# The Role of Chronotype in the Participation and Performance of South African and Dutch Marathon Runners

By

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Dissertation presented in full fulfilment of the requirements for the degree of

MSc (Medicine) Exercise Science

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# I. Declarations

I, Rob Henst, am hereby presenting this dissertation in full fulfilment of the requirements for my degree.

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## II. Candidates' contributions

Dr Dale Rae and Dr Laura Roden conceptualised this project in 2012. After introducing their perception of this project to me, I assisted in the project conception and initiation. From February to March 2013 I wrote the research proposal during which time Drs Rae and Roden reviewed the documents. We submitted the proposal to the UCT Faculty of Health Sciences' Human Research Ethics Committee (HREC) on the 1st March 2013. I then used this time to write the Literature Review section of my thesis.

The HREC approved the study on the 28th March 2013. I initiated the participant recruitment by contacting gyms, running clubs and the Langebaan Country estate Weskus marathon organisation in South Africa. With much needed help from my supervisors and fellow students, I recruited all 174 South African participants in April and May 2013. I then completed the data entry and laboratory analysis of these samples while setting up and liaising with a Dutch collaborator - Dr Richard Jaspers from the VU University in Amsterdam. I also wrote my Methods section. I travelled to the Netherlands on the 30th of July 2013 and, with the help of my father, recruited the 181 Dutch participants during September and October 2013 from gyms and the Lage Landen marathon of Eindhoven. I then analysed the buccal cell samples in the myology lab of Dr Richard Jaspers in the Netherlands. I travelled back to South Africa in December 2013 to finalise data analysis of the South African samples. From March to June 2014 I analysed the data and produced the graphs, which were then reviewed by my supervisors. The last step of the project was synthesising the thesis, which I did between July and August 2014. The thesis was submitted on the 8th of September 2014.

# III. Acknowledgements

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Thank you, Dr Richard Jaspers for agreeing to collaborate with us and allowing me to do a part of my analysis in the myology lab. I hope we can benefit from this collaboration more often in the future.

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Thank you, Guus Baan, Carla Offringa and Gerard de Wit for your time assisting me, online when I was in South Africa, as well as in the lab, when I was in the Netherlands.

Thank you, Adri Winckler, Dianna Pryce and Salega Tape for helping me out when needed. This includes the time I desperately needed a modified acceptance letter for the South African Embassy in the Netherlands to obtain my study permit, as well as numerous enrolment controls for the Dutch governmental student financing office.

Thank you, everyone from the Rhythms and Blooms lab and the UCT/MRC Research unit for Exercise Science & Sports Medicine for the fruitful discussions and small talk, either study related or not.

Finally, Thank you, everyone whose name has not been mentioned in this section, but who has contributed in one way or another, deliberately or not.

I thank all of you.

# IV. List of abbreviations

| | |
|---|---|
| AMPA | α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid |
| ANOVA | Analysis Of Variance |
| ARNT | Aryl Hydrocarbon Receptor Nuclear Translocator |
| BALM | Basic Language Morningness scale |
| BMAL | Brain and Muscle ARNT-Like |
| BMI | Body Mass Index |
| BSA | Bovine Serum Albumin |
| CaM | Calmodulin |
| CaMKII | Calmodulin Kinase II |
| cAMP | Cyclic Adenosine Monophosphate |
| cGMP | Cyclic Guanosine Monophosphate |
| CIRENS | Circadian Energy Scale |
| CK1δ | Casein Kinase 1 Delta (also CSNK1D) |
| CK1ε | Casein Kinase 1 Epsilon (also CSNK1E) |
| CLOCK | Clock Locomotor Output Cycle Kaput |
| CMEP | Children's Morningness-Eveningness Preference |
| CONNL | Control group, the Netherlands |
| CONSA | Control group, South Africa |
| CR | Circadian Rhythm |
| CRE | cAMP Response Elements |
| CREB | cAMP Response Element Binding Protein |
| CRM1 | Chromosome Region Maintenance 1 |
| CRY | Cryptochrome |
| CSM | Composite Scale of Morningness |
| CSNK1D | Casein Kinase 1 Delta (also CK1δ) |
| CSNK1E | Casein Kinase 1 Epsilon (also CK1ε) |
| CTQ/CTI | Circadian Type Questionnaire/Inventory |
| DARP-32 | Dopamine- and cAMP-Regulated Neuronal Phosphoprotein 32 |
| DET | Definite Evening-Type |
| DMT | Definite Morning-Type |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxynucleic Triphosphate |

| | |
|---|---|
| DSPS | Delayed Sleep Phase Syndrome |
| DST | Daylight Saving Time |
| DTS | Diurnal Type Scale |
| EA | Evening-Active |
| ECB | Ethische Comissie Bewegingswetenschappen |
| EDTA | Ethylenediaminetetraacetic Acid |
| EMG | Electromyography |
| ET | Evening-type |
| FASPS | Familial Advanced Sleep Phase Syndrome |
| GC | guanyl cyclase |
| GCP | Good Clinical Practice |
| Glu | Glutamate |
| GPCR | G-Protein Coupled Receptor |
| HÖ-MEQ | Horne-Östberg Morningness-Eveningness Personality Questionnaire |
| HREC | Human Research Ethics Committee |
| $IP_3R$ | Inositol Triphosphate Receptor |
| MA | Morning-Active |
| MAPK | Mitogen-Activated Protein Kinase |
| MCTQ | Munich Chronotype Questionnaire |
| MET | Moderate Evening-Type |
| METc | Medisch Ethische Toetsingscommissie |
| MMT | Moderate Morning-Type |
| MRC | Medical Research Council |
| $MSF_{(sf)}$ | Mid-Sleep on Free-days |
| MT | Morning-type |
| MTNR1A | Melatonin Receptor 1A |
| MTNR1B | Melatonin Receptor 1B |
| NMDA-R | N-Methyl-D-Aspartate Receptor |
| nNOS | Neuronal Nitric Oxide Synthase |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| NT | Neither-type |
| PACAP | Pituitary Adenylate Cyclase-Activating Peptide |
| PAS | Per-Arnt-Sim Protein Domain Signal Sensor |
| PASA | Per-Arnt-Sim Protein Domain Signal Sensor A |

| | |
|---|---|
| PASB | Per-Arnt-Sim Protein Domain Signal Sensor B |
| PB | Personal Best |
| pCREB | Phosphorylated CREB |
| PER | Period protein |
| *PER3* | *PERIOD3 gene* |
| PKA | cAMP-dependent Protein Kinase |
| PKG | cGMP-dependent Protein Kinase |
| RHT | Retinohypothalamic Tract |
| rMEQ | Reduced Morningness-Eveningness Personality Questionnaire |
| ROR | Retinoic Acid-related Orphan Receptor |
| RPE | Rating of Perceived Exertion |
| RRE | ROR Response Element |
| RUNNL | Runners group, the Netherlands |
| RUNSA | Runners group, South Africa |
| RyR | Ryanodine Receptor |
| SAD | Seasonal Affective Disorder |
| SCN | Suprachiasmatic Nucleus |
| SMD | Standardized Mean Difference |
| SNP | Single Nucleotide Polymorphism |
| SSISA | Sport Science Institute of South Africa |
| TAE | TRIS Acetate EDTA |
| ToD | Time-of-Day |
| TRIS | Tris(hydroxymethyl)aminomethane |
| UCT | University of Cape Town |
| UV | Ultra-Violet |
| VNTR | Variable Number Tandem Repeat |
| VU | Vrije Universiteit |
| Xpo1 | Exportin 1 |

# V. Abstract

**Introduction**

Our circadian rhythms are internal biological rhythms of approximately (circa) 24-hours (dies) allowing us to synchronize our internal biological "clock" with external time cues. Many innate biological functions are dependent on time-of-day, such as secreting adrenaline and cortisol in the mornings and melatonin in the evenings. The time-of-day at which these and other physiological functions are active, change or reach a certain level may influence a person's diurnal preference, i.e. preference for mornings (morning-types) or evenings (evening-types), and is referred to as '*chronotype*'. Many different factors may affect a person's chronotype, including age, sex, physical activity, ethnicity and geographical location. Certain clock-related genotypes have also been shown to be associated with chronotype. For example, some studies have found that the 5-repeat allele of the *PER3* variable number tandem repeat (VNTR) polymorphism (*PER3*$^5$) is associated with a preference for mornings. Recent research has shown a high prevalence of morning-types and *PER3*$^5$ VNTR allele carriers in trained South African runners, cyclists and triathletes. It was proposed that the early morning start-times of these endurance events might select people with a preference for mornings, since morning-types may cope better with rising early and being physically active in the early morning. Alternatively, the habitual early waking for training or endurance events may have conditioned the athletes to adapt to become morning-types. However, the geographical location of South Africa (i.e. climate and day length) and the fact that each group was physically active may also have contributed to this finding. Comparison of South African and Dutch runners would allow us to explore the effects of race start time and geography on this observation, since marathons in The Netherlands on average start at 11:41, and since the two countries differ significantly in latitude and as such have noticeable differences in daylight exposure.

**Aims**

The aims of this study were 1) to compare the *PER3* VNTR genotype and chronotype distribution of South African and Dutch recreational marathon runners and active but non-competitive controls; 2) to investigate the relationship between the *PER3* VNTR genotype and chronotype in both the Dutch and South African samples; and 3) to determine whether marathon race time is associated with chronotype and *PER3* VNTR genotype in Dutch and South African marathon runners.

**Methods**

Ninety-five trained South African male marathon runners, 97 South African male active but non-competitive controls, 90 trained Dutch male marathon runners and 98 Dutch male active but non-competitive controls completed a questionnaire capturing demographics, training and race history, including personal best and most recent full and half-marathon race time (if applicable) and the Horne-Östberg morningness-eveningness personality questionnaire (HÖ-MEQ, a tool to assess a person's chronotype). Each participant provided a buccal cell swab from with total genomic DNA was extracted to determine his *PER3* VNTR polymorphism genotype. The official race time from each runner who completed the designated marathons in South Africa or the Netherlands was collected from the event websites.

**Results**

The South African and Dutch runners were more morning-orientated than their respective control groups and the South African runners were more morning-orientated than the Dutch runners. The *PER3* VNTR polymorphism distribution was similar between the four groups and was not associated with chronotype. The marathon performance of the morning-type South African runners was better than the evening-types, and a higher HÖ-MEQ score (morningness) correlated with better personal best and most recent half-marathon race time. Similar observations were not found in the Dutch runners.

**Discussion**

Since a higher prevalence of morning-types in South African marathon runners compared to Dutch marathon runners was found, it is proposed that the early marathon start-times in South Africa may favour morning-types, who are able to cope with those early morning start times. Alternatively, one could argue that through repetitive early-morning racing (i.e. participating in competitive running events), the chronotype of South African runners may be conditioned to that of a morning-type over time. It is proposed that this ability to cope with early morning marathon start times may lead to better marathon performances for morning-types than neither-types and evening-types in the South African running group. This effect does not occur in the Netherlands, where marathons start later in the morning and do thus not favour a certain chronotype. The difference in daylight exposure between the two countries as a function of latitude does not seem to affect chronotype, since the active but non-competitive control groups did not differ significantly between South Africa and the Netherlands. Unlike the findings from a previous study, the $PER3^5$ allele was not more prevalent among the South African runners, but rather the distribution was in line with what has been described in most, but not all, other populations. No association between the $PER3^5$ VNTR

polymorphism and chronotype was found in any of the four groups. Since the four groups investigated in this study comprised physically active individuals, it is proposed that this lack of association may be due to the habituation effects of physical activity and early morning start times of marathon events (for only the South African runners). Conceivably, this habituation may even shift the diurnal preference of those with the *PER3*$^{4/5}$ and *PER3*$^{4/4}$ VNTR genotypes towards morningness, disassociating any relationship between chronotype and the *PER3* VNTR genotype.

**Conclusion**

The early morning start time of South African marathon events may favour morning-types, due to their ability to cope with being physically active in the early morning. We propose that the *PER3* VNTR genotype cannot solely explain the higher prevalence of morning-types in the South African runners in this study, however, it is very likely that the *PER3* VNTR genotype does play an important role in the chronotype distributions found in the study of Kunorozva *et al.* (2012). Since the *PER3* VNTR genotype was not associated with chronotype in any of the four groups, it is proposed that habituation to early-morning marathon racing may be the causal effect of the high number of morning-types in the South African runners group, and the apparent disassociation between chronotype and the *PER3* VNTR genotype. We also propose that the habituation effect of physical activity and training time-of-day on chronotype in the other groups may dissociate the *PER3* VNTR genotype with chronotype in a similar manner to which the early-morning start times of South African endurance events dissociates the two. No effect of geographical location on chronotype was found when comparing the Dutch and the South African groups. The morning-orientated South African runners seem to perform better in marathon running than the more evening-orientated runners do, which may be caused by their ability to cope with these early-morning marathon events. Further studies may explore whether marathon performance in later chronotypes can be improved by training-based habituation.

# Chapter 1: Introduction

**1.1 Circadian rhythms: our internal wristwatches**

A businessman who has an appointment with a client at 15:00 needs to know the time so that he can initiate a certain action to get there punctually. Thus, he wears a mechanical wristwatch to tell him what time it is. Our body also has to initiate and schedule certain actions over the course of a day, such as inducing sleep and rest (1), raising alertness (2), starting digestion (3), or suppressing bowel movement during sleep (4). For this purpose, our body has a system consisting of millions of little wristwatches, all controlled by one main clock. This so-called 'master oscillator' is located in the suprachiasmatic nucleus (SCN) in the hypothalamus (5). Just like in a normal wristwatch, this system 'ticks' in cycles of 24 hours. However, no mechanical wristwatch is 100% accurate. Its cycle either delays or advances slightly, perhaps only a few nanoseconds a day. So it is with our biological clock, which has a 'circadian' rhythm (CR), i.e. an approximately 24-hour period. 'Circadian' is derived from the Latin phrase "circa diem", which means "about a day". Just like a watch, whose inaccuracy can be reduced by regularly resetting the clock by twisting the crown, so can CRs be entrained by molecular components interacting with external time cues (see **section 1.3**).

As in humans, CRs are observed in other animals (6), plants (7), insects (8), fungi (9) and cyanobacteria (10). In fact, it is thought that most organisms on Earth have CRs. In mammals, the CRs influence a vast number of biological activities such as core body temperature (11), metabolism (12), DNA synthesis (13), cell regeneration (14), brain wave activity (15) and the regulation of plasma hormone levels such as melatonin (16) and corticosterone (17). The reason why organisms have CRs is speculated about. Evolutionary-wise, having CRs gives organisms a better chance of survival. For example, if a nocturnal rodent was to leave his safe burrow during broad daylight, he would be an exceptionally easy prey for other animals (18). SCN-lesioned Siberian chipmunks (*Eutamias sibiricus*) showed irregular and shorter hibernation bouts, which sometimes commenced during summer (19). Although not hazardous in lab conditions, in the field these chipmunks may not have survived, suggesting the evolutionary advantage of having CRs. The reproduction-mediated fitness in the midge *Chironomus thummi* is enhanced when eclosion at lower temperatures takes place during daytime, while eclosion normally coincides with dusk at high temperatures (20). This suggests that having a circadian rhythm in the timing of eclosion provides an evolutionary advantage.

**1.2 Circadian oscillation: the 'balance wheel' that keeps the time**

Just like a mechanical wristwatch consists of an interacting network of mechanical components that are necessary for maintaining the time, our CRs consist of a network of clock genes and proteins to

maintain the time. These molecular 'cog wheels' of CRs in mammals are understood to comprise autoregulatory feedback loops of core "clock" genes and proteins. These include Brain and Muscle Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT)-like 1 (BMAL1), Circadian Locomotor Output Cycles Kaput (CLOCK), Period1 (PER1), Period2 (PER2), Cryptochrome1 (CRY1) and Cryptochrome2 (CRY2). These genes and proteins play an important role in CRs and the mechanism has been well described as outlined in **Figure 1.1** (21).

Briefly, the basic helix-loop-helix PAS-domain containing transcription factors BMAL1 and CLOCK activate transcription of the *PER* and *CRY* genes. Upon translation, the PER and CRY proteins form heterodimers and translocate to the nucleus to interact with the CLOCK-BMAL1 complex and inhibit the transcription of the *PER* and *CRY* genes. The PER-CRY repressor complex is degraded after a certain period of time and a new cycle of transcription is activated by CLOCK-BMAL1 (21). The length of one cycle determines the circadian rhythmicity and thus the endogenous circadian period, which was found to be 24 hours and 11 min (± 16 min) in length in 24 people when living in free-running conditions, devoid of external time cues (22).

The CLOCK-BMAL1 complex also activates the transcription of the *REV-ERBα* and *REV-ERBβ* genes (23). The REV-ERBs are components in the secondary autoregulatory feedback loop that represses *BMAL1* transcription and competes with a retinoic acid-related orphan receptor (ROR) to bind ROR response elements (RREs) in the *BMAL1* promoter (24). Other crucial steps for determining circadian periodicity are the post-translational modifications and degradations of circadian clock proteins. Casein kinases 1 delta (CK1δ) and 1 epsilon (CK1ε) phosphorylate PER (and CRY) to target them for polyubiquitylation by the β-TrCP1 and FBXL3 ubiquitin ligase complexes (25-27). The PER and CRY proteins are then degraded by the 26S proteosomal pathway (21). In mice, newly synthesized mPER2 is translocated into the nucleus, by virtue of the nucleus localisation sequence (NLS). Once in the nucleus, the mPER2 is transported back into the cytoplasm through the chromosome region maintenance 1 (CRM1) or Exportin1 (Xpo1) nuclear export system. The process repeats itself over and over until mPER2 either binds to mCry1 or mCry2 or gets polyubiquitylated in the cytoplasm and degraded as described above (28).

