the West (χ^2=0.47, p=0.789).

The relationship between *PER3* genotype and chronotype was also investigated within the white ancestry group in both the Eastern and Western populations, Figure 3.16.

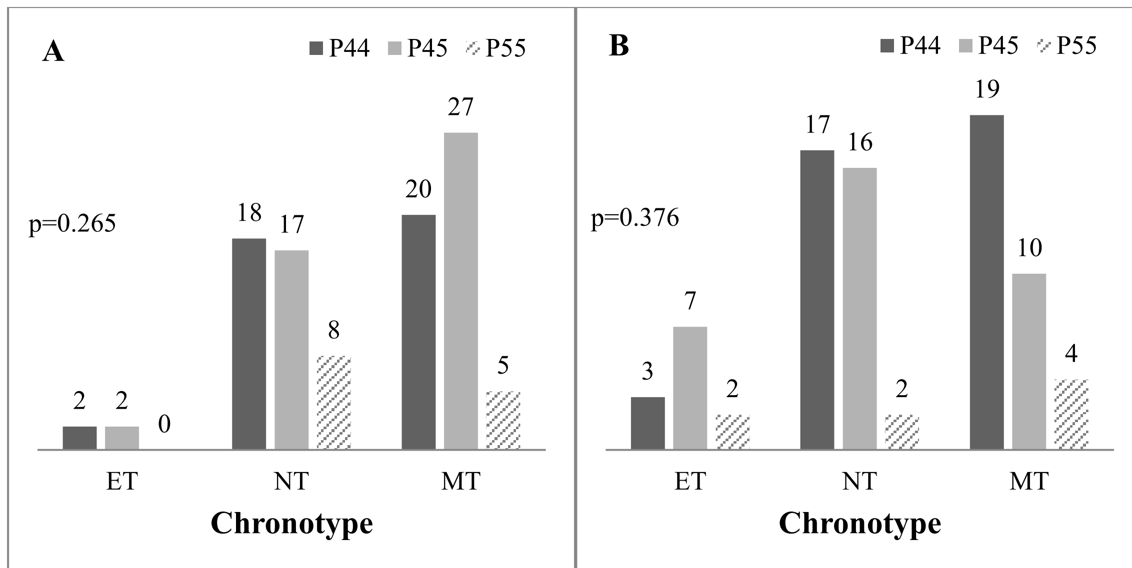


Figure 3.16. Relationship between *PER3* genotype and chronotype within the White ancestry group in the Eastern (A) and Western (B) populations: Correlation analyses were performed to investigate a relationship between *PER3* genotype and H-Ö score in the Eastern (n=99) and Western (n=80) populations within the white ancestry group. MT (morning-type), NT (neither-type) and ET (evening-type), P44, P45, and P55 represent the *PER3* genotypes $PER3^{4/4}$, $PER3^{4/5}$ and $PER3^{5/5}$ respectively. Kruskal-Wallis ANOVA by ranks was used for analysis in the Eastern population and a one way ANOVA was used for analysis in the Western population.

No association was found between *PER3* genotype and chronotype neither in the East (p=0.265) nor in the West (F=1.156, p=0.376). There was also no significant bias in distribution of chronotype in the East (χ^2=2.42, p=0.298) or in the West (χ^2=0.58, p=0.747).

Chapter 4

4.0 DISCUSSION

4.1 Chronotype and the environment

The comparison of chronotype distribution in populations from the East and West of South Africa revealed a significantly higher proportion of morning-types in the East. Three fifths (60.6%) of the population in the Eastern region had a preference for mornings whereas in the West it was only two fifths (40.5%). The neither-types were in the majority in the West as they accounted for 50.8% of the population. Evening-types were the least in both populations accounting for less than 5% in the East and just above 8% in the West (Figure 3.1). The western population was made up of participants living in Cape Town at 34°5.3'S/18°35.3'E. Participants from the Eastern region were recruited from Pietermaritzburg at 29°36.0'S/30°22.8'E and Johannesburg at 26°12.2'S/28°2.8'E. All three towns are in the subtropics, and although Cape Town is at the lowest latitude the mean yearly temperature ranges are similar. Cape Town has a photoperiod ranging from approximately 9h and 53min in the winter to 14h and 25min in the summer and its average yearly temperatures range from approximately 7°C to 28°C. Pietermaritzburg and Johannesburg have similar day lengths to Cape Town, approximately 14h 30min and 13h 47min in summer, and 10h 14min and 10h 29min in winter respectively. However, the sunrise and sunset times in Pietermaritzburg and Johannesburg differ from those of Cape Town (Table 4.1). The mean yearly temperature ranges from 6˚C to 30˚C in Pietermaritzburg and 3˚C to 26˚C in Johannesburg.