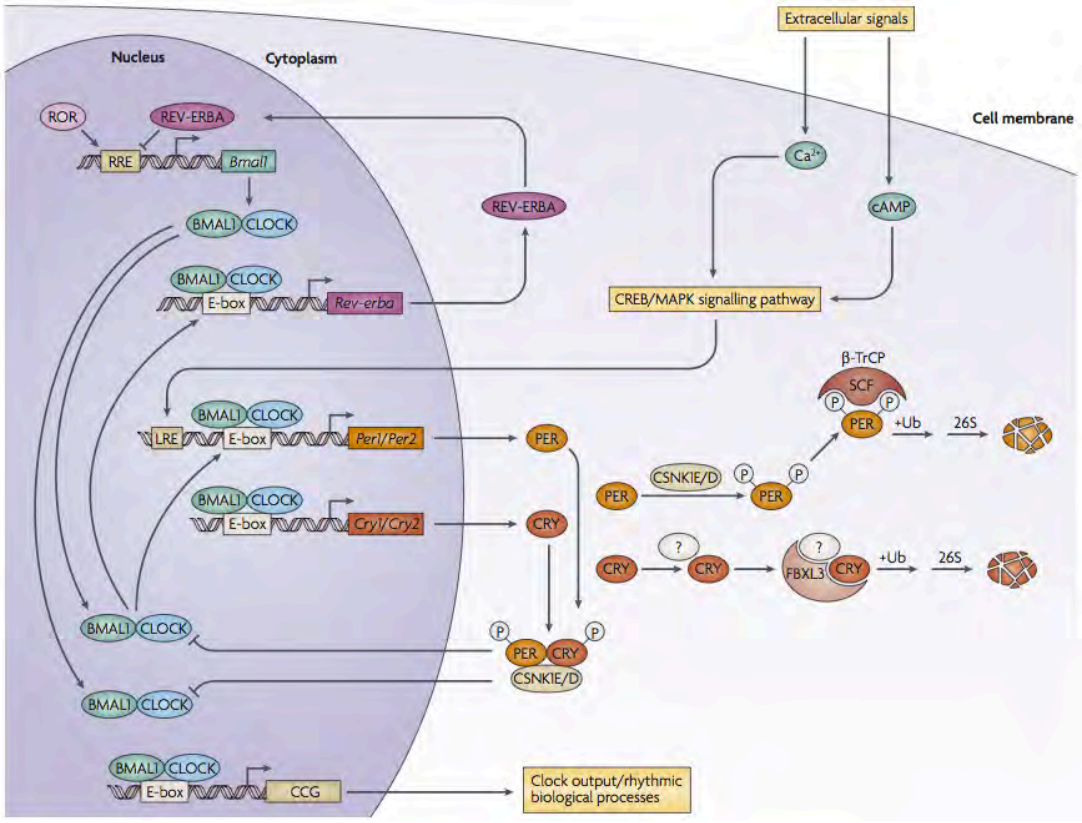


**Figure 1.1 - *The mechanism of the transcriptional-translational feedback of the mammalian circadian clock***. *The basic helix-loop-helix PAS-domain containing transcription factors BMAL1 and CLOCK activate transcription of the Per and Cry genes. Upon translation, the PER and CRY proteins form heterodimers and translocate to the nucleus to interact with the CLOCK-BMAL1 complex and inhibit the transcription of the Per and Cry genes. The PER-CRY repressor complex is degraded after a certain period of time and a new cycle of transcription is activated by CLOCK-BMAL1. The CLOCK-BMAL1 complex also activates the transcription of the Rev-erbα gene. REV-ERBα is a component in the secondary autoregulatory feedback loop that represses Bmal1 transcription and competes with a retinoic acid-related orphan receptor (ROR) to bind ROR response elements (RREs) in the Bmal promoter. Other crucial steps for determining circadian periodicity are the post-translational modifications and degradations of circadian clock proteins. Casein kinase 1 delta (CSNK1D) and Casein kinase 1 epsilon (CSNK1E) phosphorylate PER (and CRY) to target them for polyubiquitylation by the β-TrCP1 and FBXL3 ubiquitin ligase complexes. The PER and CRY proteins are then degraded by the 26S proteosomal pathway. CCG, clock-controlled genes; CREB, cAMP response element-binding; E-box, CACgTg/T consensus sequence; MAPK, mitogen-activated protein kinase; SCF, SCF E3 ubiquitin ligase; Ub, ubiquitin **(21)**. Figure reproduced, with permission, from reference **(21)***

### 1.3 Circadian entrainment

The circadian cycle, outlined in the previous section (**section 1.2**), can oscillate independently of exogenous influences, in which case it is said to be "free-running". However, in order for it to function as a useable clock, it is entrained to external time. This is similar to how a wristwatch is free-running and would only be useful as a timer rather than to tell the time, if it cannot be adjusted to the current time. Depending on the accuracy of your watch, it needs to be reset after a certain amount of time, which is why each mechanical watch has a twistable reset button, called the

'crown'. The 'balance wheel' or oscillator of CRs - also called the pacemaker - is entrained (reset) by multiple external time cues. These exogenous time cues themselves are rhythmic and are called 'zeitgebers' - or time givers (11). The Earth's dark-light cycle for instance, rhythmically repeats itself every 24 hours, and may be the most important zeitgeber in humans.

### 1.3.1 The Earth's dark-light cycle as zeitgeber

The Earth's light-dark cycles alone are enough to entrain the human circadian rhythm and may thus be considered the most important zeitgeber in humans (29). Generally, the free-running endogenous period (i.e. tau, τ) in humans is usually (but not always) slightly longer than 24 hours (22). When in free-running conditions, if an endogenous circadian period is 24.18 hours, the onset of a particular activity or circadian period will be advanced by 10 min and 48 sec each day (**Figure 1.2)**. For an individual that has shorter circadian periods, for example 23.88 hours, the circadian period will delayed by 7 min each day (22).

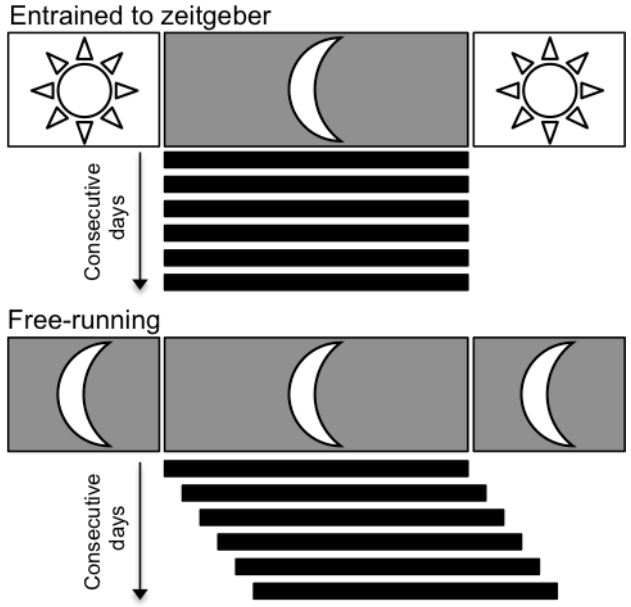


**Figure 1.2 – *The light-entrained and free-running circadian rhythm***. *While entrained, the endogenous period is synchronous with the external time cues, in this example: light-dark cycles. In the absence of this time cue (dark-dark cycles) the circadian rhythm is free-running, however it keeps its endogenous cycle. The endogenous period (black bar) in this example could be sleep or a decreased locomotor activity. Figure reproduced, with permission, from reference (30).*

Most cells in mammals have their own circadian oscillator which is synchronized with other cells within the same tissue (5). These synchronized oscillators are not reset by light cues, but by other non-photic zeitgebers, which will be discussed in **section 1.3.3**. These oscillations are called 'peripheral' and are controlled by the master oscillator in the SCN (5,31). Under *In vitro* conditions, the CRs in the peripheral tissues of rats eventually damp out in the absence of an entraining agent.

However, the SCN entrains the circadian oscillations in peripheral tissues after an abrupt shift in the environmental light cycle (32).

The master oscillator in the SCN is mainly entrained by blue light (see **section 1.3.3**), which regulates the secretion of melatonin in blood and the brain's cerebrospinal fluid (33). Melatonin's presence and/or synthesis is also described in other tissues such as the retina (34), Harderian gland (35), gastrointestinal track (36), testes (37) and lymphocytes (38). Circulating melatonin levels peak at night and are low during day (39). In mammals, melatonin triggers several pathways by binding to melatonin receptors: melatonin receptor 1A (MTNR1A) and melatonin receptor 1B (MTNR1B), which can be found in areas of the central nervous system and peripheral target tissues including retina, pars tuberalis, cerebral, peripheral arteries, kidneys, pancreas, adrenal cortex, testes and immune cells [reviewed in (40)]. Melatonin thus distributes the message of time from the SCN to the periphery, allowing the entrainment and synchronisation of the CR throughout the body (41).

### 1.3.2 Non-photic zeitgebers

Although the light-dark cycle is thought to be the most important and best understood zeitgeber in mammals, other non-photic zeitgebers are known to exist. For example, an experiment in 1971 found that social cues are sufficient to entrain the human CR for at least four consecutive days (42). Likewise, the circadian phase in male golden hamsters advances when pairs of animals interact for half an hour in the middle of the hamster's subjective day, and a phase delay was found when the interaction took place in the late subjective night (43).

Food availability and other food related cues are external time cues also able to entrain the CRs. Researchers have been able to entrain CRs in mice by inducing variations in food availability without affecting the SCN. They proposed that another clock, a peripheral food-entrained clock, is responsible for this entrainment (44). Another publication mentions one or more food-entrained oscillator(s) that together with food-related cues may entrain clock gene cycles in the SCN, either with a direct effect, or indirectly via a different neural or peripheral site (45).

Physical activity can also induce phase-shifting of the CR oscillator, which is especially well studied in rodents (46,47). A study on mice suggested that the peripheral CR in the skeletal muscles and lungs, fully re-entrain when the mice had access to a running wheel. This did not occur in mice that did not have access to a running wheel (32). This highlights the importance of physical activity on circadian entrainment of peripheral tissues. Furthermore, a study on sixteen nightshift workers, of

which eight exercised for 15 minute every hour and eight did not, revealed that exercise decreased the time to re-entrain to a 9 hour phase delay (48). Plasma corticosterone, which is one of the hormones believed to reset the peripheral circadian clocks, increases with acute and chronic voluntary exercise (49-51). This could mean that exercise itself resets peripheral CRs, albeit indirectly. In adult males and females for example, acute modest exercise for 2.5 hours in the night had the same phase-shifting effect on the CRs of melatonin and thyrotropin secretion as bright light under constant routine conditions (52,53). Another study on CR phase shifts by exercise concluded that a longer duration (more than 1 hour) of repeated daily exercise may be necessary for reliable phase-shifting of the human circadian system and that high-intensity exercise in the early evening may induce phase advances (54). When participants were under a forced sleep-wake cycle, with two bouts of exercise during the subjective day, plasma melatonin rhythm was significantly phase advanced compared to subjects in the same environment but without exercise (55). These findings suggest that physical exercise accelerates phase advance shifts of CRs.

Daily cycles of ambient temperature have also been shown to act as a zeitgeber for mammalian circadian rhythms in heterothermic mammals such as the little pocket mouse (*Perognathus longimembris*) (56), the velvety free-tailed bat (*Molossus ater*) (57), the Syrian hamster (*Mesocricetus auratus*) (58) and rats (59), but also in various normothermic mammals including the squirrel monkey (60), the stripe-faced dunnart (*Sminthopsis macroura*) (61) and the antelope ground squirrel (*Ammospermophilus leucurus*) (58). However, the sensitivity of the pacemaking systems to ambient temperature varies largely between species and individuals (58), which is the result of the relatively weak zeitgeber property of ambient temperature cycles (62).

### 1.3.3 The 'crown' that sets the time

Although setting the time on a mechanical watch may be as easy as twisting the crown, resetting a molecular clock proves to be more complicated. As mentioned above (**section 1.3.1**), the master oscillator in the SCN is mainly entrained by blue light, naturally emitted by the sun. This blue light then acts on the photopigment melanopsin in the ganglion cells, located on the eye's retina (63). As a result, the neurotransmitters glutamate, aspartate, pituitary adenylate cyclase-activating peptide (PACAP) and possibly substance P are transmitted through the monosynaptic retinohypothalamic tract (RHT) to the SCN (30,64). Receptors in the SCN, including the N-methyl-D-aspartate receptor (NMDA-R), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic receptors receive these signals. Various second messengers including calcium, released in response to an NMDA-induced calcium influx, triggered by the released glutamate, activate various signal

transduction cascades. The cascades include the calmodulin kinase II (CaMKII), neuronal nitric oxide (NO) synthase (nNOS) activity, cyclic adenosine monophosphate (cAMP)- and cyclic guanosine monophosphate (cGMP)-dependent protein kinases (PKG) and mitogen-activated protein kinase (MAPK) (see **Figure 1.3**).

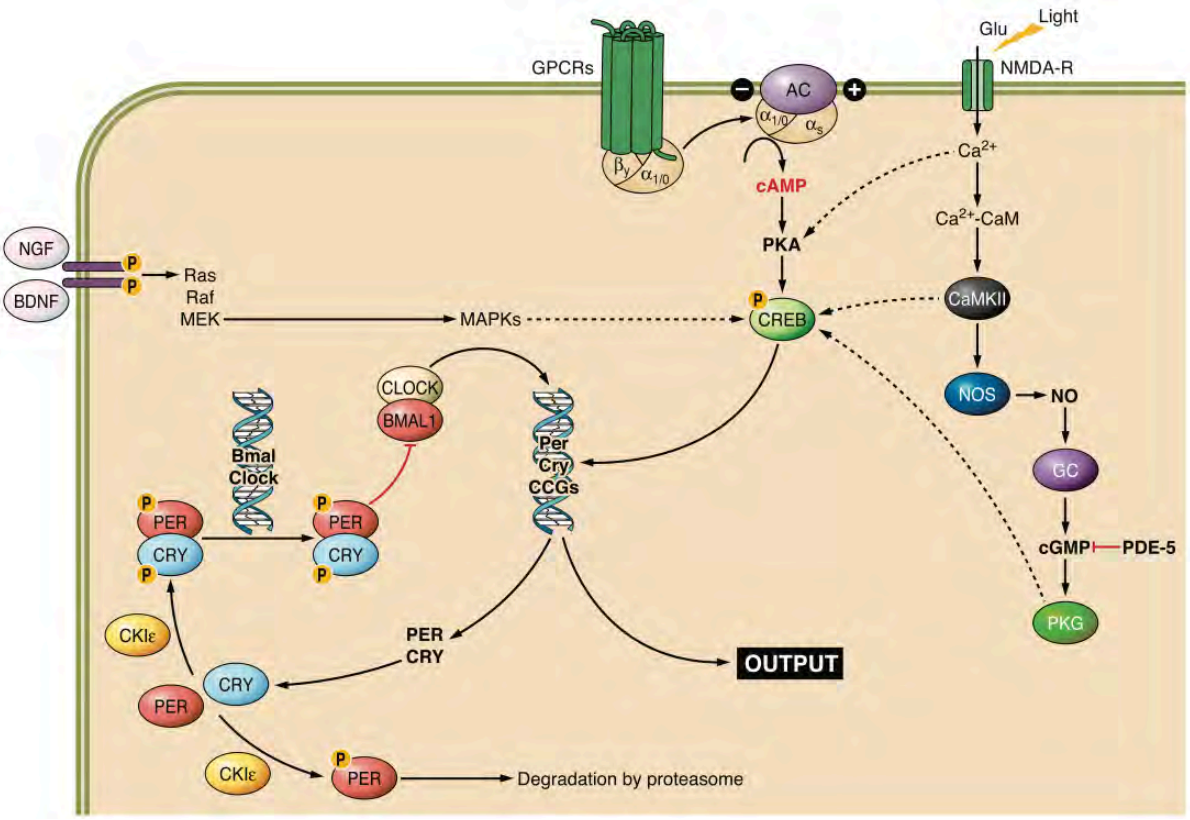


**Figure 1.3** – ***The circadian entrainment mechanism*** *(30)**.** Glutamate (Glu), the molecular signal for light, induces a signal transduction cascade by binding to the N-methyl-D-aspartate receptor (NMDA-R). Respectively, the cascade includes calmodulin (CaM) kinase II (CaMKII), neuronal nitric oxide (NO) synthase (nNOS) activity, soluble guanylyl cyclase (GC) and cGMP-dependent protein kinases (PKG), which then activates cAMP response element binding protein (CREB). CREB can also be phosphorylated by cAMP-dependent protein kinase (PKA). G-protein coupled receptor (GPCR) activates adynyl cyclase, which produces cAMP. PKA is then activated by the cAMP. Also mitogen-activated protein kinases (MAPK) are thought to phosphorylate CREB. Ultimately, phophorylated CREB binds to cAMP response elements of per1 and per2, activating their transcription. This causes the circadian clock to phase advance, hence entraining the rhythm to the light. cAMP; cyclic adenosine monophosphate, NMDA-R; N-methyl D-aspartate receptor; $Ca^{2+}$; calcium, NOS: neuronal nitric oxide synthase, cGMP; cyclic guanosine monophosphate, PDE-5; phosphodiesterase 5, NGF; nerve growth factor, BDNF; brain-derived neurotrophic factor, Ras; rat sarcoma GTPase, Raf; rat fibrosarcoma kinase, MEK; mitogen activated protein kinase kinase, Per; period, Cry; cryptochrome, CCGs; clock controlled genes, CKIε; casein kinase Iε, Bmal; Muscle Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT)-like. Figure reproduced, with permission, from reference (30).*

It is thought that MAPK and PKG then phosphorylate cAMP response element binding protein (CREB). Phosphorylated CREB (pCREB) then binds to a cAMP response element (CRE) in the promoter region of the *PER1* and *PER2* genes, activating their transcription. When the PER1 and PER2 proteins are available, the molecular phase of the circadian clock shifts, allowing the entrainment of the CRs (30).

When light exposure occurs in the early subjective night in rats, a phase delay is initiated (65). When this same stimulus occurs in the late night or early morning, a phase advance is triggered (see **Figure 1.4**). Whether a phase delay or a phase advance occurs, is likely to be dependent on the phase of the molecular feedback loop of the circadian cycle especially with respect to PER. PER levels peak during the early night (66), thus the induction of PER by light in the early night may be negligible and have a smaller effect on the circadian cycle than a light-induced increase in PER levels during late night or early morning, when the PER levels are at a nadir (30). Alternatively, there is evidence suggesting that different pathways exist for advancing or delaying the biological clock, and that these may be dependent on the time of day of light exposure. After a light pulse given during late night, glutamate initiates the activation of cGMP mediated by the NMDA-R according to the pathway described above (65). This signal transduction cascade results in a phase advance. However, when a light pulse is given during the early night, it seems that another mechanism is responsible for the phase shift of the circadian clock (65), since glutamate follows a different pathway that eventually activates ryanodine receptors (RyRs) which results in the release of $Ca^{2+}$ from the endoplasmatic reticulum (ER). While the precise mechanism is not yet known, and possibly NO plays an important role in RyR activation (65), the result is that this early-night pathway results in a phase delay rather than a phase advance (65).

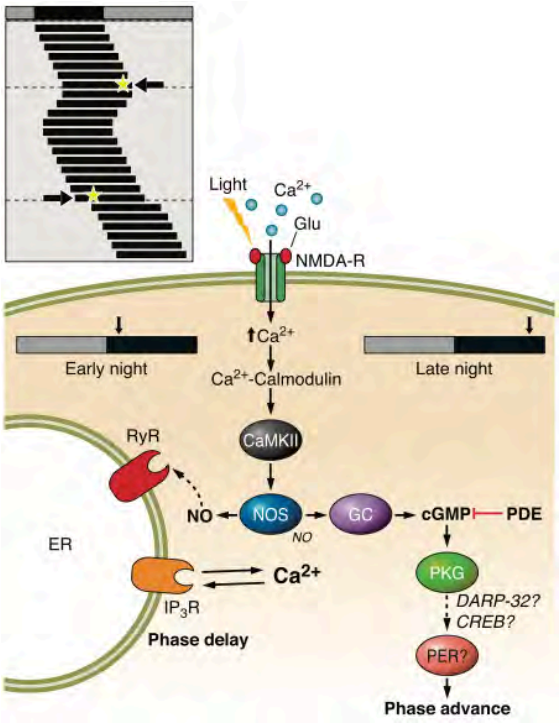


**Figure 1.4** – ***The proposed mechanism of how light effects phase shifts during early and late night*** *(30). As a result of a light pulse during late night, glutamate (Glu) communicates with the N-methyl-D-aspartate receptor (NMDA-R) to induce a signal transduction pathway to entrain the circadian rhythm by phase advancing (see*

***Figure 1.3**). However, during early night, nitric oxide (NO), a product from another cascade induced by Glu, activates ryanodine receptor (RyRs). As a result, the intracellular calcium ($Ca^{2+}$) rises which initiates a phase delay by a yet unknown mechanism. CaMKII; calmodulin kinase II, NOS; nitric oxide synthase, GC; guanylyl cyclase, cGMP; cyclic guanosine monophosphate, PDE; phosphodiesterase, PKG; cGMP-dependent kinase, DARP-32; dopamine- and cAMP-regulated neuronal phosphoprotein, CREB; cAMP response element binding protein, PER; period, $IP_3R$; inositol triphosphate receptor. (65). Figure reproduced, with permission, from reference (30).*

**1.4 Chronotype**

If the businessman from **section 1.1** looks at his wristwatch without initiating a certain action to be in time for his appointment, he might as well not wear a watch at all. The reason he wears a watch in the first place is to live according his time-schedule. However, mechanical wristwatches are free-running, which means that no two wristwatches run exactly synchronously. While the businessman may be on time according to his wristwatch, he might be late according to his client's wristwatch. Similarly, inter-individual differences in our own endogenous periods lead to differences in phase shifts in biological time display (i.e. the circadian output), which may be behavioral in nature. These slight variations lead to a person's chronotype, which is the characteristic of a person reflecting his/her diurnal preference. Chronotype is usually divided into three categories: MTs, ETs and an intermediate type or neither-types category (NTs). While these categories may be discrete, they are positioned adjacent to one another such that chronotype is really reflective of a spectrum of scores, ranging from extreme evening-types on one end to extreme morning-types at the opposite end. MTs or 'Larks' get up and go to bed earlier, while ETs or 'Owls' prefer to wake up and retire later. The NTs, positioned midway between the MTs and ETs, experience a more intermediate preference for waking up and retiring. These characteristics also express themselves in activity habits such as the time of day an individual prefers to carry out certain tasks or participate in physical activity (67-69).

From the early 1930's on, many studies have been done on the characterization of MTs and ETs. It has been found that many aspects of behavior and physiology differ between the two extremes. For example, ETs are more tolerable to sleep deprivation and are more prone to stress (69,70). Additionally, subjective alertness, subjective fatigue, mid-activity point, vigilance performance and detection performance in MTs also peak earlier than in ETs. In MTs, body temperature and heart rate (both rest and strain) peak earlier compared to ETs. There is a difference of up to 2 hours and 11 minutes in the body temperature minimum rhythm between extreme morning- and evening- types (71). Urinary adrenaline peaks earlier in ETs but the amplitude is larger in MTs, the amplitude in subjective alertness is also higher in MTs, while the amplitude in oral temperature is higher in ETs (reviewed by Kerkhof, 1985, (69)). These studies combined show that

there is a deeper biological mechanism that drives chronotype, apart from being solely a psychological preference.

### 1.4.1 Chronotype assessment tools

Chronotype assessment tools, typically comprising subjective questionnaires, are used to assess whether your biological wristwatch is advanced or delayed relative to societal time. Most tools provide a person with a score that places them in a certain chronotype category (e.g. MT or ET).

The most widely used chronotype tool is the Horne-Östberg Morningness-Eveningness Personality Questionnaire (HÖ-MEQ) (67). This questionnaire consists of 19 questions to assess where the *preferred* time of mental and physical activity and sleep lies. The questionnaire scores individuals on a scale from 16 to 86, based on which, they are place in one of the five chronotype categories: definite evening-types (DET, score of 16-30); moderate evening-types (MET, score of 31-41); neither-types (NT, score of 42-58); moderate morning-types (MMT, score of 59-69) or definite morning-types (DMT, score of 70-86). The 'definite' and 'moderate' categories are sometimes grouped together, so that only three groups are described: MT, NT and ET. Adan and Almirall (1991) introduced a shortened version of the HÖ-MEQ (rMEQ). Using just 5 out of the 19 original HÖ-MEQ questions, the tested subjects receive a score ranging from 4 (DET) to 25 (DMT) (72). The Children's Morningness-Eveningness Preference (CMEP) scale by Carskadon *et al.* (1993) was revised for use with children and only contained 10 out of the original 19 questions. The CMEP was written so that young children would understand the questions (73).

Another widely used chronotype assessment tool is the Composite Scale of Morningness (CSM) (74). The CSM consists of 13 questions regarding the time that the assessed individual actually wakes up, goes to bed, and prefers physical and mental activities and subjective alertness. The CSM provides an individual with a score ranging from 13 to 55, on which evening-types score lower (13 to 27) and morning-types score higher (41 to 55). The CSM has been validated by numerous studies in different languages, such as Italian (75), Thai (76), French (77), Spanish (78), Dutch, and Portuguese (79). Brown used the CSM and simplified it to a questionnaire at a seventh grade (age: 12-13 y) reading level and called it the Basic Language Morningness scale (BALM) (80).

The Munich Chronotype Questionnaire (MCTQ) is yet another chronotype assessment tool that determines ones chronotype by questions based on the actual times that the individual wakes up and discriminates between working days and free days. As a result, the chronotype is calculated

and is given as the time of mid-sleep (MSF, half way between onset and end) on free days. Free days are days where the individual is not restricted by social obligations and does not suffer from any accumulated sleep debt (81). The individuals are categorized as extreme early-types, moderate early-types, slight early-types, normal-types, slight late-types, moderate late-types and extreme late-types. The MSF correlates well ($|r|>0.7$) with the HÖ-MEQ score in a Dutch student population (n=2,481, mean age not reported) (82).

The Circadian Energy Scale (CIRENS) is also used to describe chronotypes and was first introduced in 2011 (83). It is based on the participants' energy level (a measure of how energetic the participants feel) in the morning, afternoon (optional) and evening. Participants rate their energy levels as very low, low, moderate, high or very high. Subtracting the morning energy score from the evening energy score, gives a score ranging from -4 to 4. The individuals are categorized as morning-types if the score is equal to or lower than -2, neither-types if the score is equal to or between -1 and 1 and evening-types if the score is equal to or higher than 2. The CIRENS is validated with the $MSF_{SC}$ from the MCTQ (r=0.32) and the MEQ (r=-0.70) (83).

Folkard *et al.* (1979) introduced the Circadian Type Questionnaire/Inventory (CTQ/CTI) (84). The CTQ/CTI measures three different factors that tell how well an individual adapts to shiftwork, namely rigidity (or flexibility) of sleeping habits, ability to overcome drowsiness (vigorous/languid types) and morningness (74). The CTQ/CTI was later validated and a different scale construction was suggested, however still not recommended (85).

The Diurnal Type Scale (DTS) developed by Torsvall and Åkerstedt categorizes a person as being morning-active (MA) or evening-active (EA) (86). The DTS was developed to be as short as possible, since the authors proposed that alternative scales often prove to be too long to add to the major questionnaire. The DST consists of only seven questions and each answer is scored with 1 to 4 points. The total group was then split into three equal groups namely: MA, EA and intermediate, based on the scores. The DTS is validated on 375 shift and day workers, and only focuses on sleep/wake habits (86).

One of the most important differences between the HÖ-MEQ and the other tools is that the HÖ-MEQ focuses on preferred times of activity and sleep, rather than actual times. This is crucial, because the actual time for individuals in a social-economic routine (generally between the ages of 20 and 65 years) is mostly decided by work and family commitments, not by the individual's actual preference. It has been shown that individuals within this range of age, accumulate a sleep-debt for

which they compensate on free days (weekends) by lengthening their sleep by several hours (81). This means that two individuals, of which one has a preference for mornings and the other for evenings, but both have the same work commitments, might score similarly using tools such as the CSM that assesses the actual times. Hence we choose to assess the participant's chronotype using the HÖ-MEQ chronotype assessment tool.

It has previously been proposed that in a study for assessing chronotypes among large populations, it would be appropriate to identify specific cut-off scores for each cultural and age group, because morningness scores vary across cultures and ages (69,87,88). Being a MT or ET is always relative to other persons within the same group albeit by age, sex or culture. In order to make an accurate assumption of how a certain factor contributes to an individual's chronotype, it is important to know what other factors influence chronotype so that those can be taken into account when explaining an individual's chronotype. These factors will be discussed in the following section (**1.4.2**).

### 1.4.2 Other factors that influence chronotype

Chronotype is the product of many environmental and intrinsic factors, which will now be discussed in this section. Although we may not yet know all factors that influence chronotype, this section deals with the most important factors. Apart from genetic variations in clock-genes, which will be discussed in **section 1.5**, other genetic factors that may influence chronotype are sex and ethnicity. These factors, together with non-genetic factors such as age, participating in night-time shift work, being physically active and environmental factors including the effect of latitude, and seasonal changes contribute to a person's chronotype.

#### 1.4.2.1 Sex

Since CRs have large genetic components it is likely that any genetic factor, including sex, influences chronotype. However, there are contradicting conclusions from studies that have investigated the difference in chronotype between males and females. For example, a study in New Zealand concluded that diurnal preference is independent of gender in 2526 participants aged 30 – 49 years (89). Additionally, no sex-effect on the chronotype distribution of 413 males and 262 females was found in a cohort of subjects aged 20 – 35 years (90). Similarly, sex was not significantly related to the rMEQ score (see **section 1.4.1**) in a sample of 330 males and 578 females (91). However, in a study of 2135 University students, a difference was found between males and females. Whereas women scored 49.6 on the HÖ-MEQ, men scored significantly lower, namely 46.8. The findings suggest that women are more likely to be MT (92). However, these results must be interpreted with

caution since this small statistical significant difference may possibly be a type I error, due to the large sample size. Another study on 61 recreational athletes (age: 21.6 y) also showed that males were more likely to be evening-types than females (93).

During puberty chronotype shifts from morningness to eveningness, peaks at a certain age, and then shifts back to a preference for mornings (see **section 1.4.2.3**). This pattern is different for males and females. Females reach their eveningness peak earlier (19.5 y) than males do (20.9 y) and are thus more skewed to morningness than males until both sexes reach the age of 50 y (94). In conclusion, chronotype does seem to be affected by sex, especially when age is considered.

#### 1.4.2.2 Ethnicity

In this section, ethnicity is defined as any classification within the human species that is related to common ancestral, cultural, social or national experiences. In this section 'ethnicity' may also be used to describe the different *genetic* classifications between individual humans, often defined as 'race'. These genetic differences between ethnic groups may exist due to evolutionary factors of differentiation such as isolation, drift and random fixation of the material particles which control heredity (the genes), changes in the structure of these particles, hybridization, and natural selection (95). We use the terms 'genetic ethnicity' and 'race' as stated by experts of the United Nations Economic and Security Council on problems of race in 1951 (95). Examples of such racial classifications are African, European (or Caucasian), Asian and mixed ancestral. The classification terms are dynamic, not static. These classes were not the same in the past as they are at present, and there is every reason to believe that they will change in the future (95). The genetic differences between these ethnicities express themselves in physical characteristics such as skin and hair colour and anatomy. Of the 0.1% of DNA that varies among individuals, 85-90% genetic variation is found within the three old world populations (Africa, Asia and Europe), while the genetic variation between these three groups is approximately 10-15% higher (96). This shows that genetic variations between these groups are larger than genetic differences within these groups. Thus, I think it is justified to allow genetic-based ethnic classification for research purposes.

Different ethnic populations have different genetically beneficial properties to survive in the location their ancestors grew up. For example, variations in skin colour were formed by selective pressure based on environment and geographical location. People that lived closer to the equator and thus are exposed to higher levels of sunlight benefit from a dark skin as it prevents sun burn, skin cancer, the photolysis of folate and damage to sweat glands (97,98). People that live at higher or

lower latitudes have a lighter skin, because of 1) the absence of selection or 2) because of the evolutionary advantage of being able to create more vitamin D to prevent rickets (99). Thus, if skin colour, changes as a function of latitude, it is very likely that other genes also differs between ethnicities. These differences could be polymorphisms in clock-genes that influence chronotype through the circadian system, which will be discussed in detail in **section 1.5**. Selective pressure might have formed more morning-gene carriers closer to the equator because getting up in the early morning may be more beneficial for survival (think of the heat load produced by physical activity in sunlight). Those that live in colder areas further from the Earth's equator might benefit from getting up later to avoid the cold of the night.

Takao *et al.* (2009) discovered different polymorphisms in circadian clock genes and differences in ocular photosensitivity between Caucasian and Asian infants. The authors proposed that ethnic differences might characterize the circadian photosensitivity (100), which might in turn affect chronotype. Likewise, when adjusted for age, CMEP scores (see **section 1.4.1**) were significantly higher in an Asian cohort of children (n=113, age: 13.7 ±1.9 y) compared to an African American sample (n=86, age: 10.6 ±2.3 y), but did not differ from a Caucasian (n=642, age: 13.3 ±2.4 y) or a Hispanic (n=34, age: 11.9 ±2.7 y) sample (101). These finding suggests that variety in diurnal preference between some ethnicity groups in children do exist. However, in a study on a small cohort of 410 participants, the HÖ-MEQ score did not differ by ethnic heritage (102). Also, Paine *et al.* (2006) studied the effect of being of Māori descent on chronotype and found that no statistically significant difference existed between Māori and non-Māori groups (89).

Apart from genetic ethnicity or 'race', other ethnic classifications may play a role in the chronotype of an ethnic group. For example, Evans *et al*. (2011) reported the chronotype category distribution of an United States Amish cohort (males, n=332, age: 41.9 ±13.2 y and females, age: 44.9 ±14.3 y), was found to mainly contain morning-types (77% MT, 1% ET and 78% MT, 1% ET for the males and female respectively (103). While another United State cohort (males, n=101, age: 25.2 ±5.3 y and females, n=71, age: 25.1 ±5.4 y) was mainly categorized as NT (61%, vs. 27% ET and only 12% MT) (71). Although age is very likely a confounding factor, the Amish culture and tradition of not using artificial light and getting up at sunrise, may play a significant role in this observation, providing evidence that ethnicity (based on religion, culture and tradition) may influence chronotype.

The chronotype distributions of various populations around the world are presented in **Table 1.1**. The various studies presented in that table represent cohorts of different countries, but also different ages, latitudes and distributions of male and female participants, which are all factors that

may affect genotype. For that reason it is inaccurate to determine whether chronotype is affected by each individual's origin, based upon those publications.

**Table 1.1 – *The chronotype category distribution of different cohorts around the world.*** *The list is sorted by frequency of morning-types. Chronotype categories are determined as per Horne-Östberg morningness-eveningness personality questionnaire cut-offs, unless stated otherwise.*

| Country | Latitude | MT (%) | NT (%) | ET (%) | Sample size (n) | Sex (% male) | age (y) | Specialties | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **U.S.A.** | 41° North | 79 | 20 | 1 | 293 | 0 | 44.9 ±14.3 | Amish people | (103) |
| | | 77 | 22 | 1 | 332 | 100 | 41.9 ±13.2 | | |
| **New Zealand** | 41° South | 50 | 44 | 6 | 2526 | 42 | 30-49 | - | (89) |
| **South Africa** | 33° South | 42 | 51 | 7 | 93 | 100 | 32.9 ±8.1 | Active controls | (104) |
| **Spain** | 40° North | 42 | 24 | 34 | 1495 | 18 | 39.4 ±12.3 | Obese people, adjusted MEQ-scale | (105) |
| **France** | 47° North | 40 | 49 | 11 | 2007 | NA | 42.7 | rMEQ | (106) |
| **Spain** | 40° North | 22 | 60 | 18 | 908 | 36 | 24.9 ±6.4 | rMEQ, workers and University Students | (91) |
| **Italy** | 42° North | 18 | 60 | 22 | 1256 | 49 | 18-30 | University Students | (92) |
| **Spain** | 40° North | 12 | 60 | 28 | 879 | 48 | 18-30 | | |
| **U.S.A.** | 40° North | 12 | 61 | 27 | 172 | 59 | 25.2 | - | (71) |
| **Italy & Spain†** | 42° North | 11 | 65 | 23 | 1041 | 39.2 | 22.8 ±2.9 | rMEQ, University Students | (107) |
| **Germany** | 52° North | 9 | 62 | 29 | 112 | 100 | NA | Young twins | (108) |
| **Lebanon** | 33° North | 7 | 68 | 25 | 540 | 49.4 | 19.9 ±1.5 | University Students | (109) |

†Chronotype category distribution per country is unknown

MT: morning-type, NT: neither-type, ET: evening-type, rMEQ: reduced Horne-Östberg morningness-eveningness personality questionnaire

### 1.4.2.3 Age

Age is yet another factor that might influence chronotype, such that younger people tend to be more skewed to eveningness and older people are more skewed to morningness (69). Two intrinsic characteristics of the ageing process in healthy people are changes in sleep consolidation and sleep structure. These changes include a reduction in the strength of the circadian drive for sleep in the morning hours and a sleep dependent decline in sleep consolidation in older people (64+ years of age). A result of these changes is an apparent internal circadian advance (relative to the body's temperature rhythm and melatonin oscillation) of the propensity to awaken from sleep (110). This suggests a shift in older people, which results in a chronotype that is more skewed to morningness.

In a studies on shift workers, chronotype was affected by age wherein older workers scored higher on the HÖ-MEQ than younger workers (111,112). Likewise, a study on the rMEQ score (see

**section 1.4.1**) of 1,041 younger people between the ages of 18 and 30 years (age: 22.83 ± 2.92 y) revealed that 11.3% were MTs, 65.3% were NTs and 23.4% were ETs (107). Similarly, a smaller cohort of 172 participants of a similar age group (age: 25.2 ± 5.4 y) showed a MT-ET distribution of 12% and 27% respectively (71). Both studies found a much higher number of ETs compared to an older population of 2,526 participants between the ages of 30 and 49 years. Only 6% was ET and 50% was MT (89). A comparison of the effect of age on chronotype between two studies must be made with caution, as many confounding factors exist (e.g. latitude). Within this age range, the HÖ-MEQ score shifted by 1.6 points for every 5 years of ageing.