The approximate 30-45min difference in maximal summer photoperiod between Johannesburg/Pietermaritzburg and Cape Town can be attributed to the 5-6° difference in latitude, since summer photoperiod increases with latitude (Randler, 2008a). This difference may also have an effect on chronotype. Borisenkov (2010) reported that adolescents in the republic of Komi (Russia) residing in towns 4.5^{o} apart in terms of latitude showed a significantly higher frequency of evening-chronotypes in the more Northerly town. Furthermore, on comparing a population from Central Europe at a lower latitude to a population residing in the Komi Republic, Borisenkov (2010) again observed a significantly higher frequency of late chronotypes at the higher latitude. This was attributed to photoperiod. The findings of Borisenkov (2010) in the two towns of Komi Republic are comparable to the findings of the current study as the towns in South Africa are also within

the same climate zone but differ in latitude. However, there is another perhaps stronger influence on chronotype which needs consideration.

There is an approximately 12° difference in longitude between Cape Town and Pietermaritzburg which is 80% of a time zone (15°). As time zones of 15° were arrived at by Sir Sandford Flemming because the Earth turns 15° (360° ÷ 24) every hour, this results in a considerable difference in the times of sunrise and sunset observed between these cities. There is a continuum of sunrise (Roenneberg *et al*., 2007a) from East to West with the sun rising and setting earlier in the East than the West throughout the year.

Table 4.1. Sunrise and sunset times for Cape Town, Johannesburg and Pietermaritzburg at the summer and winter solstices. *The time difference relative to Cape Town is indicated in parentheses.*

	Cape Town	**Johannesburg**	**Pietermaritzburg**
December 21			
Sunrise	05h31	05h12 (-19 min)	04h55 (-36 min)
Sunset	19h57	18h59 (-62 min)	18h58 (-61 min)
June 21			
Sunrise	07h51	06h54 (-57 min)	06h48 (-63 min)
Sunset	17h43	17h24 (-19 min)	17h06 (-37 min)

Roenneberg *et al*. (2007a) reported a strong correlation between sunrise time and chronotype in a large German population divided into longitudinal bins. A study conducted by Randler (2008b) reported that a population of adolescents in the eastern part of Germany were less evening oriented than a matched population of adolescents in the West living on similar social schedules during the week. He suggested that a 20-30min difference in sunrise time from May to August may contribute to this difference. Borisenkov *et al*. (2010) reported that in a cohort of school children and college students aged 11 to 23 living in four towns located between 40° latitude north and the polar circle, those living in the town that was the furthest West and North had the greatest shift to evening-chronotype. Borisenkov *et al*. (2010) also observed that from South to North and from East to West there was a significant phase delay in sleep-wake rhythm. The question is why do we observe these changes?

Photoperiod varies with latitude as well as with the seasons throughout the year. This results in fluctuations in sunlight exposure which is the strongest of *zeitgebers* according to current knowledge (Pedrazzoli *et al*., 2007). Randler, (2008a) reported that adolescents residing in the tropics where more morning oriented than those living in the subtropics and the temperate zone. In the tropics there is a smaller fluctuation in photoperiod and there is little variation in sunlight abundance and temperature throughout the year. Therefore, the constancy of light emitted by the sun reinforces its potency as a *zeitgeber* (Pedrazzoli *et al*., 2007) able to entrain the circadian oscillator to an earlier phase. In contrast, the variation in photoperiod in the subtropics (e.g. Cape Town) throughout the year may lead to a weaker entrainment of the circadian oscillator to sun rise time. The variation in day length in Johannesburg is similar to what was reported for São Paulo (Brazil) by (Pereira *et al.*, 2005).

In this study, the difference in chronotype is very clear from East to West which suggests that the endogenous phase of the majority of the Western population is delayed relative to clock time in comparison to the Eastern population. People in both the East and West are expected to work and/or attend school at the same. This may have a negative impact on adolescents in particular in the Western part of the country. Previous reports have shown that as children enter adolescence and go through to young adulthood they become evening-chronotypes (Randler, 2008a) before they start to shift toward morningness (Roenneberg *et al*., 2004). If this is further exacerbated by living in the Western part of the country as observed in the adult population, it may put them at a disadvantage. Their endogenous clocks entrained to local sunrise and sunset times will prepare them to wake up at a later clock hour than their social schedule will allow. In addition to this their endogenous clocks and later sunset times will compel them to sleep at a later clock time than they should in order to get enough sleep. This ultimately results in continual misalignment. This trend has been observed in European adolescents (Wittmann *et al*., 2006). The study by Randler (2008a) revealed that adolescents in the subtropics were considerably later evening-types than those in temperate climate zones. The disparity between social time and endogenous time in the West of South Africa may also lead to lower productivity in workers particularly in winter when the sun only rises at 07h50 and the majority of people have to depend on artificial light in the morning. This may result in a phase delay as artificial light is a weak *zeitgeber* (Wright *et al*., 2013) and lower levels of alertness during the time of day when one is supposed to be at one's most productive (Kerkhof and Van Dongen, 1996). This finding serves as confirmation that sunrise which is determined by longitude is indeed a strong *zeitgeber*.

4.2 Effect of age, sex and ethnicity on chronotype

Age is a factor that affects chronotype (Robilliard *et al*., 2002; Jones *et al*., 2007).The current study attempted to limit the impact of this variable by studying participants between the ages of 25 and 55 because between the ages of 11 to 21 there is a steady shift from early to late chronotype (Borisenkov *et al*., 2010; Randler, 2008a) and individuals above the age of 55 show a tendency towards morningness (Carciofo *et al*., 2012). A significant positive correlation between age and chronotype was observed in the combined Eastern and Western populations (see Figure 3.3), which concurs with what is reported in literature (Robilliard *et al*., 2002). The mean age of the Eastern population (39.1±8.8, age ± SD) was significantly higher (p=0.00005) than that of the Western population (35.6±8.2, age ± SD) which may have contributed to the observed chronotype distribution of more morning-types in the Eastern population.