Another study used the MCTQ described in **section 1.4.1** to characterize people between the ages of 8 and 94 years regarding their self-assessed chronotype based on the midpoint of sleep on free days (MSF, i.e. sleep without social obligations) and adjusted for individual average sleep need ($MSF_{SC}$) (94). The authors showed that males between the ages of 8 to 20.9 years (proposed adolescence) show an increasing $MSF_{SC}$, which indicates an increasing eveningness chronotype. As age progresses after the age of 20.9 years, the $MSF_{SC}$ displayed a reversed pattern, and changed to an earlier chronotype (94). Adolescents tend to stay up later in the evening and sleep longer in the morning than preadolescents do (73,101,113). This shift towards eveningness may be biologically mediated and linked to puberty.

Finally, a study on 908 participants showed chronotype differences between younger and older age groups, with the younger group scoring lower (evenings) and older group scoring higher (mornings) (91). Apart from the explanation given earlier in this section, these finding may also be caused by changes in occupation or domestic arrangements that are co-factored by age (114).

#### 1.4.2.4 Night-time shift work

Since chronotype is associated with wake and bedtime, any factor that influences wake and bedtime might have an effect on chronotype (67,109). This could mean that shift-workers, that often have extreme wake and bedtimes, may also have extreme chronotypes. For example, night shift workers that were categorized as MTs, averagely slept for 4 hours and 43 min on average, while those that were categorized as ETs only slept for 4 hours and 2 min. Both chronotypes slept significantly shorter than morning and afternoon shift workers, that slept at least 7 hours, regardless of their chronotype (115). This study shows how night shift work can disturb sleep and may thus also alter chronotype.

In another study, 32% of 174 permanent health care day-time shift workers were categorized as ETs, while 75% and 68% of 55 and 73 permanent evening and night-time shift workers were ETs respectively (116). For participants with shift rotations, the percentage of ET shift workers dropped to 29% (rotating days and evening shifts) and 53% (relief or combined shifts with nights) (116). This suggests that when the shift workers are active in the evening or at night, a higher preference for evenings is likely. More evidence is found in a study of 44 male automobile workers, where early-late two-shift workers were found to be more morning orientated and night-shifts workers who were more orientated to evenings (117). Also Furnham *et al.* (1999) found that, in a study on 312 television workers, night workers were more orientated towards evenings than day workers (118). Additionally, in a study on 561 male shift workers, a two-team, two-shift system cohort consisted of 50% MTs and 21% ETs, and a continuous three-team, three-shift system cohort consisted of 43% MTs and 14% ETs (111), which does not quite support the earlier mentioned findings of a higher prevalence of ETs in these cohorts. However, the results from these findings are different from most other shift-work studies, due to the fact that this cohort does not consist of any night-time shift workers (111). Another study found that individuals that changed from shift work to day work became more morning orientated then they were before (86), again suggesting that (night-time) shift work does affect chronotype.

When comparing a cohort of shift workers to a cohort of day-workers from another study, similar results are found. For example, a study on 431 Korean male night-time shift workers with the age range of 17-59 years (mean age 34.3 years) showed that the group of night-time shift workers consisted of 25% MTs and 9% ETs (111). While a similar age group of non-shift workers (~39 years, n=2,526) from a general cohort from New Zealand consists of 50% MTs and only 6% ETs (89). This either shows that night-time shift workers have a larger tendency to eveningness and a smaller tendency to morningness or that evening-types are more likely to choose shift-work. Other factors may play a role in the difference between these two groups. The two samples live on different latitudes, which might affect chronotype too (see **section 1.4.2.6**). Also, the shift workers sample consists of only male participants, while the New Zealand sample consists of both male and female participants. Sex can also affect the average chronotype categories in the group (see **section 1.4.2.1**).

Surprisingly, 25 nurses on rotating shift (mornings, evening and night shifts) were more morning-orientated than seven morning-only shift nurses (119) These results are possibly a type I error due to the low sample size. Antunes *et al.* (2010) did not find a correlation between chronotype and night-time shift work either, however, the sample size was also small (n=14) and thus may not represent a realistic sample of the night-time shift work population (120). In general, therefore, it

seemed that night-time shift work is associated with eveningness. It must be kept in mind however, that ETs may choose night shift work more readily than MTs, which may also explain the association between night shift work and the ET chronotype.

#### 1.4.2.5 Physical activity

As doing nighttime shift-work may have an effect on one's chronotype because of the daily repeating routines, so may exercise. Apart from professional athletes who train during the day, most athletic individuals work during the day and only have their mornings and evenings available for exercise. This might cause these athletes to get up earlier or go to bed later in order to exercise, thus potentially affecting their chronotype. Those who train in the early mornings may also be exposed to sunlight earlier than those who remain indoors. This can affect the synchronization of the circadian clock in the SCN and may thus affect their chronotype (29) (see **section 1.3**).

Additionally, bouts of 15 min exercise each hour during night shift facilitates temperature rhythm phase shifts (48), which may thus affect an individuals chronotype. Plasma melatonin rhythm of participants that are under a forced sleep-wake cycle, was significantly phase advanced by two bouts of exercise during the subjective day, compared to participants in the same environment but without exercise (55). These findings suggest that physical exercise accelerates phase advance shifts in the CR.

More descriptive studies have found high numbers of MTs in athletic individuals (104,121). For example, Kunorozva *et al.* (2012) found that in a sample of runners, cyclists, triathletes and active but non-competitive controls, 77% of the MTs trained in the morning and only 11% trained in the evening (104) (see **Figure 1.5**). The authors stated that these findings could be explained by: 1) MTs may select these sports more often than ETs do because of the early time at which endurance events in these sports are usually scheduled and 2) by the adaptation that may take place after having to get up early in the mornings to exercise or to participate in competitive sporting events (104). Either way, the association between morningness and individual endurance activity was found to be evident.

Alternatively, a stronger preference towards morningness was associated with less time spent in physical inactivity (e.g. watching TV and playing on the computer) and other health related behaviors (122). Schaal *et al.* (2010) found that adolescents that are more physically active are more morning-oriented. Furthermore, morning-oriented pupils gave more positive attributes to increasing

physical activity and were more likely to choose physical activity over physical inactivity such as watching an interesting television program than their more evening-oriented contemporaries (123). In another study on adolescents, physical health was also associated with morningness (124). The MTs in a group of 678 pupils (age: 13.8 y) spent more time reading books and engaging in physical activity, and spent less times watching television and less time at the computer than ETs did (125). Cavallera *et al.* (2011) also found that higher scores on eveningness disposition corresponded to fewer hours of sport activity (93). These studies thus all show an association between healthy life style decisions and morningness, which may in turn explain the link between physical activity and morningness. For example, a person who chooses to participate in physical activity may also make other healthy life-style decisions such as increasing sleep length, which may result in an earlier bedtime.

Lastly, exercise may have an effect on chronotype because of its beneficial effect on sleep. The American National Sleep Foundation recommends that people "*exercise daily*" to increase sleep hygiene (126). Benefits include a decrease in sleep disruptions, shorter time to fall asleep and an increase in slow-wave sleep. However, this exercise should be aerobic, moderate to high intensity, last at least one hour (longer in trained athletes) and be conducted 4-8 hours before bedtime (127-129). Furthermore, Youngstedt *et al.* (2005) have suggested using nighttime and morning exercise to treat those with ASPS and DSPS respectively (127). Both syndromes are associated with extreme chronotypes and this 'treatment' might as well be effective to consciously or unconsciously change chronotypes to be less extreme.

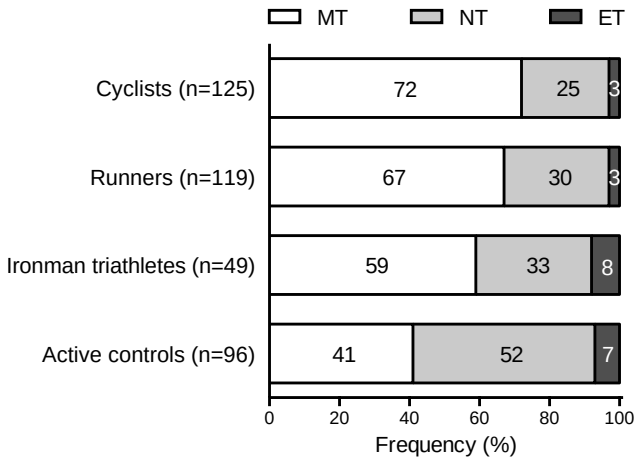


**Figure 1.5 – *The chronotype distributions of South African runners, cyclists, Ironman triathletes and an active control group*** *(104). The three individual sport endurance athlete groups are much more likely to be morning-type (MT) than the active controls (p<0.001). NT; neither-type, ET; evening-type.*

The marathon is a sporting event for which the participating athletes run a distance of 42,195 meters. Since running is such a popular sport, many athletes participate in marathons all over the world. Training is utterly important for those that participate in marathon running. Based on the fitness level and goal, recreational marathon runners train about 50 to 70 km per week on average, which equals 6 to 8 hours each week (130,131). Training for at least 50 to 70 min each day (130) requires planning and time management for non-professional athletes who have work obligations. As mentioned before, most people work during standard office hours (~09:00 – 17:00) and may only be available to train in the evening or morning. Thus timing of exercise can influence a person's chronotype, or alternatively, a person's chronotype may influence the time of day at which that person chooses to train. To conclude, the timing of exercise, whether someone exercises at all, and the intensity or nature of the exercise may all contribute to a person's chronotype.

#### 1.4.2.6 Latitude

As a function of latitude, sunlight intensity and photoperiod (time from daily beginning of blue-light emission to daily ending) change and are known to affect CR entrainment. Specifically, a longer photoperiod has a larger effect than shorter exposures, constant light exposure eventually disrupts the circadian cycle, and higher light intensity is more effective than lower light intensity [reviewed by Duffy *et al.* (2008) (132)]. The effect of bright natural light on circadian advances appears to be larger in later chronotypes, synchronizing the CR so that the circadian phase becomes closer to that of earlier chronotypes (133). In *Drosophila melanogaster*, the splicing of *PER* mRNA, one of the key components in the genetic CR, is affected by day-time length and temperature, which both change as a function of latitude (134). A positive correlation was found between DSPS and the *PER3*$^{4}$ homozygotes in a study with participants from London (51° North) while a positive correlation between DSPS and *PER3*$^{5}$ was found in participants from São Paulo (23° South) (135). This suggests that latitude has an effect on the correlation between the molecular components of the CR and diurnal preference, which was concluded by Pereira *et al.* (2004) (136). However, there is no evidence that the *PER3* VNTR genotype, shows a distributional pattern that suggests an evolutionary selection based on geographical location (137), although other clock genes might.

**Table 1.1** shows the chronotype category distributions of populations around the world that have previously been published including the latitude of were each group is recruited. The table shows that chronotype distribution varies between the groups. Comparing the data of these eleven publications based on latitude must be done with caution since many confounding factors that may influence chronotype exist. Factors that are often reported are sex, age and specialities (e.g. studied

on a cohort of students or obese people), and are included in the table. Although a pattern may seem absent, it is evident that the chronotype distribution varies greatly between the different populations around the world, although age may be contributing or even be the main reason of this finding. Other latitude-associated differences in chronotype may be based on cultural or societal factors.

The population studied by Kunorozva *et al.* (2012) consisted of participants from South Africa living at a latitude of 34° South. The authors hypothesized that this more moderate latitude, which implies greater sunlight exposure, could be the reason why not only the individual sport endurance athletes from the study (cyclists, runners and Ironman triathletes) but also the active controls had a stronger tendency to morningness than European (Italian and Spanish, 42° and 40° North respectively) and American (Michigan and Albuquerque, 42° and 41° North) samples (71,104,107,138). These differences might be attributed to the fact that South Africa is closer to the equator and thus has more sunlight and higher sunlight intensity. This is supported by the fact that a New Zealand (Wellington, 41° South) sample showed a chronotype distribution similar to the Kunorozva *et al.* (2012) South African control sample (89).

Randler *et al.* (2008) found that adolescents who lived in the tropics were more oriented to morningness than those who lived in the subtropics (139). This finding is in agreement with the finding of Smith *et al.* (2002) who found that University students in warmer climates (e.g. Spain and Columbia) are more likely to be MT than University students from colder climates (e.g. England and the Netherlands), and suggested that people that live in warmer climates should start their day earlier to cope with the increasing heat throughout the day (79).

**1.5 How 'cog wheels' affect time display**

Since chronotype is the product of the relativity of the circadian period to the environmental time, any factor that influences the synchronization between these two, such as the molecular 'cog wheels' or clock-genes and proteins may affect chronotype. Imagine how having an altered cogwheel may slow down or speed up the circadian rhythm, thus making it either longer or shorter respectively. Any circadian process that has a longer circadian period will happen later than it would have on a shorter circadian period. Evidence for this theory was provided by Duffy *et al.* (2001) who found an association between a person's intrinsic circadian period length and chronotype, with ETs having longer circadian periods (140). For example, a longer circadian period delays the onset of secretion of melatonin in the evening, which results in a delayed propensity for sleep (141). That

genetics play a large role in one's chronotype became evident in a study on the chronotype of 8,753 adult twin-pairs. The authors revealed that genetics accounted for approximately 50% of chronotype (142).

### 1.5.1 Clock genes polymorphisms and associated phenotypes

Several genes have been found to be associated with preferences for mornings or evenings. For example, a 3111C single nucleotide polymorphism (SNP) in the 3'-untranslated region of the *CLOCK* gene was found to correlate with a lower HÖ-MEQ score suggesting a higher level of eveningness in both American (n=410, age: 50.0 ±7.9 y) and Japanese adults (n=421, age: 35.5 ±0.5 y, 28% male and 65% shift worker) (102,143). However, these findings could not be replicated in a sample from the United Kingdom (n=484, age: 35.0 ±13.0 y, 45% male) (144). Therefore, the exact mechanism of the correlation between the $CLOCK^{3111C}$ SNP and eveningness, if any, remains unknown.

A variation in another gene that is known for its role in the transcriptional-translational feedback loop of the circadian clock, *PER2*, may also influence chronotype. Extreme morning-types in a cohort of 484 volunteers (age not reported) from the United Kingdom had a significantly higher frequency of the $PER2^{111G}$ allele (rs2304672) (145). Variation in rs2304672 has also been associated with bipolar disorder in a different study in a cohort of Canadians and Americans (n=423, no age reported) (146). Additionally, in a study on 299 Korean students (age: 22.9 ±2.1 y, 64% male), another *PER2* variation, the G3853A SNP (rs934945), was also found to correlate with morningness (147). Likewise, a mutation in exon 17 of *PER2*, resulting in the substitution of a conserved serine to a glycine (S662G) in the CKIε binding region of *hPER2* that reduces phosphorylation of the protein *in vitro*, was found to be associated with familial advanced sleep phase syndrome (FASPS) in an American family (148). However, these findings were not replicated in two Japanese FASPS families (149). Finally, another *PER2* mutation, the $PER2^{A10870G}$ SNP was associated with morningness in 189 patients with winter depression (i.e. seasonal affective disorder, SAD) (150). In that study, genetic variations in *ARNTl* and *NPAS2* were also associated with SAD. Combined, these studies show how different mutations in solely the *PER2* clock gene can affect chronotype or can even be associated with advanced sleep phase disorders.

A T2434C polymorphism in exon 18 of the *PER1* gene was associated with an extreme preference for mornings in 240 male and female adults from the United Kingdom of which 80 were classified as MTs (age: 37.1 ±10.6 y, 45% male), another 80 were classified as ETs (age: 41.6 ±16.3 y, 45% male) and the remaining 80 were classified as NT (age: 41.6 ±15.4 y, 53% male) (151). This

suggests, that the *PER1* gene, another component of the molecular circadian clock, also affects chronotype. The *PER1* and *PER2* genes are homologues of the *PER3* gene that has also been associated with chronotype and will be discussed in the next section.

### 1.5.2 The *PERIOD3* gene

The *PERIOD* gene was first described in the 3B region of the X chromosome in *Drosophila* (152,153). Three ethylmethanesulfonate-induced mutations caused arrhythmic, shortened and prolonged circadian periods of activity in *Drosophila melanogaster*, suggesting that this gene affects circadian rhythm generation and was thus named *Period*. Three mammalian *Period* homologues were later discovered in mice and in humans, named *PER1, PER2* and *PER3* (154-156). *PER1* and *PER2* have been described as regulators of CRs and the proposed mechanism in mammals is described in **section 1.2** (21,157). Studies on *mouse (m)PER3* revealed only a minor circadian response, when compared to the mutations in *mPER1* and *mPER2* (158). In *mPER3*-deficient mice, the locomotor activity rhythm was normal and was thus found to be unnecessary for mammalian CR generation. However, the free-running circadian period was 30 minutes shorter compared to wild-type mice, suggesting that *mPER3* does influence phenotype (158). Another study found that *PER3*-deficient mice have shortened circadian periods in the peripheral oscillators in the pituitary, liver, lungs, adrenal glands, oesophagus, aorta, thymus and arcuate complex, but not in the SCN, kidney, colon, spleen and the white adipose tissue surrounding the adrenal glands (159). The study shows that in mice, *mPER3* is a key player in the CRs of peripheral oscillators of specific tissues, but not in the master oscillator.

In humans, a variable number tandem repeat (VNTR) polymorphism in the *PER3* gene is linked to extreme diurnal preference. The longer allele of the gene (*PER3*$^5$) has been associated with morning preference, and the shorter (*PER3*$^4$) with evening preference and delayed sleep phase syndrome (DSPS) (90,135,160). Also, a strong association with the *PER3*$^4$ allele and the *PER3*$^5$ allele for respectively ET/NT and MT chronotype was observed by Kunorozva *et al.* (2012) (104).

To explain a possible mechanism of this morning-phenotype, a schematic of the human PER3 protein is presented in **Figure 1.6** (135). Exon 18 of the PER3 protein contains a polymorphic repeat domain with either four or five repeats of 18 amino-acids, similar to that found in PER2 (148). This region contains a number of potential casein kinase 1 ε (CK1ε) phosphorylation sites, containing a serine and threonine rich region, which can be phosphorylated in all three PER proteins (161). Upon phosphorylation by CK1ε and CK1δ the PER1 and PER2 proteins are rapidly degraded via the ubiquitin-proteasome pathway (25,26), and a similar mechanism may be present for PER3. CK1ε and

CK1δ phosphorylation also induces the nuclear translocation of the PER proteins (161). Thus, the PER3$^{4}$ repeat protein has fewer potential phosphorylation sites than the PER3$^{5}$ protein and thus may be translocated to the nucleus and degraded at a lower rate than PER3$^{5}$ proteins (161). This differential turnover may alter the kinetics of the circadian cycle and may thus have an effect on the circadian period length. Shorter circadian lengths may result in a morning-orientated chronotype, which has been discussed in detail in **section 1.4**.