Sex did not appear to influence chronotype in this study as there were no significant differences in mean chronotype between males and females nor were any differing trends in chronotype observed (refer to Figure 3.2). Certain studies have found a difference (Vink *et al.*, 2001) and others have not (Choub *et al.*, 2011), but Randler (2007) observed that differences between the sexes were noticeable in larger populations. Randler (2007) used Natale and Danesi (2002) with a sample size of 1319 participants as an example of a population large enough to detect significant differences between the chronotype scores of males and females.

The distribution of ancestry groups between regions was significantly different (Table 3.1). The Western region had a higher proportion of the mixed ancestry group than of the Indian ancestry group whereas the Eastern population had the reverse. Nonetheless, mean H-Ö score did not differ significantly between populations of different ancestry when they were grouped without regard to region (Figure 3.4). This result, nevertheless, cannot be generalised to the wider South African population because the numbers of black, Indian and mixed ancestry participants were very low and as a result require further investigation. However, this finding concurs with that of Paine *et al.* (2006) who reported that ethnicity did not influence chronotype.

4.3 Clock genes and ethnicity

It was interesting to note the *PER2* AA genotype was present only in individuals of white ancestry. The black ancestry group had the highest proportion of the GG genotype. This may be because black Africans are the oldest known lineage of modern man (Schuster *et al*., 2010) and therefore have a higher proportion of the G allele which is the ancestral allele (Cruciani *et al*., 2008). The GA genotype frequency was similar in the white, Indian and mixed ancestry groups but the black ancestry group had the lowest frequency of this genotype. The GG genotype was at the lowest frequency in the white ancestry group. The white and black ancestry groups had the most dissimilar *PER2* genotype frequency distributions (Figure 3.5). The differences in genotype frequency distribution among ancestry groups resulted in corresponding differences in allele frequencies. The A allele had a frequency of 0.10 in the black ancestry group and 0.31 in the white ancestry group. The Indian and mixed ancestry groups had similar frequencies of A and G alleles between them (Figure 3.10). These observations may be attributed to the low numbers of participants in the black, Indian and mixed ancestry groups. Despite the low representation of the black, Indian and mixed ancestry groups, the differences observed in *PER2* genotype and distribution may be intrinsic differences due to ethnicity. Entries in the 1000 genome browser (NCBI Genbank) of other world populations have shown AA genotype frequency as low as 0.091 with the frequency of the A allele being 0.182 in an Asian population. Another Asian population had 0.023 as the AA genotype frequency and 0.279 for the A allele. In two separate Sub-Saharan African populations the AA genotype and A allele frequencies were both 0.0 with only the GG genotype being represented.

Ojeda *et al*. (2013) investigated the *PER2 G3853A* SNP in a population in Bogotá Columbia which had a European genetic background with some historical admixture with Amerindians, and reported the following genotype frequencies: GG 46.9%, GA 41.1% and AA 12%. Of the four ancestries in the current study, this was most similar to the white ancestry group which had 11.7% of the AA genotype (Figure 3.5A). Lee *et al*. (2011) observed allele frequencies in the *PER2 G3853A* polymorphism genotypes similar to those reported by Ojeda *et al*. (2013). Barbosa *et al*. (2010) observed that among studies conducted involving clock genes in different populations, similarities in allelic frequencies intra-group were common within Caucasian or Asian studies however, this was not the case when comparisons were made between different ethnic groups. The observations in the current study of the *PER2* genotype

serve to confirm the observations of Barbosa *et al*. (2010) concerning the differences in circadian gene genotype distribution among different ethnicities. In addition a study conducted by Cruciani *et al*. (2008) examined eleven populations for five *PER2* SNPS including the *PER2 G3853A* and reported on the frequencies of the observed haplotypes in these populations. They did not observe any association between latitude and haplotype but however did observe high levels of inter-population genetic differentiation for the *PER2* gene. Therefore they suggest that *PER2* may have been subjected to geographically restricted positive selection. They also observed that the G allele was by far the dominant allele in the two African populations they studied.

No significant differences in *PER3* VNTR genotype and allele frequencies were observed between the ancestry groups (see Figure 3.5B). Each genotype was represented in each ancestry group, with the $PER3^{5/5}$ genotype being the least represented across all groups. The $PER3^{5}$ allele was the minor allele in each ancestry group, which is similar to the findings of Carleglio *et al*. (2008). They screened different ethnic populations from different environments and latitudes: African American, European American, Han Chinese, Ghanaian and Papua New Guinean for the *PER3* VNTR polymorphism. They observed that the $PER3^{4}$ allele was the major allele in all but the Papua New Guinean population. The $PER3^{5}$ allele frequency was between 0.34 and 0.41 in the European American, African American and Ghanaian populations. This is similar to what was observed in all the ancestry groups in the current study (Figure 3.10B). The Papua New Guinean population was divided into subpopulations and the $PER3^{5}$ was observed to be the major allele in all but one population, however the numbers of these populations were very low, ranging from 4 to 23, which reduces the strength of the observations. Carleglio *et al*. (2008) stated that their Ghanaian and Papua New Guinean populations both reside close to the equator at similar latitudes where photoperiod is almost constant throughout the year, yet their allele frequencies were very different. They posited that clock genes may not necessarily play a role in the processes involved in adaptation to different photoperiods. They further speculated that rather the light input pathway may be optimised by natural selection depending on one's latitude or photoperiod because light is the most important synchroniser of the circadian oscillator. Despite reported differences in clock gene allele frequencies in different world populations, the circadian oscillator functions in a similar way in different ethnic groups (Smith *et al*., 2009). This indicates that the differences in clock gene allele frequency do not have a significant effect on the functioning of the circadian clock.

Nadkarni *et al*. (2005) also screened different populations around the world for the *PER3* VNTR. They screened a South African Bantu group and the $PER3^4$ allele frequency was 0.652 which is slightly higher than the black ancestry group (0.598) in the current study. An Indian population was also screened and the $PER3^4$ allele frequency was 0.576 which was lower than the Indian ancestry group (0.646) in the current study. Nadkarni *et al*. (2005) also looked at this allele in some European groups among them Danish and English which had 0.664 and 0.683 frequencies respectively which was similar to what was observed in the current study for the white ancestry group (0.672) (Figure 3.10B). The *PER3* VNTR genotype distribution within the control population of the Kunorozva *et al*. (2012) study was similar to those observed in the WA group in both the Eastern and Western regions of the current study.

4.4 Clock genes and region

Genotype and allele frequencies between the Eastern and Western populations did not differ significantly for either the *PER2* (p=0.121 and p=0.051) or *PER3* (p=0.672 and p=0.250) genes (Figure 3.6) despite the differences in population ancestry between the regions. Although the main difference was in the proportions of the mixed and Indian ancestry groups, the genotype frequency distributions within these two groups were similar and as a result the low representation of mixed ancestry in the East and of Indian ancestry in the West did not influence genotype distribution. To eliminate any differences due to ancestry a comparison was made between East and West excluding the black, Indian, and mixed ancestry groups. No significant difference was then observed in *PER2* genotype frequency between East and West, p=0.256 (Figure 3.7A). This can be attributed to the comparison being made within one ancestry group even if they were separated geographically. The *PER2* allele frequency distributions between Eastern and Western populations were at the borderline of significance (p=0.051), Figure 3.9A, when all population groups were included, and this was probably due to differences in ethnic composition of the two regions. Therefore, the differences seen in the *PER2* gene where not region specific but rather originated from the difference in ancestry group composition between the regions. The *PER2* and *PER3* genotypes when combined showed no significant difference in distribution between East and West (Figure 3.8). The *PER2* allele frequency in a Caucasian Columbian population was 0.33 for the A allele and 0.67 for the G allele which was comparable to the A allele frequency in the East (0.31) and in

the West (0.27) in the white ancestry group in the current study (see Figure 3.11A). The *PER3* VNTR allele distributions in Caucasians from different parts of the world are similar. Barbosa *et al.* (2010) reported frequencies of 0.69 and 0.31 for the $PER3^4$ and $PER3^5$ alleles respectively in a Caucasian population residing in Brazil. Osland *et al.* (2011) reported that their Norwegian population had an allele frequency of 0.67 for $PER3^4$ and 0.33 for $PER3^5$. Kunorozva *et al*. (2012) in their control population (South African) reported 0.38 for the $PER3^4$ allele and 0.62 for the $PER3^5$. These are similar to what was observed in the East with 0.36 for the $PER3^4$ and 0.64 for the $PER3^5$ alleles and in the West 0.71 and 0.29 for the $PER3^4$ and $PER3^5$ alleles respectively (see Figure 3.11B). This demonstrates that ethnicities maintain their allele frequencies despite migration. This confirms the hypothesis that no difference in clock gene genotype or allele frequency would exist between Eastern and Western parts of South Africa.

4.5 Genotype–phenotype relations

The chronotype distribution for the *PER2* AA and GA genotypes showed no significant difference between East and West, (Figure 3.13A, p=0.072 and p=0.461 respectively). In the case of the AA genotype it may be due to the low number of carriers. Conversely the GG genotype showed a significant difference in chronotype distribution between the Eastern and Western populations (p=0.025) similar to the overall chronotype distribution in the East and West (see Figure 3.1). It seems longitude and latitude exert influence on this particular genotype since the other two genotypes did not differ in chronotype distribution from East to West. Lee *et al.* (2011) using the CSM to determine chronotype found a significant difference in mean chronotype among the different *PER2 G3853A* genotype carriers in their population. Ojeda *et al*. (2013) who also used CSM to assess chronotype did not find a significant difference in mean chronotype among the carriers of the different *PER2* genotypes. Lee *et al*. (2011) and Ojeda *et al*. (2013) did not look at the separate chronotype categories but rather looked at the average chronotype score for each genotype. Carpen *et al*. (2005) also investigated the relationship between the *PER2 G3853A* polymorphism and chronotype using the H-Ö MEQ. The allele frequencies in extreme subsets of morning and evening-types as well as in neither-types of their total population where examined. They found no significant difference in allele frequency among the three diurnal preference groups.