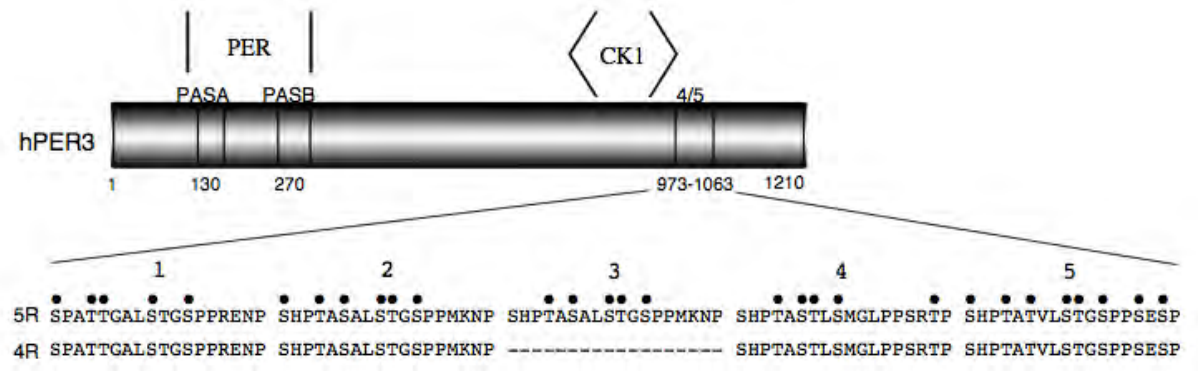


**Figure 1.6** – ***The Human PERIOD3 protein*** *(135). The amino acid sequence details are shown for the 4/5 variable number tandem repeat (VNTR) region. Each filled black circle represents a predicted CK1ε and CK1δ phosphorylation site, which increases PER3 degradation and nuclear translocation. It is hypothesized that the fewer CK1ε and CK1δ phosphorylation sites in the 4-repeat allele may result in a lower turnover rate of PER3 and thus a longer circadian period. Figure reproduced, with permission, from reference (135).*

Besides chronotype, other phenotypes result from this *PER3* VNTR polymorphism. For example, the *PER3$^{5}$* allele is associated with an earlier age for the onset of bipolar disorder type I and the *PER3$^{4}$* allele with a later age (162). Individuals homozygous for the *PER3$^{5}$* allele showed more slow-wave sleep, have elevated sympathetic predominance and have a reduction of parasympathetic activity compared to individuals that are homozygous for the *PER3$^{4}$* allele. The effect of the *PER3* VNTR polymorphism on the sympathovagal balance in cardiac control in non-rapid eye movement is similar to the effect of sleep deprivation (163). *PER3$^{5}$* homozygotes perform worse on executive function tests between 06:00 and 08:00 during a ~40 hour constant routine than *PER3$^{4}$* homozygotes. This suggests that the *PER3* VNTR polymorphism mediates effects on sleep deprivation and thus homeostatic sleep pressure (164). Furthermore, structural polymorphisms within the *PER3* gene are associated with DSPS (160). The links between these phenotypes and the *PER3* VNTR polymorphisms may be caused by other *PER3* VNTR-associated phenotypes such as chronotype or additionally these phenotypes may affect a person's chronotype.

### 1.5.2.1 The *PERIOD3* VNTR genotype distribution in worldwide ethnic populations

Although polymorphic distribution differences in one of these "chronotype-affecting clock-genes", namely the *PER3* gene, do exist between ethnically classified groups, no relation between the *PER3*$^{5}$ allele and geographical location (latitude and longitude) has been found (137). Alternative explanations of why genetic grouping in the *PER3*$^{5}$ allele may exist include genetic drift and sexual selection (165) or modern migration. **Figure 1.7** shows the *PER3* VNTR genotype distributions from various worldwide ethnic populations. The graph clearly shows that the *PER3* VNTR allele frequencies varies among different ethnic populations; and that the allele ratio is typically between 30 – 40% for the *PER3*$^{5}$ allele and 60 – 70% for the *PER3*$^{4}$ allele, with the exceptions being Yemenites, Ethiopians and Papua New Guineans.

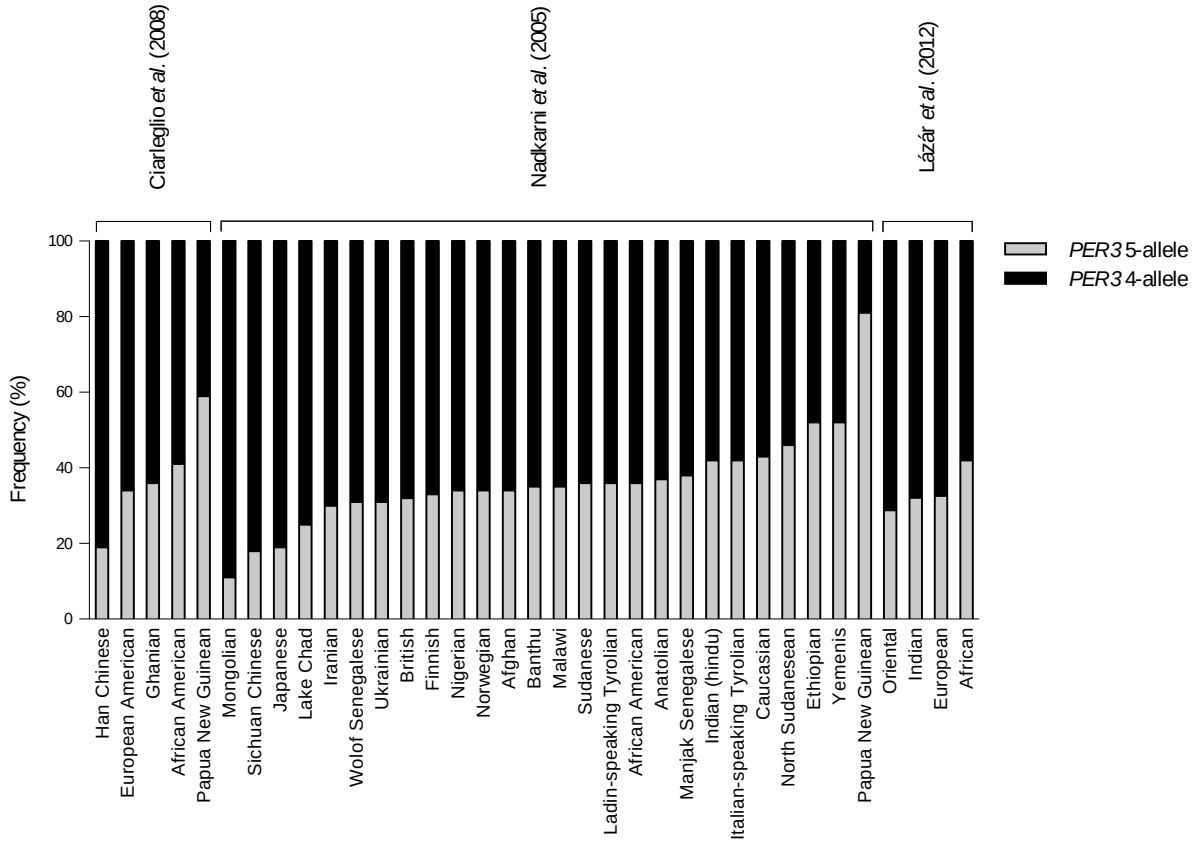


**Figure 1.7** – ***The PER3 VNTR polymorphism allele frequency distributions in various worldwide ethnic populations*** *(90,137,166). PER3 VNTR allele frequency is not related to geographical location or any other pattern that would indicate a selective pressure for the PER3 gene (137).*

## 1.6 Circadian variation in sport performance

The effect of circadian variation in exercise performance, in other words "Do athletes perform better in sport and exercise at a specific time of day?" is an area of chronobiology that has been well researched since the late 1960s. A number of physiological components that may influence exercise

performance exhibit a rhythmic variation throughout the day, with peaks being specific to the component and type of performance being measured.

Basic physiological components have been shown to vary with rhythmicity throughout the day. For example, heart rate has been shown to peak around 15:00 (167) and core temperature peaks at about 17:00 (168). Spengler *et al.* (2000) used the phase of the circadian body temperature (from 0 [nadir] to 360 degrees, 0° ≈ relative clock hour 6) to investigate whether metabolic parameters, which might also affect sport performance in terms of energy consumption, exhibit circadian variation (169). It was concluded that end tidal carbon dioxide ($P_{ET.CO2}$) and minute ventilation ($V_E$) both peak around relative clock hour 10 (60°) and that carbon dioxide production ($VCO_2$) peaks around relative clock hour 8 (30°) (169). Other studies that focus on components that may influence athletic performance suggest that reaction time peaks at 15:00 (170) as well as coordination (171). Although these basic physiological components are no direct measurements of exercise performance, they may be decisive in the outcome of certain sports, and therefore the performance of sports that rely on these components may vary diurnally.

All forms of physical activity are dependent on skeletal muscle, and are thus an important factor in exercise performance. Various studies have investigated the time-of-day effect on maximal muscle strength and peak torque. The maximal voluntary contraction of the knee extensors was found to be highest at 18:00, compared to 06:00, 10:00, 14:00 and 22:00 (172). Likewise, maximal torque of the knee extensor was found to be higher in the evening compared to the morning (173,174). Leg extension peak torque, leg flexion peak torque and peak torque ratio were also higher between 18:00 and 19:30 than they were between 08:00 and 09:00 and between 13:00 and 14:00 (175), which is in line with the results from the above mentioned studies. More evidence that muscle strength is at its highest in the late afternoon and early evening was provided by Deschenes *et al.* (1998), who found that peak torque, average power, maximal work in a single repetition and total work per set on an isometric dynamometer were higher when measured at 20:00 compared to 08:00, 12:00 or 16:00 (176). Even when taking muscle fatigue into account, peak torque seem to be higher in the early evening, as demonstrated in a study of sixteen males who were challenged to sustain an elbow flexor contraction for as long as they could with 40% of their maximal strength. Before, immediately after and ten minutes after the challenge, maximal voluntary contractions were performed (177). It was concluded that peak torque of the elbow flexors measured at 18:00 was higher than when measured at 06:00 (177).

Although evidence that maximum muscle strength and peak torque peak at a certain time-of-day is abundant, these studies do not show which component of voluntary muscle contraction, neural or muscular, is the origin of this diurnal variation. However, Guette *et al.* (2005) externally stimulated peripheral mechanisms in the leg, which showed variation during the day, while the central activation of the quadriceps remained unchanged during the day, suggesting that the diurnal variation is linked with diurnal modifications on a muscular level, rather than on neural level (172). Another study found that electromyography (EMG) parameters during a knee extension did not vary during the day, while maximum torque values were significantly higher in the evening, again suggesting that diurnal variation occurs on a muscular level (173). Besides power and torque, other neuromuscular properties seem to be affected by time-of-day too. The neuromuscular efficiency of the knee extensors after a maximal aerobic cycling until exhaustion was higher in the evening than in the morning, possibly due to a time-of-day effect on the muscle involvement in the pedalling processes (174).

As muscle strength and peak torque are only pieces in a jigsaw of how a person performs in a sport situation, tests that measure more complete aspects of physical performance may be a better measure of absolute sport performance. For example, a Wingate test on eleven male students revealed that peak power, mean power, total work done and oxygen uptake were higher at 18:00 compared to 06:00 in a group of eleven male students (178). These findings do not agree with Kin-Isler *et al.* (2006), who found that peak and mean power in 14 male students during a Wingate test were higher at 13:00 than at 09:00 and 18:00. However, the authors stated that a vigorous warm-up or lack of motivation and psychological drive may be the causal effects (179). The rating of perceived exertion (RPE) in nine male runners during a 30-minute run at lactate threshold speed, was significantly elevated in the morning (07:00 – 09:00) compared to the evening (18:00 – 21:00) (180), which may result in a better running performance in the evening, as a result from be able to run at a higher lactate threshold for a given RPE.

The maximal force and muscular power during three 5-second accelerations on a bicycle, following a 12-minute cycle at 50% of $VO_2$max, were higher in the afternoon (17:00 – 19:00) than in the morning (07:00 – 09:00) (181), showing that maximal force and maximum muscular power is higher in the afternoon, even when fatigue is considered. However, these findings were contradictory to a study which showed that peak power during five 6-second maximal cycle sprints was better in the morning than in the evening, while this difference faded away after the second to the fifth repetition, suggesting that the beneficial effect on exercise in the evening may fail to advantage performance during repeated sprints (182). This may suggest that time-of-day effects on

maximal cycling sprints do fade away when fatigue is considered. Another study that focused on cyclists and fatigue was that of Bessot *et al.* (2006), in which the mean time to exhaustion recorded at 18:00 was greater than at 06:00, which was paired with a higher blood lactate concentration (183). This suggests that cyclists might perform better in the evening, since it would take longer for the cyclists to become fatigued. Another study that focused on recovery found that the decrease in peak torque after 6-second maximal sprints during an evening session (17:00–19:00) was larger than the decrease in a morning session (08:00–10:00), suggesting that recovery patterns of neuromuscular function are slower in the evening compared to the morning (184). Thus, when investigating actual exercise performance, rather than the performance of single exercise components, the diurnal variation remains.

Interestingly, there is now some evidence that the diurnal variations of maximum muscle strength and peak torque are dynamic, rather than static, meaning that they can change and are not set on a certain time-of-day. Evidence for this theory was provided by Sedliak *et al.* (2007), who found that the maximal knee extensor torque in healthy untrained men was higher in the afternoon in a group of men who trained in the afternoons compared to a group that trained in the mornings (185). This study suggests that training time-of-day affects the diurnal variation of knee extensor maximal torque, and may thus also affect the diurnal variation in the maximum muscle strength and peak torque of other muscles. Muscular anaerobic performance as determined by the squat jump, countermovement jump, Wingate and 1-repetition maximum during leg extension, leg curl and squat tests were 3 - 18% higher between 17:00 and 18:00 compared to 07:00 – 08:00 (186). While the difference persisted when participants specifically trained for these tests in the evenings or did not train at all, the difference disappeared when the participants trained in the morning (186). This shows that the adaptation to strength training is greater when performed at the time-of-day at which training was scheduled than at other times. Based on the findings of these studies, training time-of-day influences the diurnal variation of muscle strength and peak torque, and possibly other physiological components in exercise performance.

Ultimately, these time-of-day dependent peaks in performance of exercise components may translate themselves into a better overall sport performance. For example, Baxter and Reilly (1983) found evidence that swimming performance in a 100 m and 400 m sprint peaked in the evening [between 17:00 and 22:00 (187)], which is in line with the diurnal variation of the peaks in basic physiological components, muscle strength and torque (167,168,172,173). During 16.1 km cycling time trials at 07:30 and 17:30, the mean cycling time was found to be significantly slower in the

morning, even when the athletes were MTs (188). Again this shows performance may be better in the late afternoon or early evening, parallel with the peaks in physical exercise components.

Also more sophisticated tasks including those related to soccer, tennis and badminton seem to be influenced by time-of-day. For example, soccer juggling performance and chip test performance were both subject to time-of-day effects and were found to peak at 16:00 (171). The wall-volley performance and dribbling time were highest at 20:00, suggesting that soccer players may perform at their best between 16:00 and 20:00 (171). Likewise, serve speed, but not serve accuracy, in six competitive tennis players was found to be higher at 18:00 compared to 09:00 and 14:00 (189). Furthermore, the accuracy of badminton serves was higher at 14:00 than at 08:00 or 20:00, while the consistency of the badminton serve was not affected by time-of-day (190). To conclude, the time-of-day at which these peaks in performance of sophisticated tasks occur are thus in line with the diurnal variation of performance-peaks of basic physiological or exercise components.

Since the diurnal variations in the above-mentioned biological factors are likely to be driven largely by one's innate circadian rhythms, and since chronotype reflects individual differences between innate circadian rhythms, it is reasonable to believe that diurnal variation in exercise performance may be at least in part chronotype-dependent. Only a few studies have investigated the effect of chronotype on the diurnal variation in sport performance components. For example, a study of Burgoon *et al.* (1992) on twenty-six males that were MTs, NTs and ETs, who performed a maximal treadmill test (Bruce protocol) in the morning (07:30 – 08:30) and in the evening (19:30 – 20:30), concluded that the maximum exercise performance was better in the afternoon, but did not differ between the chronotypes (191). However, the heart rate recovery (beat-by-beat heart rate decrease) for the first 30 s after a 3-min cycle ergometer exercise test at 80% of ventilatory threshold did appear to be different among chronotypes. Namely, heart rate recovery was worse in the morning for ETs and also worse than those values of MTs (192), suggesting that physiology may be different between chronotypes when tested at specific times-of-day. Additionally, Brown *et al.* (2008) found that MTs performed better in a 2000 m rowing time-trial in the morning than in the evening (193). This suggests an advantage for MTs that have a game or race scheduled in the morning.

To conclude, various physical performance components that are affected by time-of-day, include muscle strength (172-175), rating of perceived exertion (180), time-trial performance (187,188), time to exhaustion (183) and recovery patterns of neuromuscular function (184), and may be important components in marathon performance. Marathon running performance is also related to the velocity at which blood lactate accumulates to a concentration of 4 $mmol \cdot l^{-1}$ and the ability to

run at a pace close to that velocity during the marathon (194). Since the concentration of blood lactate at the point of exhaustion is higher in the evening (18:00) than in the morning (06:00) (183), suggesting that the ability to tolerate a higher blood lactate concentration may be better in the evening. Thus based on this information, marathon running performance may be better in the evening than in the early morning. Since individual variation in these time-of-day effects exist, and because these time-of-day effects may be associated with a person's chronotype, marathon-running performance at a specific time-of-day may depend on a person's chronotype.

**1.7 Summary and conclusions**

Kunozorva *et al.* (2012) found that Caucasian males in South Africa who participate in individual endurance events such as running, cycling and triathlons are more likely to be morning-types (MTs) and carry the *PER3*$^{5}$ VNTR allele than active but non-competitive gym goers (104). The authors proposed that this finding could be explained by the athlete's ability to cope with early morning events. That is, MTs and people with clock genes that are associated with preferences for mornings, such as the *PER3*$^{5}$ VNTR allele, may find it easier to cope with the early start of South African endurance events (generally between 05:00 and 08:00), and thus may be more likely to choose to participate in such events. These athletes may also choose to schedule their training at the same time-of-day at which the physical task (e.g. a marathon) takes place, which has proven to be beneficial for cycling performance (195). On the other hand, evening-types (ETs) or persons with genotypes associated with evening behaviour may chose sports that do not require getting up at a certain time they do not feel comfortable with. Therefore this situation could create a selection and leave a group containing more MTs and NTs and fewer ETs in the individual endurance athlete population.

However, Kunorozva *et al.* (2012) found that active but non-competitive South African controls were also more morning-orientated than what has previously been described in other populations around the world (104). Since these controls do not participate in endurance events and are thus not exposed to early morning start times, Kunorozva *et al.* (2012) could only speculate about whether this observation could be explained by (i) the physically active nature of the participants or (ii) the geographical location of South Africa. Physical activity has been associated with a preference for mornings in studies on pupils and adolescents (122-124) and it has been shown that physical activity can phase advance body temperature rhythm and melatonin rhythm (48,55), which may in turn affect chronotype. Since sunlight is thought to be the most important zeitgeber for circadian

rhythm entrainment, chronotype may also be affected by light intensity and photoperiod (132), both known to vary by geographical location. Thus making the level of physical activity of the participants and the geographical location of South Africa plausible explanations for the higher prevalence of MTs in the study of Kunorozva *et al.* (2012). This present study is designed to continue where Kunorozva *et al.* (2012) left off, and to answer the question whether physical activity or geographical location may explain the higher prevalence of morning-orientated people in South African individual-sport endurance athletes and active controls.