Chronotype distribution between the Eastern and Western populations was compared among each of the *PER3* genotypes (Figure 3.13B). No significant differences were observed in the *PER3*$^{4/4}$ (p=0.442), and *PER3*$^{5/5}$ (p=0.688) genotypes. The *PER3*$^{4/5}$ showed significant differences in chronotype distribution between East and West, p=0.016. The chronotype distribution for this genotype in the Eastern and Western populations is similar to those in the *PER2* GG genotype. This indicates that longitude and latitude also exert their influence on this particular *PER3* genotype. Voinescu and Coogan (2012) found no differential distribution of chronotype in the *PER3* VNTR genotypes in the population they examined. Voinescu and Coogan (2012) reported a lower proportion of morning-types in their study than in both the Eastern and Western populations in the current study. Furthermore each of their *PER3* genotypes had a greater proportion of evening-types than did either population in the current study (that is, Eastern or Western populations). Osland *et al*. (2011) reported chronotype frequency for each *PER3* genotype in only 7% of the extreme morning and evening-types and 7% of the neither-types of their Norwegian population. However, they did not report on chronotype distribution for each genotype. For the *PER3*$^{4/4}$ genotype the evening-types accounted for a greater proportion of their population than both the Eastern and Western populations of the current study. For the *PER3*$^{4/5}$ and *PER3*$^{5/5}$ genotypes reported by Osland *et al*. (2011), the morning and neither-types accounted for lower proportions of their population than in either the Eastern or Western populations of the current study. In contrast the evening-types with the *PER3*$^{4/5}$ and *PER3*$^{5/5}$ genotypes in the current study accounted for much smaller proportions of the two populations than the evening-types reported by Osland *et al*. (2011). There seems to be a tendency towards lower chronotype scores in the Osland *et al*. (2011) and Voinescu and Coogan (2012) populations. In the case of Voinescu and Coogan (2012) this may be a bias resulting from the population having sleep problems since it has been reported that evening-types report more problems with sleep than do morning-types (Merikanto *et al*., 2012). For the population studied by Osland *et al*. (2011) the observed proportion of evening-types may be due to the effect of latitude taking into consideration the geographical position of Norway.

Barclay *et al*. (2011) used the H–Ö MEQ to assess chronotype in a large European population and found no significant difference in average chronotype score among the *PER3* genotypes. They did not compare chronotype distribution among the different *PER3* VNTR genotypes. Archer *et al*. (2003), Pereira *et al*. (2005) and Jones *et al*. (2007) similar to Carpen *et al*. (2005) examined the individual alleles in subsets of the population with extreme preference

as well as neither-type preference. The three studies reported significant differences in allele frequency in the morning and evening preference groups.

No association was found between *PER2* genotype and individual chronotype in the Eastern population (p=0.263) or in the Western population (p=0.806). No bias was observed in chronotype distribution in the East (χ^2=3.8, p=0.146) or in the West (χ^2=1.7, p=0.431) (Figure 3.14). Even when this relationship was investigated in the white ancestry group only no relationship between chronotype and genotype was observed in the Eastern population (p=0.169) or in the Western population (p=0.912), see Figure 3.16. These results show that the *PER2* genotypes do not have a significant relationship with chronotype in this study's populations. They further suggest that ethnicity did not influence this relationship. When Ojeda *et al*. (2013) carried out a similar investigation they reported an association with morning alertness and activity planning and the *PER2 G3853A* genotype which are subscales of the CSM. *Lee et al*. (2011) reported an association between the *PER2* G allele and higher total CSM scores (preference for morning) as well as with the morningness and activity planning subscales. Carpen *et al*. (2005) also investigated the *PER2* G3853A polymorphism for a relationship with chronotype. They found no association between this polymorphism and chronotype using the H-Ö MEQ. Information on the ethnic background was not provided therefore no comparisons can be drawn between this study and theirs based on ethnicity. Lee *et al*. (2011) and Ojeda *et al*. (2013) both found correlation between the *PER2* polymorphism and certain aspects of the CSM. On the other hand Carpen *et al* (2005) and this study did not. This may be due to differences in the tools used to assess chronotype. It may also be that the polymorphism does not exert a strong influence on diurnal preference.

The *PER3* VNTR showed no association between genotype and individual chronotype in the East (p=0.118) or in the West (p=0.647). Nor was there any bias observed in the trend of chronotype in the East (χ^2=3.8, p=0.149) or in the West (χ^2=1.3, p=0.510), see Figure 3.14. Osland *et al*. (2011) also found that there was no association between the *PER3* VNTR polymorphism and diurnal preference. Their population was at least 90% native Norwegian and even after investigating the five percentile of extreme scorers (similar to Archer *et al*., 2003) they were unable to find an association. This contrasts with the findings of Kunorozva *et al*. (2012) who found an association between genotype and chronotype in a very specialised subset of the South African population. They reported that no individual with the $PER3^{5/5}$ and $PER3^{4/5}$ genotype scored as an evening-type. The individuals bearing the

PER3$^{4/4}$ genotype scored as neither or evening-types except for two participants who scored as morning-types. However, they investigated only active Caucasian males whereas the current study investigated a mixed population. Archer *et al*. (2003) reported a significantly higher frequency of the *PER3*^{5} allele in the morning-types in comparison to the evening-types. The information on the ethnic background of the participants was not provided. A similar study carried out in a Brazilian population on the *PER3* VNTR also found a significantly higher representation of the *PER3*^{5} allele among the morning-types than among the evening-types (Pereira *et al*., 2005).

4.6 Conclusion

As was hypothesised there was a greater percentage of people with morning preference in the East than in the West. There was also a lower proportion of evening-types in the East than in the West. This finding was despite the significant difference in distribution of the ancestry groups between the regions, and was also evident when the stratification was removed. This demonstrated the influence of longitude and latitude on chronotype. Morning-types have been reported to be better adapted to the temporal organisation of society. They have fewer complaints of insufficient sleep, insomnia and less sleep medication use than evening-types (Merikanto *et al*., 2012). Morning-types experience less mental exhaustion in the evening and feel psychologically better at the time of the assessment and in reviewing the previous week than do evening-types (Wittmann *et al*., 2006). Hidalgo *et al*., (2009) reported an association between the incidence of depressive states and evening preference which was not observed among participants with morning or neither preference. Additionally, Kitamura *et al*. (2010) confirmed the association between evening preference and depressive states independent of sleep time, sleep quality, sleep timing and sleep debt. These have previously been suggested by Wittmann *et al*. (2006) to contribute to poor psychological well-being in individuals with evening preference.

If we take the current study to be representative of South Africa, the majority of the population on the Eastern side of the country have morning preference whereas in the West the majority have neither morning nor evening preference. While several studies have been conducted on morning- and evening-types not much has been reported on sleep problems, or psychological well-being in literature on neither-types. Therefore there is no evidence that

their diurnal preference puts them at a disadvantage socially or otherwise in comparison to morning-types. For this reason it cannot be concluded that the majority of people on the Western side of the country encounter difficulties adhering to the imposed common social schedules of the timing of school and work. Nevertheless because there is a greater proportion of evening-types in the West, more people in this region will potentially encounter difficulties adhering to the temporal dictates of work and school.

Further work could be done in order to ascertain the differential influences of longitude and latitude on diurnal preference, populations that are at closer longitudes and latitudes to Cape Town could be investigated. Populations in the towns of Springbok (29°30.0'S/17°53.0'E) or Nababeep (29°35.5'S/17°47.0'E), and Port Elizabeth (33°54.5'S/25°31.9'E) for instance would be suitable. This would enable a comparison between the two influences on chronotype. Then, in order to the determine the impact of the scheduling of work and school on the populations in the Eastern and Western regions assessment of indicators such as sleep quality, psychological well-being, and performance can be carried out. These assessments need to be carried out with regard to seasons, particularly summer and winter which result in the most and least exposure to sunlight respectively and have the greatest differences in temperature. This would give evidence for or against the necessity of instituting measures such as separate time zones or daylight saving to improve the general well-being of the population in the Western part of South Africa.

The other hypothesis was also confirmed as no significant difference in the distribution of the *PER2* or *PER3* genotypes was observed between East and West. This was despite the fact that the *PER2* AA genotype was only observed in the white ancestry group. Smith *et al*. (2009) reported that African Americans had a significantly shorter endogenous period than subjects of other races. The other races included Asians, Caucasians and another unspecified race(s). Smith *et al*. (2009) further reported that African Americans had larger phase advances and smaller phase delays relative to Caucasians. They hypothesised that these differences in endogenous period between the races were selected for based on latitude, suggesting that Caucasian populations with the longer endogenous period were suited to live at higher latitudes where greater seasonal changes in photoperiod occur. Whereas African populations originating relatively closer to the equator have shorter endogenous periods suited for the more constant photoperiod. Given this reasoning, perhaps the AA genotype is associated with or somehow contributes to this longer endogenous period as previous work

has demonstrated the role of *PER2* in the length of the endogenous period (Toh *et al*., 2001). The *PER3* genotype unlike that of *PER2* did not show significant differences in distribution among the different ancestry groups. This would suggest as postulated by Ciarleglio *et al*. (2008) that no differential selection has occurred for this polymorphism. Furthermore its role in sleep homeostasis as reported by Viola *et al.* (2007) is likely to be similar in all ethnicities and therefore does not necessitate differential selection based on environment.