**1.8 Aims and hypotheses**

In this study, we seek to understand whether the bias towards morningness observed in the South African runners in the study of Kunorozva *et al.* (2012) was specific to South African runners, for who the marathon start time is early in the morning. We therefore have compared South African marathon runners with Dutch marathon runners, who have marathon start times later in the day. We also seek to understand whether the high proportion of morning-types in both the runners and active control groups described by Kunorozva *et al.* (2012) can be explained by the geographical location of South African. To address this we compared South African runners and controls to Dutch runners and controls.

The main goal of this study is to answer the question: ***"*Can the observation that South African individual-sport endurance athletes were primarily morning-orientated be explained by geographical location, the *PER3* VNTR genotype, or the timing of the competitive sporting events in South Africa**?"

The aims of this study are:

1. To determine and compare the chronotype and *PER3* VNTR genotype distributions of South African Caucasian male marathon runners to a control population of active but non-competitive South African Caucasian males.
2. To determine and compare the chronotype and *PER3* VNTR genotype distributions of Dutch Caucasian male marathon runners to a control population of active but non-competitive Dutch Caucasian males.
3. To compare the chronotype and *PER3* VNTR genotype data of the South African and Dutch runner groups.

4. To compare the chronotype and *PER3* VNTR genotype data of the South African and Dutch control groups
5. To investigate the relationship between the *PER3* VNTR genotype and chronotype in both the Dutch and South African samples.
6. To determine whether marathon race time is correlated to chronotype or *PER3* VNTR genotype in Dutch and South African marathon runners.

We hypothesize that the diurnal preference of runners and controls from South Africa may be more skewed towards morningness compared to runners and controls from the Netherlands. This may, at least in part, be due to the difference in climate, photoperiod and light intensity, which changes as a function of latitude. Furthermore, we hypothesize that the Dutch marathon runners may not display the same bias towards morningness observed in their South African counterparts, because endurance races in South Africa tend to start before 07:00, which may select people that can cope with early mornings, while those in the Netherlands have start times ranging from 09:00 to 16:00, which is favourable for all chronotypes.

We have chosen to study males of European descent to minimise differences in genetics due to ancestry. We thus hypothesize that if South African runners are more skewed to morningness than the Dutch runners, and that the frequency of the $PER3^{5}$ allele (associated with morningness) will be higher in the South African running sample compared to the Dutch runners as a result of *PER3* VNTR genotype based selection.

Finally, we hypothesise that there may be a correlation between chronotype score and marathon race time, since morning-types may better cope with early morning marathon events, especially in the South African running group.

# Chapter 2: Materials and Methods

**2.1 Participants**

Ninety-five South African Caucasian male marathon runners who had trained for marathons and had participated in at least one marathon event in the past 12 months were recruited for the South African runners group (RUNSA). The participants were recruited via running clubs, newsletters, and the Langebaan country estate Weskus marathon expo. Fifty-three p*er cent* of the participants finished the Langebaan Country Estate Weskus Marathon, which took place on the 20th of April 2013 in Langebaan, South Africa.

Ninety-seven South African Caucasian males were recruited as controls (CONSA group). Eligible volunteers for this group exercised for at least one hour, twice per week. The exercise could not be running and the participants for this group could not have participated in any competitive event for the past three years. The South African control sample was recruited at the Sport Science Institute of South Africa (SSISA) gym in Newlands, Cape Town and at the Old Mutual Gym in Pinelands, Cape Town in April and May 2013.

Similarly, 90 Dutch Caucasian male marathon runners who had trained for marathons and had participated in at least one marathon event in the past 12 months were recruited for the Netherlands runners group (RUNNL). Recruitment took place at the Lage Landen marathon Eindhoven expo 2013 and the TATA Consultancy Service Marathon expo in Amsterdam 2013. Sixty-four *per cent* of the RUNNL group finished the Lage Landen marathon of Eindhoven (the Netherlands) on the 13th of October 2013.

The Dutch control sample (CONNL) comprised 98 participants with the same inclusion and exclusion criteria as for the CONSA group. They were actively recruited at the Achmea Health Center in Oss, Achmea Health Center in Alkmaar, VU sports centre gym, Basic Fit and Puur Fitness in Oss, Squash & Fit Centre in Schaijk and Basic Fit in Uden, in the Netherlands.

All participants were apparently healthy and between the ages of 25 and 50 years, since chronotype is related to age (142). Caucasians have specifically been chosen for this study since the frequency distribution of the VNTR polymorphism within *PER3* has been shown to be ethnic-sensitive (137). Similarly, only males were included in this study since chronotype is gender-specific (92,144,196). The participants were categorized as being South African or Dutch if they had lived in their respective countries for at least 12 months. We expect that a 12-month period is sufficient for the circadian rhythm to adapt to the environmental factors. The South African participants were recruited in April and May 2013 (i.e. autumn in South Africa) since chronotype scores may be

affected by season (103). Similarly, the Dutch participants were recruited in October and November 2013 (i.e. autumn in the Netherlands).

The participants did not receive any remuneration and there was no direct benefit to the volunteers for participating in this study. On completion of the study participants received feedback relating to their personal preferenece for either mornings or evenings as established by the HÖ-MEQ as well as the general results of the study. Only the Dutch participants received personal feedback regarding their *PER3* VNTR genotype as permitted by the Ethische Comissie Bewegingswetenschappen (ECB ref: 2013-40)

### 2.2 General study design

The investigator (same person for both the South African and Dutch components of this study) explained the aim of the study, all procedures as well as the risks and benefits of participation in detail to the volunteers. This was done in English for the South African participants and in Dutch for the volunteers from the Netherlands. All the questions that the participants had were answered during this occasion. Alternatively, the participants were allowed to ask any questions over email or telephone. When the volunteers agreed to participate, they signed an informed consent document (**Appendix 1**) in a language appropriate to their country of residence (i.e. English for South Africa and Dutch for the Netherlands). Secondly, the participants were asked to complete a questionnaire with four sections, namely: A) personal information, B) medical history and medication and/or supplement use, C) running training and race history (RUN groups only), D) exercise history (CON groups only), and E) the HÖ-MEQ (**Appendix 2**). Participants donated a buccal cell sample for subsequent genetic analysis. Genetic analysis for the South African participants took place at the UCT/MRC Research Unit for Exercise Science and Sports Medicine at the Sport Science Institute of South Africa in Newlands and the UCT department of Molecular and Cell Biology, Rondebosch. Genetic material collected from the Dutch participants was analysed at the Faculty of Exercise Science at the VU University of Amsterdam, the Netherlands.

### 2.3 Detailed testing procedures

#### 2.3.1 Anthropometry

The self reported body mass in kilograms (kg) and height in centimetres (cm) was acquired from the participants. Body mass index (BMI) was calculated by dividing the body mass (kg) by the square of the height ($m^2$) (197). Outcome variables were mass, height and BMI ($kg \cdot m^{-2}$).

### 2.3.2 Chronotype

The HÖ-MEQ (67) was used to determine the chronotype of all participants (**Appendix 2**). This questionnaire is the most widely used self-report measure of diurnal preference or chronotype (198). It consists of 19 questions which address each participant's preferred rising and bed times and preferred times of physical and mental activity. Low scores (16-41) indicate a preference for evenings and high scores (59-86) indicate a preference for mornings. Based on the scores obtained, individuals were placed into one of five chronotype categories: definite evening-type (DET) (16-30), moderate evening-type (MET) (31-41), neither-type (NT) (42-58), moderate morning-type (MMT) (59-69), and definite morning-type (DMT) (70-86) (67). Outcome variables were HÖ-MEQ score and chronotype category.

### 2.3.3 Genomic DNA extraction

The participants donated a buccal cell sample using a buccal swab (SK-2S, Isohelix, Cell Projects, Kent, UK). Total genomic DNA was extracted using a kit that was specially designed for the SK-2S buccal swabs (DDK, Isohelix, Cell Projects, Kent, UK). The DNA pellet was dissolved in 60µl TE buffer (1mM Tris, 1mM EDTA, pH 8.0).

### 2.3.4 *PERIOD3* genotyping

The VNTR polymorphism in exon 18 of the *PER3* gene was amplified by polymerase chain reaction (PCR) using MyTaq™ DNA Polymerase, MyTaq™ reaction buffer and the following primers:
Forward: (*PER3*F) 5'-CAAAATTTTATGACACTACCAGAATGGCTCAC-3',
Reverse: (*PER3*R) 5'-AACCTTGTACTTCCACATCAGTGCCTGG-3' (135,160).
Each 25µl PCR reaction mix contained ~100ng genomic DNA, 1U (0,2µl) DNA Polymerase, 1mM deoxynucleotide triphosphates (dNTPs), 3mM $MgCl_2$, stabilizers and enhancers, 10pM *PER3*F and 10pM *PER3*R. The following PCR cycling conditions were used: initial denaturation at 94°C for 5min, 35 cycles of denaturation at 94°C for 45s, annealing at 59°C (as determined by primer design) for 45s and extension at 72°C for 1min followed with a final extension cycle at 72°C for 5min.

Fifteen microliters of the PCR product was then restricted with 3U (0,3µl) *Nco*I (R0193S, New England BioLabs inc., UK) in 2,5µl 10X NEBuffer 3.1 (100mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$, 100µg·$ml^{-2}$ BSA, pH 7.9) and 7,2µl sterile demineralised water for at least 2h at 37°C. The resulting fragments, 600, 420, 380 and 200 base pairs in size, were resolved on a 2% (w/v) agarose gel in 1X Tris Acetate EDTA (TAE) buffer (40mM Tris, 0,11% (v/v) glacial acetic acid and 1mM EDTA) and visualized under ultra-violet (UV) light after gel staining (SYBR® Safe DNA Gel Stain, Invitrogen™).

Based on the bands present, individuals were assigned one of three genotypes: *PER3*$^{4/4}$, *PER3*$^{4/5}$ *or* *PER3*$^{5/5}$, where the numbers 4 and 5 represent the *PER3*$^{4}$ and *PER3*$^{5}$ repeats respectively of the VNTR polymorphism. A M1037T polymorphism for the *PER3*$^{4}$ allele exists and is not restricted by NcoI, thus resulting in an undigested 600 bp band. NcoI restriction was solely performed to accurately distinguish between the three *PER3* VNTR polymorphism genotypes and the M1037T polymorphism was not of interest to this study. All samples were analysed and read by two independent investigators to ensure no reading errors were made. Outcome variables were the *PER3* VNTR polymorphism genotypes as shown in **Table 2.1**. The *PER3* VNTR allele distribution was calculated by: *%allele*$^{a}$*= %homozygous*$^{aa}$ *genotype + 0.5(%heterozygous*$^{aA}$ *genotype).*

**Table 2.1 – *The possible outcome variables for the PER3 VNTR and M1037T polymorphism genotypes****.*** *The first column shows the three PER3 genotypes 55, 45 and 44. The second column represents the M1037T polymorphism and its combination with the PER3 VNTR genotype. The third to sixth columns represent the fragment size of each possible outcome variable. Bp: base pairs.*

| *PER3* VNTR Genotype | M1037T Genotype | Fragment size (bp) | | | |
|---|---|---|---|---|---|
| | | 600 | 420 | 380 | 200 |
| *55* | 5M 5M | | • | | • |
| *45* | 4M 5M | | • | • | • |
| *45* | 4T 5M | • | • | | • |
| *44* | 4T 4M | • | | • | • |
| *44* | 4M 4M | | | • | • |
| *44* | 4T 4T | • | | | |

### 2.3.5 Marathon race time and performance

The official race time from each runner who completed the designated marathons in South Africa or the Netherlands was collected from the event websites: www.strandlopers.co.za for the Langebaan Country Estate Weskus marathon (South Africa) and www.marathoneindhoven.nl for the Lage Landen marathon (the Netherlands). The corrected race time (i.e. time from crossing the start line to crossing the finish line) was used if available. The outcome variable is the 42.195m race time (in minutes and seconds, min:s).

## 2.4 Statistical analysis

Sample size was determined using the nomogram method described by Altman *et al.* (199). The standardized mean difference (SMD) was calculated from the values of the Kunorozva *et al.* (2012) study (104). A statistical significance was found with a proportional difference for *PER3*$^{5}$ VNTR of 0.58

for runners (p1) and 0.38 for the active controls (p2). The SMD was calculated to be 0.40. The α level was set to 0.05 and the power to 0.80. Using Altman's nomogram, the total sample size was proposed to be 180, or 90 for each group. The drop out ratio from unusable buccal cell swabs and uncompleted questionnaires was estimated to 10%, which sets the sample size to ~100 for each group.

All data collected is reported as the mean ± standard deviation if the data were normally distributed, or median and 25$^{th}$ and 75$^{th}$ percentile if the data were not normally distributed. Normal distribution was measured with the Shapiro-Wilk normality test. A one-way analysis of variance (ANOVA) was used to compare the descriptive characteristics and chronotype scores of the groups for normally distributed data. A Bonferroni-adjusted α-level was used to interpreted post-hoc tests. Non-parametric data and data with unequal sample sizes between four groups were analysed with the Kruskal-Wallis one-way ANOVA and a post-hoc Kruskal-Wallis ANOVA with an adjusted α-level was used to determine which two groups differed from each other (200). A Mann-Whitney U test was used to determine the probability of significance between two independent groups. The chronotype, genotype and allele distributions of the groups were analysed using Fisher's exact test and the genotype data were checked for the Hardy-Weinberg equilibrium (201). Cramer's V were calculated as effect sizes for Fisher's exact tests. The difference in training time-of-day between midweeks and weekends for each of the four groups was calculated with the Wilcoxon signed-rank test for non-parametric data. Correlations were performed using either Pearson's product-moment correlation (normally distributed data) or a Spearman's rank correlation (non-normally distributed data). Statistical significance was accepted when $p<0.05$. Data were analysed using Stata v.12 (StataCorp, Texas, USA).

**2.5 Ethical considerations**

This study was performed in accordance with the principles of the Declaration of Helsinki (October 2013, Fortaleza, Brazil), the International Conference on Harmonization, the European Good Clinical Practice (GCP) guidelines, the South African GCP guidelines, the laws of South Africa and the laws of the Netherlands. The study was covered by the University of Cape Town's no-fault insurance policy. Participants were not included unless they had signed an informed consent form (**Appendix 1**), after the investigator had provided substantial verbal and written explanation of the study, including risk factors. Participants were informed that their participation in the study was entirely voluntary and that they had the right to withdraw from the study and have their DNA sample destroyed at any time

without stating a reason. All the information collected during the trial was stored in a computer database in a secure facility, was kept confidential and will only be used for scientific purposes. The participants' anonymity was ensured at all times. The DNA collected for this study was only used for the purpose described in **section 2.3.3** and **section 2.3.4**. Approval from the Human Research Ethics Committee of the Faculty of Health Sciences at the University of Cape Town (HREC ref: 170/2013), Ethische Comissie Bewegingswetenschappen VU University (ECB ref: 2013-40) and the Medisch Ethische Toetsingscommissie VU medisch centrum (METc ref: 2013/217) was obtained.

# Chapter 3: Results

### 3.1 Participant characteristics

#### 3.1.1 General

The general characteristics of the RUNSA, CONSA, RUNNL and CONNL groups are shown in **Table 3.1**. All four groups were similar in age ($p$=0.243). The RUNNL and CONNL groups were taller than the RUNSA group ($p$=0.003). The CONSA and CONNL groups weighed more than the RUNSA and RUNNL groups ($p$<0.001). Finally, the RUNNL group had a lower BMI than the RUNSA, CONSA and CONNL groups ($p$<0.001).

**Table 3.1** – **General characteristics of the RUNSA, CONSA, RUNNL and CONNL groups**.

| | RUNSA | CONSA | RUNNL | CONNL | *p*-value |
|---|---|---|---|---|---|
| Age (y) | **38** (12) *95* | **36** (15) *97* | **40** (12) *90* | **36** (15) *98* | *0.243* |
| Height (cm) | **180.0** ±7.1 *95* | **182.5** ±7.6 *96* | **183.1** ±7.1 *89*[a] | **183.7** ±7.6 *98*[a] | *0.003* |
| Weight (kg) | **80** (14) *95* | **86** (15) *97*[a] | **76** (9) *90*[b] | **84** (16) *98*[ac] | *<0.001* |
| BMI (kg·m$^{-2}$) | **24.3** (3.3) *95* | **25.3** (4.5) *96* | **22.5** (2.2) *89*[ab] | **25.1** (3.8) *98*[c] | *<0.001* |

*RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands, CONNL: controls the Netherlands, BMI: body mass index. Data are presented as **median** (interquartile range) sample size or **mean** ±standard deviation sample size. The probability of significance was calculated with the Kruskal-Wallis one-way ANOVA or the one-way ANOVA. Specific differences between groups, determined using post-hoc analyses, are indicated with superscript letters.*

[a] *Significantly different to RUNSA (p<0.05)*

[b] *Significantly different to CONSA (p<0.05)*

[c] *Significantly different to RUNNL (p<0.05)*

#### 3.1.2 Training habits

The training characteristics of all participants during the past three months are presented in **Table 3.2**. The RUNSA group had trained for fewer consecutive years than the CONSA and CONNL groups ($p$<0.001). Likewise, the RUNNL group had trained for fewer consecutive years than both control groups ($p$<0.001). Both RUN groups trained for more days per week than their respective control groups ($p$<0.001). The RUNSA group trained for more hours per week than the CONSA, RUNNL and CONNL groups did ($p$<0.001). Also, the RUNNL group had trained for more hours per week than both the CONSA and CONNL groups ($p$<0.001). Finally, the RUNSA group had more training kilometres per week compared to the RUNNL group ($p$<0.001).

**Table 3.2** – **Training characteristics for each of the four groups**.

| | RUNSA | CONSA | RUNNL | CONNL | *p*-value |
|---|---|---|---|---|---|
| Training (y) | **6.0** (12.0) *95* | **12.0**(20.5) *96*[a] | **7.0** (10.0) *90*[b] | **15.0** (21.0) *98*[ac] | *<0.001* |
| Training (d·wk$^{-1}$) | **5.0** (1.5) *95* | **3.0** (1.0) *96*[a] | **3.5** (1.0) *90*[a] | **3.0** (2.0) *98*[ac] | *<0.001* |
| Training (h·wk$^{-1}$) | **7.0** (3.0) *93* | **4.0** (3.0) *96*[a] | **6.0** (2.5) *89*[ab] | **4.5** (3.0) *98*[ac] | *<0.001* |
| Training (km·wk$^{-1}$) | **65** (30) *95* | - - - | **50** (30) *89* | - - - | *<0.001* |

*RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands, CONNL: controls the Netherlands. Data are presented as* ***median*** *(interquartile range) sample size. The Kruskal-Wallis one-way ANOVA was used to determine the level of significance, except for training (km·wk$^{-1}$), for which the Mann-Whitney U test was used. Specific differences between groups, determined using post-hoc analyses, are indicated with superscript letters.*

[a] *Significantly different to RUNSA (p<0.05)*

[b] *Significantly different to CONSA (p<0.05)*

[c] *Significantly different to RUNNL (p<0.05)*

### 3.1.3 Training time-of-day

The time-of-day at which each group actually trains and prefers to train are shown in **Table 3.3**. In the midweek (i.e. Monday – Friday), the actual training times of both RUNNL and CONNL groups were later than those for the RUNSA and CONSA groups ($p$<0.001). The weekend actual training time-of-day for the RUNSA group was earlier than that of the CONSA, RUNNL and CONNL groups ($p$<0.001). A similar finding was observed for the preferred training time-of-day, in which the RUNSA group would prefer to train earlier than the CONSA, RUNNL and CONNL groups ($p$<0.001).

Each group's actual midweek and weekend training times-of-day are different to the preferred training times-of-day. In all four groups, the actual training time-of-day during the midweek is later than it is on the weekends ($p$<0.001). In RUNSA, RUNNL and CONNL, the actual midweek training time is later than the preferred training time-of-day ($p$<0.001). Finally, the actual training time on the weekend is different from the preferred training time-of-day in CONNL ($p$<0.001), but not in the other groups.

**Table 3.3 – Actual training time of day (ToD) during the midweek and on the weekend, and the preferred training ToD of the four groups.**

| | RUNSA | CONSA | RUNNL | CONNL | *p*-value |
|---|---|---|---|---|---|
| Midweek (h:min) | **11:45** (9:50) *81* | **14:24** (6:00) *93* | **18:28** (4:04) *86*[ab] | **18:57** (3:50) *95*[ab] | *<0.001* |
| Weekend (h:min) | **06:28** (1:26) *75* | **09:07** (4:04) *50*[a] | **10:04** (2:52) *83*[a] | **09:50** (1:55) *42*[a] | *<0.001* |
| Preferred (h:min) | **06:57** (4:33) *68* | **12:00** (7:40) *78*[a] | **10:04** (1:55) *69*[a] | **11:02** (8:53) *87*[a] | *<0.001* |
| *p*-value | *<0.001*[12] | *<0.001*[1] | *<0.001*[12] | *<0.001*[123] | |

*RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands, CONNL: controls the Netherlands. Data are presented as* ***median*** *time (interquartile range) sample size. The Kruskal-Wallis one-way ANOVA was used to determine the level of significance. The p-values between the groups are shown in the right hand column. The p-values within groups are shown in the bottom row. Specific differences between groups, determined by post-hoc analyses, are indicated by superscript letters or numbers.*

[a] *Significantly different to RUNSA (p<0.05)*

[b] *Significantly different to CONSA (p<0.05)*

[1] *Midweek training ToD is significantly different to weekend training ToD (p<0.05)*

[2] *Midweek training ToD is significantly different to preferred training ToD (p<0.05)*

[3] *Weekend training ToD is significantly different to preferred training ToD (p<0.05)*

The times at which each individual runner and active control actually exercises and the times at which they would prefer to exercise, are plotted for visual interpretation in **Figure 3.1**. In all three cases - the actual training time during the midweek (**Fig. 3.1.A**), the actual training time during the weekend (**Fig. 3.1.B**) and the preferred training time (**Fig. 3.1.C**) - the RUNSA group is the most morning-orientated regarding their training time-of-day. The CONSA group actually trains and prefers to train earlier than the Dutch groups – who have later actual and preferred training times. Finally, the least early training times are those of the Dutch groups, for who the actual training times are similar, while the preferred training times are earlier for the RUNNL group than for the CONNL group.

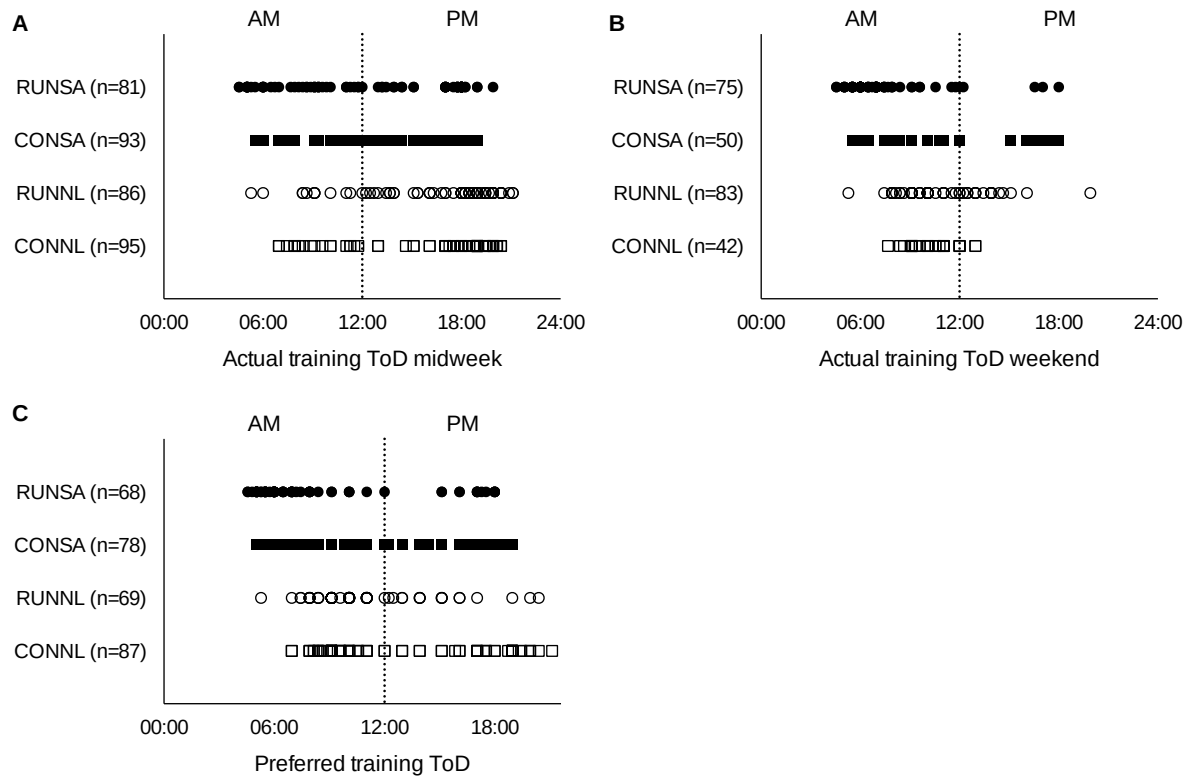


**Figure 3.1 – *The actual and preferred training time-of-day (ToD) plotted per individual.*** *A) Actual training ToD for midweek days. B) Actual training ToD for weekend days. C) Preferred training ToD. Each circle represents a running individual and each square represents an active control. The filled symbols represent South Africa, while clear symbols represent the Netherlands. RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands, CONNL: controls the Netherlands, AM: before midday, PM: after midday. Graph is presented for visual interpretation only.*

### 3.1.4 Marathon race time

The marathon details for the RUNSA and RUNNL groups are presented in **Table 3.4**. Running marathons (years) is the time in consecutive years for which the participants have been running marathons. The RUNSA group had been running for more consecutive years than the RUNNL group ($p$<0.001). Similarly, the RUNSA group had participated in more marathons in the past twelve months than the RUNNL group ($p$<0.001).

The personal best (PB) marathon race times and the PB half-marathon race times were similar in the two groups ($p$=0.201 and 0.159 respectively). However, the RUNNL group ran their recent marathon faster than the RUNSA group ($p$<0.001). Finally, the recent half-marathon race time of the RUNNL group was also faster than that of the RUNSA group ($p$=0.001). The selected marathon times (min) are shown in **table 3.4**, however, a comparison is not relevant for the purpose of this study, and thus no $p$-value is shown.

**Table 3.4 – Marathon racing details for the RUNSA and RUNNL groups.**

| | RUNSA | | | RUNNL | | | *p*-value |
|---|---|---|---|---|---|---|---|
| Marathon racing years | **4.0** | (6.0) | *95* | **2.0** | (4.0) | *90* | <0.001 |
| No. of marathons (past year) | **4.0** | (4.0) | *99* | **1.0** | (1.0) | *90* | <0.001 |
| Selected marathon time (min) | **238.7** | ±33.7 | *50* | **210.5** | ±30.2 | *58* | - |
| PB marathon time (min) | **220.6** | ±32.2 | *92* | **214.1** | ±31.3 | *69* | 0.201 |
| PB half-marathon time (min) | **96.7** | ±11.6 | *90* | **94.2** | ±11.3 | *83* | 0.159 |
| Recent marathon time (min) | **235.0** | ±33.6 | *89* | **216.8** | ±29.8 | *62* | <0.001 |
| Recent half-marathon time (min) | **103.9** | ±14.1 | *85* | **97.0** | ±11.9 | *71* | 0.001 |

*Selected marathon time (min) is the race time of the selected marathon when no pre-race reason for underperformance was given. RUNSA: runners South Africa, RUNNL: runners the Netherlands, PB: personal best. Running marathons (years) is the number of consecutive years the participants had been running marathons. Data are presented as **median** (interquartile range) sample size or **mean** ±standard deviation sample size. The Mann-Whitney U test or the Student's T-test was used to determine the level of significance.*

### 3.2 Chronotype and *PER3* VNTR genotype analyses

**Figure 3.2** shows the HÖ-MEQ scores and the chronotype category frequency distributions and **Figure 3.3** the *PER3* VNTR genotype and allele frequency distributions of all four groups. Comparisons were made between (i) the two South African groups, (ii) the two Dutch groups, (iii) the two groups of runners, and (iv) the two control groups. The results are presented in this order below.

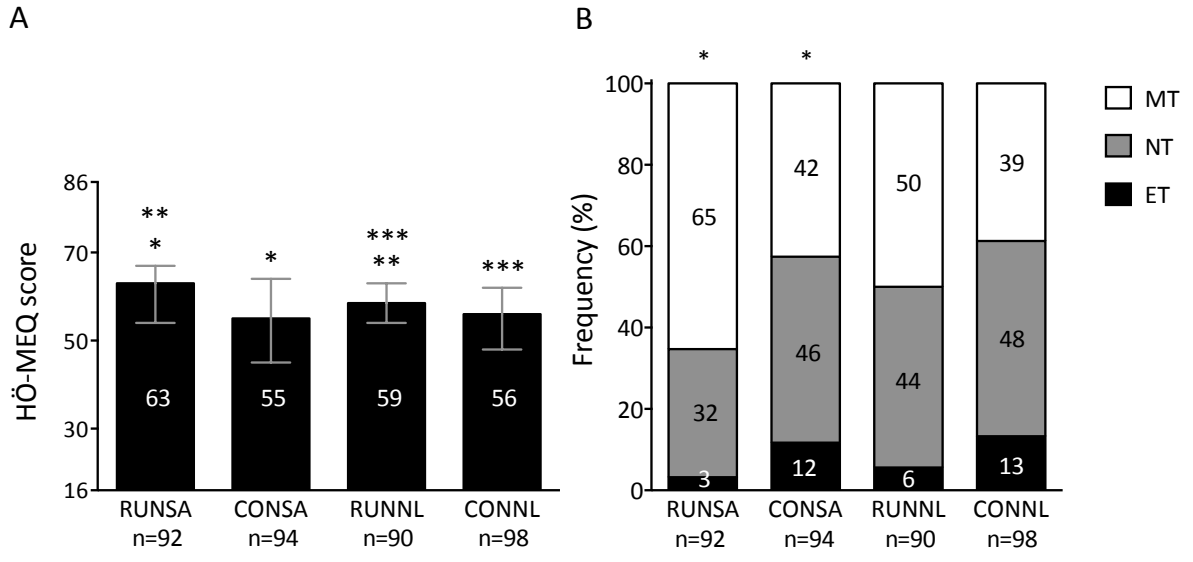


**Figure 3.2 –** ***The HÖ-MEQ scores (A) and chronotype category distributions (B) of the RUNSA, CONSA, RUNNL and CONNL groups.*** *MT: morning-type, NT: neither-type, ET: evening-type, ES: effect size, HÖ-MEQ: Horne-Östberg morningness-eveningness personality questionnaire, RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands and CONNL: controls the Netherlands. The HÖ-MEQ score data (A) are presented as medians with 25$^{th}$ and 75$^{th}$ percentiles and the chronotype distribution data (B) are presented as frequencies. The p-values were calculated using the Mann-Whitney U test (A) and the Fisher's*

*exact test (B). No p-values are shown for differences between groups with no scientific relevance. Asterisks denote statistical differences. Panel A*: RUNSA v CONSA: p<0.001, Effect-size (ES): 0.281 (moderate); A**: RUNSA v RUNNL: p=0.009, ES: 0.193 (small); A***: RUNNL v CONNL: p=0.041, ES: 0.149 (small); Panel B*: RUNSA c CONSA: p=0.004, ES: 0.160 (small).*

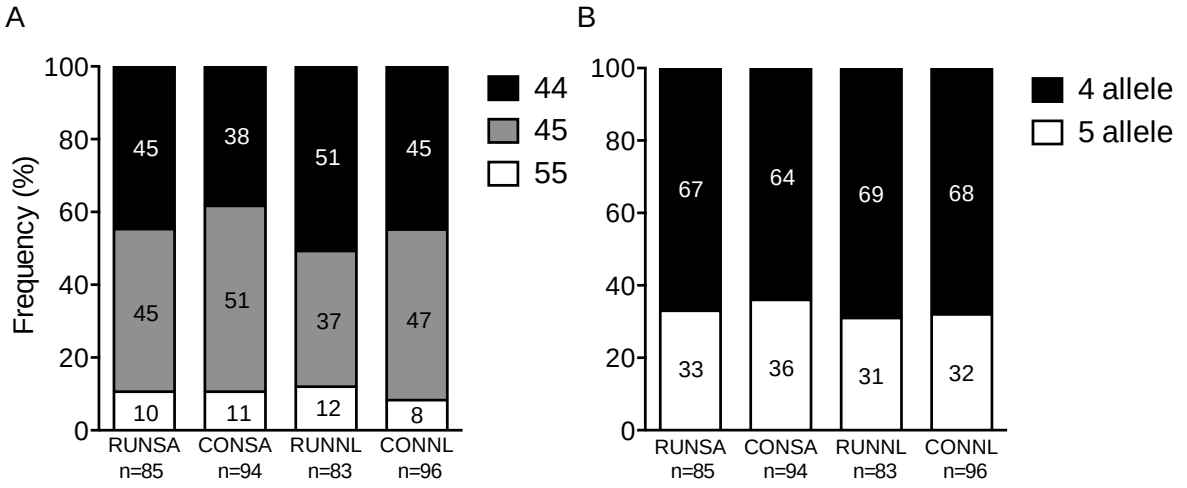


**Figure 3.3** – ***PERIOD3 VNTR polymorphism genotype (A) and allele (B) distributions for the RUNSA, CONSA, RUNNL and CONNL groups.*** *RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL, runners the Netherlands, CONSA, controls the Netherlands, 44: $PER3^{4/4}$ VNTR, 45: $PER3^{4/5}$ VNTR, 55: $PER3^{5/5}$ VNTR. Data are presented as frequencies. The p-values were calculated with the Fisher's exact test. The groups were not significantly different from each other.*

### 3.2.1 Aim 1: To compare chronotype and *PER3* VNTR genotype of the two South African groups

The HÖ-MEQ score was higher in the RUNSA group compared to the CONSA group ($p$<0.001, **Fig. 3.2A**). The effect size of this difference is moderate (ES=0.281). The two groups also showed different chronotype category distributions ($p$=0.004, **Figure 3.2B**). More MTs were observed in the RUNSA group compared to the CONSA group. Additionally, the RUNSA group had fewer NTs and ETs compared to the CONSA group. Age was associated with HÖ-MEQ score in the RUNSA group, such that older individuals scored higher on the HÖ-MEQ (ρ=0.358, $p$<0.001, n=92). This association was not found in the CONSA group (ρ=0.196, $p$=0.059, n=94).

The genotype distributions were not statistically different between the RUNSA and CONSA groups ($p$=0.696, **Figure 3.3A**) and were found to be in the Hardy-Weinberg equilibrium ($p$=0.964 and $p$=0.268 respectively). The *PER3* VNTR polymorphism allele distribution is shown in **Figure 3.3B**. Similarly, no differences were found between the two groups ($p$=0.753).

### 3.2.2 Aim 2: To compare chronotype and *PER3* VNTR genotype of the two Dutch groups

While the RUNNL group scored higher on the HÖ-MEQ than the CONNL group ($p$=0.041), the effect-size is small (ES=0.149). Furthermore, the chronotype category distributions were not statistically different between the groups ($p$=0.116, **Figure 3.2B**). HÖ-MEQ score did not correlate with age in the RUNNL group (ρ=0.099, $p$=0.354, n=90). However, a positive correlation between HÖ-MEQ score and age was observed in the CONNL group (ρ=0.224, $p$=0.027, n=98).

The *PER3* VNTR polymorphism genotype distribution was similar in the RUNNL and CONNL groups ($p$=0.391, see **Figure 3.3A**). When the *PER3* VNTR allele frequency of the groups was compared, the absence of a difference became more evident ($p$=1.000, **Figure 3.3B**). The *PER3* VNTR genotype distributions of the RUNNL and CONNL groups were also found to be in Hardy-Weinberg equilibrium ($p$=0.279 and $p$=0.292 respectively).

### 3.2.3 Aim 3: To compare chronotype and *PER3* VNTR genotype between the two groups of runners

The HÖ-MEQ score of the RUNSA group was higher than that of the RUNNL group ($p$=0.009), however the effect size is small (0.193). The chronotype category frequency distribution was not different between the groups ($p$=0.131). Similarly to the other compared groups, no differences exist in the *PER3* VNTR polymorphism genotype ($p$=0.620) or allele ($p$=0.741) distributions between the two running groups (**Figure 3.3**).

### 3.2.4 Aim 4: To compare chronotype and *PER3* VNTR genotype between the two control groups

The HÖ-MEQ scores of the CONSA and CONNL groups are not statistically different ($p$=0.952, **Figure 3.2A**), and no differences in chronotype category distributions were observed ($p$=0.899, **Figure 3.2B**). The small difference in the *PER3* VNTR polymorphism genotype distributions between the two groups was not statistically different ($p$=0.638, **Figure 3.3A**). Similarly, no statistical difference in the *PER3* allele distribution between the two groups was found ($p$=0.540, **Figure 3.3B)**.