No associations between chronotype and the *PER2* or *PER3* genotypes were found in populations from either region in the current study. While an association between *PER3* VNTR polymorphism and diurnal preference has been reported by several studies (Archer *et al*., 2003; Jones *et al*., 2007; Pereira *et al*., 2005; Kunorozva *et al*., 2012), these studies with the exception of Kunorozva *et al*. (2012) reported an association between this polymorphism and diurnal preference in subsets of extreme scorers. In the current study, the population in the Western region had the majority of participants scoring as neither-types which may be a reason for the lack of association as no differential allele frequency has been reported in this group. The Eastern region however had a majority of morning-types but still did not show any association. Jones *et al.* (2007) who reported an association between diurnal preference and the *PER3* VNTR found the strongest association in the 18 to 29 age group. They further reported that this association attenuated with age. The absence of an association in either region may be because the majority of participants were over 29 years of age with the mean ages being 39.1± 8.8 (yr ± SD) in the Eastern region and 35.6±8.2 (yr ± SD) in the West. The associations reported by Kunorozva *et al*. (2012) cannot be generalised because they looked at active Caucasian males only, a sub-section of the population. Additionally the populations examined in the two regions where of mixed ethnicities and had differences in genotype frequency which are intrinsic due to ethnicity. It was observed that chronotype was not influenced by ethnicity, consequently a correlation between genotype and phenotype may not be plausible. It is therefore possible that the *PER3* VNTR polymorphism is not associated with diurnal preference in a general population but rather certain segments of it particularly people with extreme diurnal preference. As for the *PER2 G3853A* SNP, it has been reported to associate with CSM score in a Korean population, and not yet reported to associate with H-Ö score.

Chronotype is influenced by both genetic and environmental factors. Vink *et al*. (2001) carried out an investigation in a Dutch population on the contribution of environmental and

genetic factors to the variance observed in chronotype. They estimated that genetic factors contribute 44% in adolescents and 47% in an older population (mean age mid-to late-forties) to chronotype. Hur (2007) reported a similar estimate of 45% contributed by genetic factors and 55% by environmental factors in a Korean population aged 9 to 23yr. The contributions of environmental factors (latitude and longitude) on chronotype were more visible in the *PER3*$^{4/5}$ and *PER2* GG genotype carriers than in the other genotype carriers. This finding concurs with what was suggested by Pereira *et al.* (2005) that the same genotype at different latitudes would result in different circadian phenotypes. Moreover it is possible that the relationship between clock genotype and diurnal preference is more easily detected when indigenous populations are studied. An example is the population studied by Lee *et al*. (2011) where a correlation between the *PER2* genotype and diurnal preference was observed in a general population and not just in subsets of the population with the most extreme morning-types and evening-types as was done by Carpen *et al*. (2005). It is probable that clock genes have been adapted for optimal interaction with the external environment in indigenous populations. The only population in the current study that is indigenous to South Africa was the black ancestry group and even within this group migration may have taken place within the country.

Further work in association studies should not look for associations with individual polymorphisms in clock genes but rather, similar to Ebisawa *et al*. (2001) and Cruciani *et al*. (2008), identify haplotypes among the groups of polymorphisms in the coding and/or promoter regions of the gene. These haplotypes can be examined for how they may cause changes in protein folding and therefore affect interactions with other clock proteins, or affect RNA stability and or DNA-protein interactions. This would increase knowledge towards the establishment of a causal link between the genetic and the phenotypic instead of a correlational one. This interaction should also be examined in indigenous peoples who have not participated in recent migration events.

4.7 Limitations

Sampling in this study was only done on a small segment of the population and covered a very limited geographical area. This resulted in a low representation of the native people and shift workers were also excluded from the study. Furthermore the questionnaires were in

English and therefore prevented the majority of the population from participating. The timing of sampling was disparate, as it took place from August to June (spring to winter) in Western Cape and December to April (summer to autumn) in the Eastern region. There was no assessment of indicators carried out in order to verify any real life effects of these differences in chronotype between East and West. These indicators could include: stimulant consumption, sleep quality, school/work performance and psychological well-being. The circadian clock is driven by intricate interactions of genes and proteins. The current study however, only looked at two polymorphisms in two separate genes; their assortment with other polymorphisms within the same gene was not considered. This means that the association of the particular polymorphism with diurnal preference has not been put in context with other polymorphisms in the gene. This limits what can be inferred from the findings the since the folding and interactions of the protein are a direct result of the sequence of the gene.

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Appendix A

SOUTH AFRICAN CHRONOTYPE STUDY

INFORMATION SHEET

Dear Volunteer,

Thank you for agreeing to participate in the University of Cape Town's study entitled **Chronotype and Clock gene allele frequency in the Southern African Population** to be conducted by researchers from the UCT in the Department of Molecular and Cell Biology.

Background

Human physiology, metabolism and behaviour are coordinated and regulated by circadian rhythms (i.e. 24 hour day/night cycles). Furthermore there appear to be genetically determined inter-individual differences in circadian rhythm. The time at which we sleep is governed by two clocks: the inner biological clock and the clock imposed by the outside world. These two clocks may not be synchronised, making good sleep and good health difficult. Some circadian clock genes are associated with the time at which an individual prefers to go to bed and to get up. What is the prevalence of individuals preferring to wake and sleep early (morning-types), individuals preferring to wake and sleep late (evening-types) and individuals with no strong preference (neither-types) in the South African population? We are particularly interested in comparing the population data from the Western Cape in the West with those in KwaZuluNatal in the East, because of the differences in light/dark timings within our time zone that occur due to longitudinal differences.

Therefore, the aims of this research are:

1. To describe the distribution of individuals preferring either mornings or evenings in

    (a) Western Cape Province

    (b) Kwazulu Natal Province

2. To genotype the same two populations for the variable number tandem repeat

    (VNTR) polymorphism within the *PERIOD* genes and other circadian clock associated genes.

Procedures

You will be asked to complete a General Questionnaire (personal details, medication / supplement use) and the Horne-Östberg Morningness/Eveningness personality questionnaire. The investigator will then take a buccal cell sample from the inside of your cheeks using a sterile swab. DNA will be extracted from your buccal cells 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 valuable observations of differences between individuals in the Western and Eastern parts of the country and determine whether our Society is organized in a temporal way that suits the majority of the population. We would like to ascertain whether it is

possible to match work/study schedules with diurnal preference, thus enhancing sleep, health, quality or life as well as safety and efficiency at work. Perhaps in the future the study could contribute to the establishment of two time zones in the country.

Potential Risks

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

Benefits

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

Ethical considerations

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

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

Your DNA sample will only be used for the purposes explained to you, namely to determine your genotype for circadian clock associated genes, and will be destroyed on completion of the Chronotype and clock gene allele frequency in the Southern African population study. You may request that your 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.

Investigators

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Appendix B

SOUTH AFRICAN CHRONOTYPE STUDY

INFORMED CONSENT

ALL participants to read and sign

I, the undersigned, have been fully informed about the University of Cape Town's study entitled **South African Chronotype Study** to be conducted by researchers from the Department of Molecular and Cell Biology.

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

- Buccal samples will be obtained from the insides of my cheeks using sterile swabs. DNA will be extracted from these samples to establish my genotype for polymorphisms within circadian clock 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 polymorphisms within the circadian clock genes, and will be destroyed on completion of the Chronotype 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:____

Appendix C

SOUTH AFRICAN CHRONOTYPE STUDY

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): _______________________

How many consecutive (following one after another without interruption) years have you been living in the Western Cape? ____________

Have you engaged in night-time shift work in the past three (3) months? **YES** **NO**

"If yes, please provide details of the shift times and rotation and how long you have been engaged in this type of shift work"

____

____

Medication use

What medication, if any, are you currently using? Please be sure to include any sleeping or alerting medication you may use.

Name of medication **Years taken**

____

Appendix D

HORNE-ÖSTBERG MORNINGNESS-EVENINGNESS PERSONALITY QUESTIONNAIRE

INSTRUCTIONS

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

QUESTION 1

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

Time:________

QUESTION 2

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

Time:________

QUESTION 3

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

a. Not at all dependent………………...☐

b. Slightly dependent…………………☐

c. Fairly dependent……………………☐

d. Very dependent……………………..☐

QUESTION 4

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

a. Not at all easy…………………☐

b. Slightly easy…………………...☐

c. Fairly easy……………………..☐

d. Very easy……………………...☐

QUESTION 5

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

a. Not at all alert…………☐

b. Slightly alert…………...☐

c. Fairly alert……………...☐

d. Very alert………………□

QUESTION 6

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

a. Not at all good……………□

b. Slightly good……………..□

c. Fairly good………………..□

d. Very good………………...□

QUESTION 7

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

a. Very tired………………….□

b. Slightly tired………………□

c. Fairly refreshed……………□

d. Very refreshed…………….□

QUESTION 8

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

a. Seldom or never later……………□

b. Less than one hour later.………...□

c. 1-2 hours later…………...………□

d. More than 2 hours later………….□

QUESTION 9

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

a. Would be on good form………………..□

b. Would be on reasonable form………….□

c. Would find it difficult ……………….....□

d. Would find it very difficult…………….□

QUESTION 10

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

Time:________

QUESTION 11

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

a. 8.00 am – 10.00 am……………

b. 11.00 am – 1.00 pm…………...

c. 3.00 pm – 5.00 pm…………….

d. 7.00 pm – 9.00 pm…………….

QUESTION 12

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

a. Not at all tired………………….

b. A little tired…………………….

c. Fairly tired……………….…….

d. Very tired………………………

QUESTION 13

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

a. Wake up at the usual time and not go back to sleep……..

b. Wake up at the usual time and doze…………………..….

c. Wake up at the usual time and go back to sleep…………

d. Wake up later than usual……………………………....

QUESTION 14

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

a. Would NOT go to bed until 6.00 am………………………………………

b. Nap before 4.00 am and sleep after 6.00 am………………………………

c. Sleep before 4.00 am and nap after 6.00 am……………………………….

d. Only sleep before 4.00 am and remain awake after 6.00 am………………

QUESTION 15

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

a. 8.00 am – 10.00 am………………..

b. 11.00 am – 1.00 pm………………..

c. 3.00 pm – 5.00 pm…………………

d. 7.00 pm – 9.00 pm………………....

QUESTION 16

You have decided to engage in some physical exercise. A friend suggests that you do this between 10.00 pm and 11.00 pm twice a week. How do you think you would perform?

a. Would be on good form……………………

b. Would be on reasonable form………………

c. Would find it difficult………………………

d. Would find it very difficult…………………

QUESTION 17

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

Hours:________

QUESTION 18

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

Time:_________

QUESTION 19

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

a. Morning type……………………...

b. More morning than evening……....

c. More evening than morning……….

d. Evening type……………………....