## 3.3 Aim 5: To determine whether *PER3* VNTR genotype is associated with chronotype

To assess whether the *PER3* VNTR polymorphism is associated with chronotype, all participants for whom both the HÖ-MEQ score and *PER3* VNTR genotype was known, were grouped by *PER3* VNTR genotype and the HÖ-MEQ score median was compared per genotype group. The results are shown in **Figure 3.4A**. The HÖ-MEQ scores were not different between the three genotypes ($p$=0.373), suggesting the absence of an association between the two. In **Figure 3.4B,** individual HÖ-MEQ scores were plotted against their *PER3* VNTR genotype. This representation of the data confirms that no

association exists in this sample of South African and Dutch athletes and controls. The analysis was repeated for each of the four groups independently and the data are shown in **Figure 3.5.** The HÖ-MEQ score was not different between the *PER3* VNTR genotypes in the RUNSA ($p$=0.279), CONSA ($p$=0.188), RUNNL ($p$=0.894) or CONNL ($p$=0.394) groups.

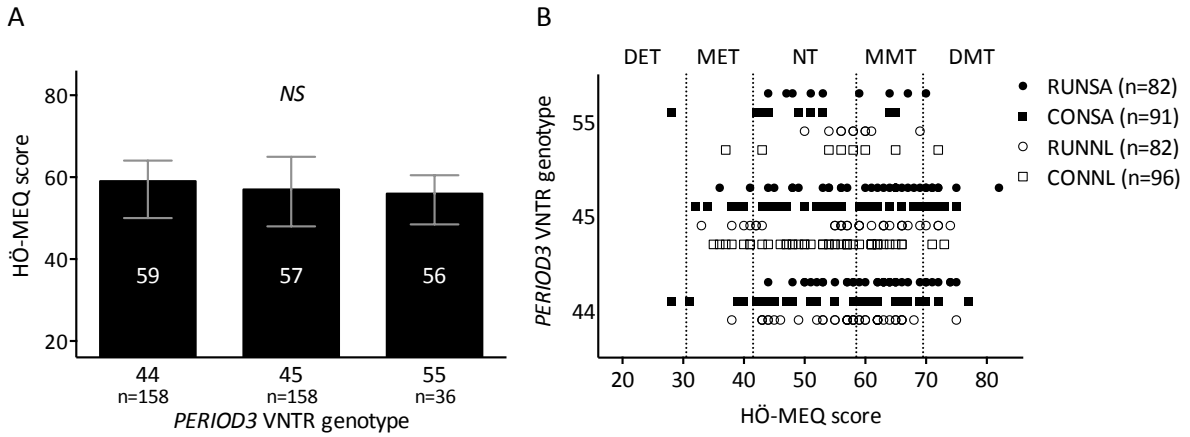


**Figure 3.4** – ***The association of the PERIOD3 VNTR genotype with chronotype. A****) HÖ-MEQ scores of the three genotype groups. Data are presented as medians with the 25$^{th}$ and 75$^{th}$ percentiles. The level of significance was calculated with the Kruskal-Wallis one-way ANOVA.* ***B****) The HÖ-MEQ scores of each individual within the four groups is plotted against his PER3 VNTR genotype. Runners are represented as circles and controls are represented as squares. South African participants are represented by filled symbols and Dutch participants are represented by open symbols. DET: definite evening-type, MET: moderate evening-type, NT: neither-type MMT: moderate morning-type, DMT: definite morning-type, RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands, CONNL: controls the Netherlands, HÖ-MEQ: Horne-Östberg morningness-eveningness personality questionnaire, 44: PER3$^{4/4}$ VNTR, 45: PER3$^{4/5}$ VNTR, 55: PER3$^{5/5}$ VNTR.*

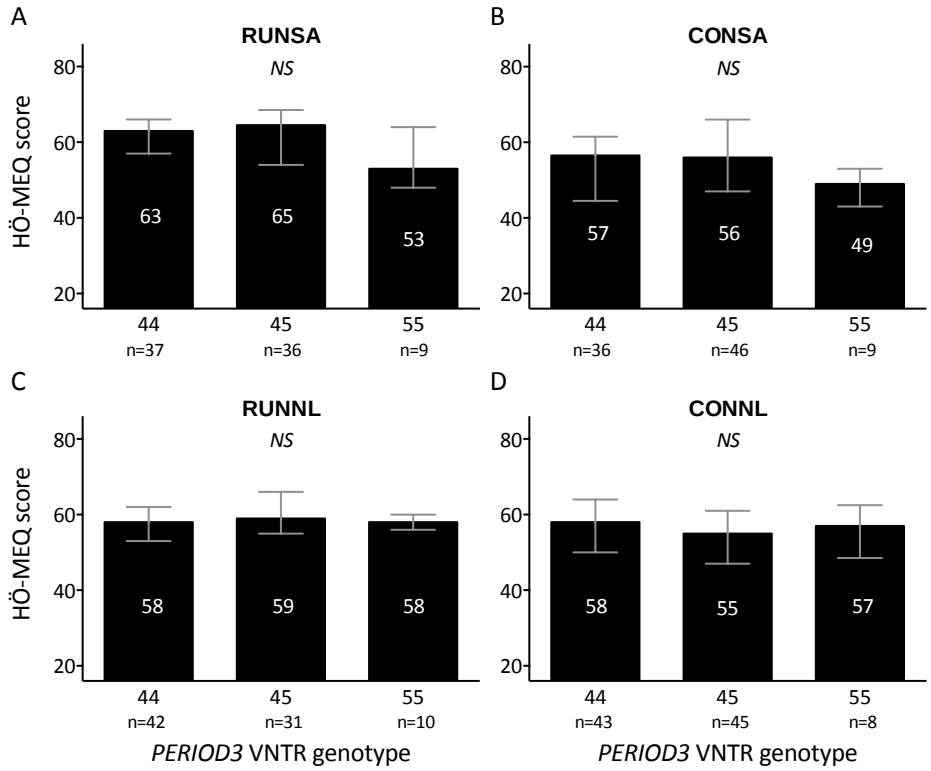


**Figure 3.5 –** ***The association of PER3 VNTR genotype with HÖ-MEQ score in the RUNSA (A), CONSA (B), RUNNL (C) and CONNL (D) groups.*** *RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands, CONNL: controls Netherlands, HÖ-MEQ: Horne-Östberg morningness-eveningness personality questionnaire, 44: PER3$^{4/4}$ VNTR, 45: PER3$^{4/5}$ VNTR, 55: PER3$^{5/5}$ VNTR. Data are presented as median HÖ-MEQ score and 25$^{th}$ and 75$^{th}$ percentile per PER3 VNTR genotype. The p-values were calculated with the Kruskal-Wallis one-way ANOVA.*

### 3.4 Aim 6: To investigate the association between chronotype, *PER3* VNTR genotype, and race time

The Langebaan Country Estate Weskus marathon race times of the RUNSA group and the Lage Landen Marathon Eindhoven race times of the RUNNL group, grouped per chronotype category are presented in **Figure 3.6**. The data show a pattern that suggests that ET chronotype is associated with poorer marathon performance and MT chronotype is associated with a better marathon performance. However, only the difference between the MTs and the ETs in the RUNSA group excluding any under-performers (runners that pre-race reported any reasons to underperform, e.g. running with friend, training-run or recovering from the flu) was found to be statistically significant ($p$=0.034) in this particular marathon. The marathon race time was not associated with chronotype in the Dutch runners group, excluding any under-performers ($p$=0.436*).*

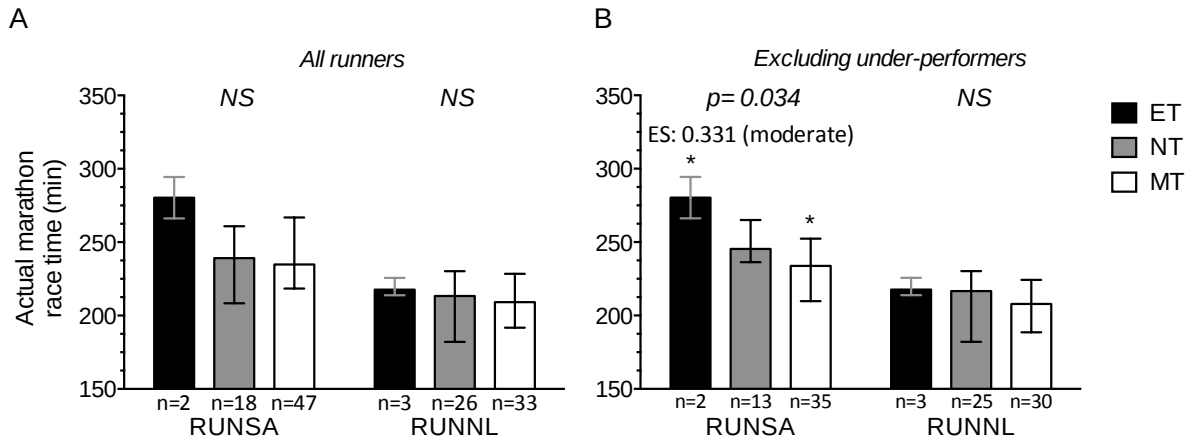


**Figure 3.6 –** ***Actual marathon race times of the RUNSA and RUNNL participants grouped by chronotype category. A)*** *All participants.* ***B)*** *Only those runners that did not report any reasons to underperform. RUNSA: runners South Africa, RUNNL: runners the Netherlands, ET: evening-type, NT: neither-type, MT: morning-type. Data are presented as median with the 25$^{th}$ and 75$^{th}$ percentiles. A Kruskal-Wallis one-way ANOVA was used to determine the p-values. 'NS' denotes not-significant p-values (p>0.05).*

All runners who provided their PB and recent full and half-marathon times were stratified for chronotype within their sample group and these data are displayed in **Figure 3.7**. The PB full marathon race time of the MTs was faster than that of the ETs in the RUNSA group ($p$=0.023), and the effect size was found to be moderate (ES: 0.320, **Figure 4.7A**). The PB full marathon race times in the RUNNL group between the chronotypes were not different ($p$=0.270, **Figure 3.7A**). The PB half-marathon race time in the RUNSA group was statistically faster in the MT group compared to the ET group ($p$=0.042, **Figure 3.7B**). Again, the PB half-marathon race times in the RUNNL groups were not different between the chronotypes ($p$=0.287, **Figure 3.7B**). The most recent full marathon race time of the MTs was also faster than that of the ETs in the RUNSA group ($p$=0.036, **Figure 3.7C**), and the effect-size was moderate (0.315). Once again, no difference in recent full marathon race time was found between the chronotypes in the RUNNL group ($p$=0.341, **Figure 3.7C**). Finally, no differences in recent half-marathon race times between the chronotypes were found in either the RUNSA or RUNNL groups ($p$=0.113 and $p$=0.243 respectively, **Figure 3.7D**).

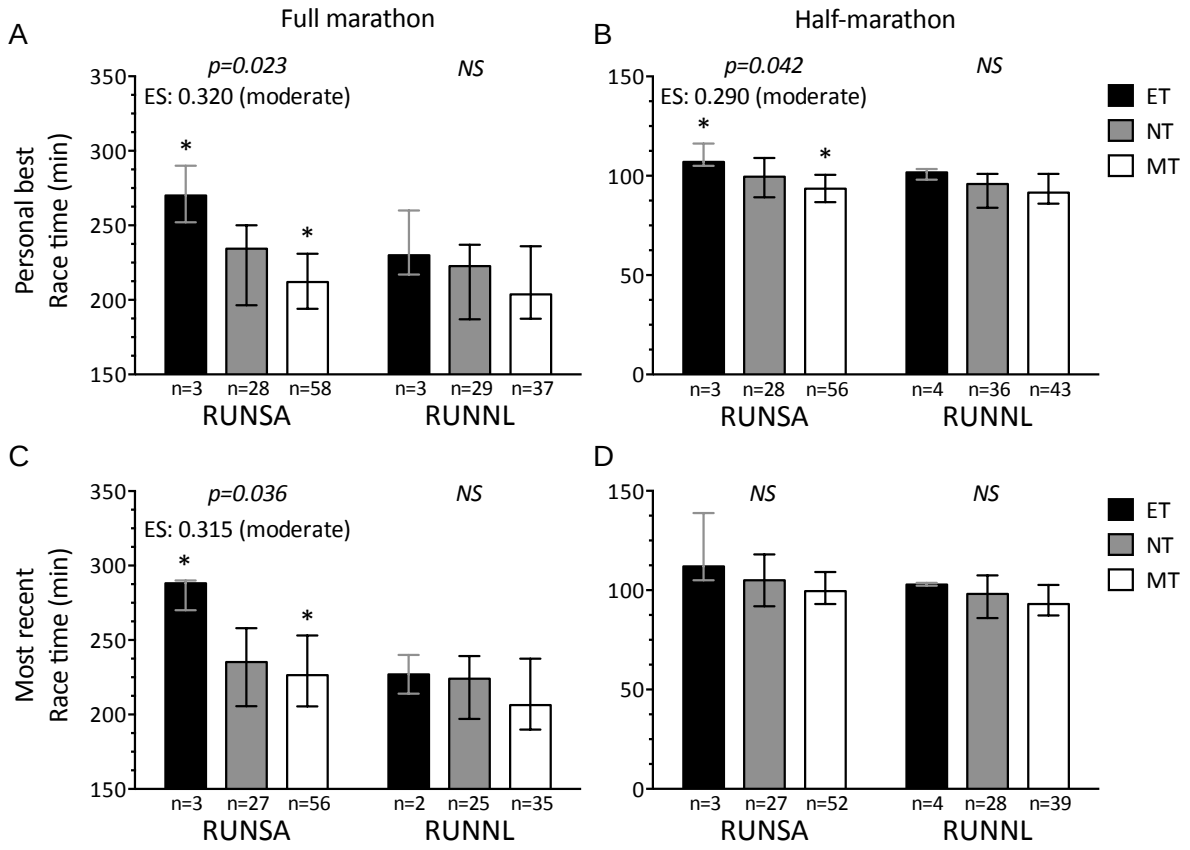


**Figure 3.7 –** ***The personal best and most recent race times for the full and half-marathon distances, stratified by chronotype for each of the two running groups. A)*** *Personal best (PB) full marathon race times.* ***B)*** *PB half-marathon race times.* ***C)*** *Most recent full marathon race times.* ***D)*** *Most recent half-marathon race times. RUNSA: runners South Africa, RUNNL: runners the Netherlands, ET: evening-types, NT: neither-types, MT: morning-types. Data are presented as the median with the 25$^{th}$ and 75$^{th}$ percentiles. The levels of significance were determined using the Kruskal-Wallis one-way ANOVA. An asterisk denotes significant differences within each group. 'NS' denotes not-significant p-values (p>0.05).*

A Spearman's correlation between HÖ-MEQ score and each of the four marathon race times was performed and is shown in **Figure 3.8**. In the RUNSA group, PB half-marathon race time was correlated with chronotype ($p$=0.006), such that a better performance was associated with a higher HÖ-MEQ score (morningness) (**Figure 3.8A**). Similarly, most recent half-marathon race time was correlated with HÖ-MEQ score ($p$=0.021) in the RUNSA group, such that faster race times were associated with higher HÖ-MEQ scores (**Figure 3.8B**). No correlations were found between HÖ-MEQ scores and any of the race times in the RUNNL group (**Figure 3.8C** and **3.8D**).

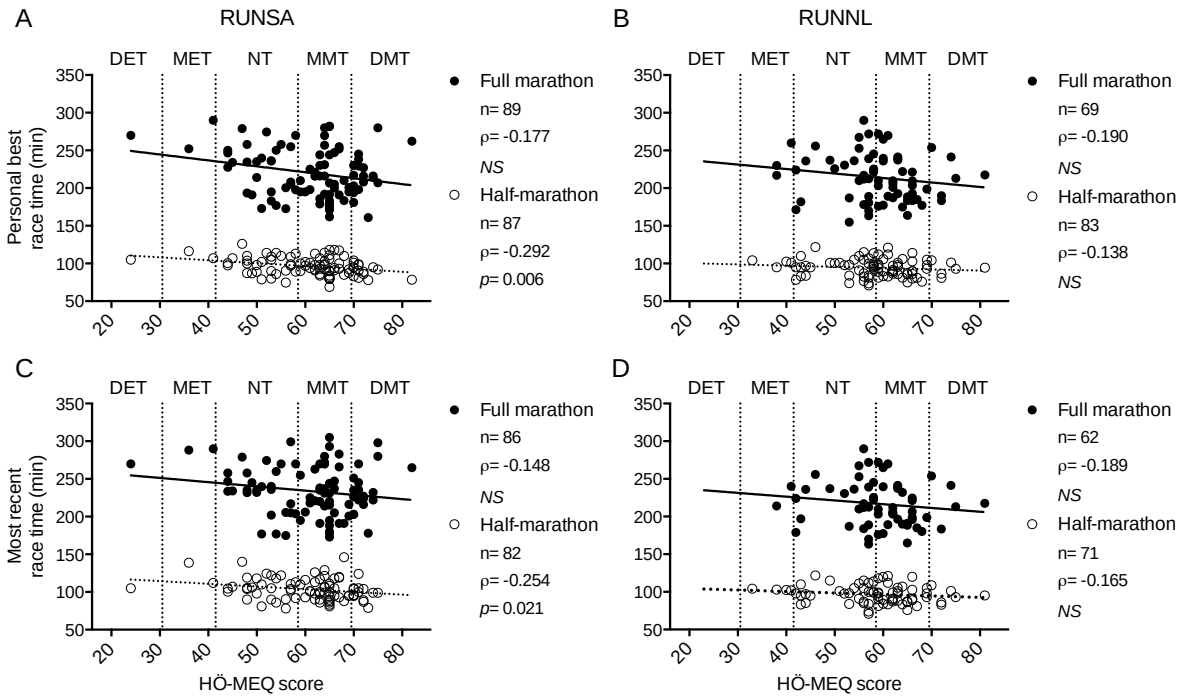


**Figure 3.8 –** ***Correlations beween personal best and most recent full and half-marathon race times and HÖ-MEQ score in the RUNSA and RUNNL groups. A)*** *Personal best (PB) race times of the South African runners group (RUNSA).* ***B)*** *PB race times of the Dutch runners group (RUNNL).* ***C)*** *Most recent race times of the RUNSA group.* ***D)*** *Most recent race times of the RUNNL group. Correlations are presented as Spearman's Rho (ρ). 'NS' denotes not-significant p-values (p>0.05).*

The Langebaan Weskus marathon race time of the RUNSA group and the Lage Landen Marathon Eindhoven of the RUNNL group, grouped per *PER3* VNTR genotype are shown in **Figure 3.9**. When all participating runners were analysed, no statistical difference in marathon race time was found between the carriers of three genotypes in either the RUNSA or RUNNL groups ($p$=0.346 and $p$=0.650 respectively). Similarly, when under-performers were removed from the analysis, still no association was found between the *PER3* VNTR genotype and marathon race time in both groups ($p$=0.111 for RUNSA and $p$=0.871 for RUNNL).

Likewise, the PB marathon race time was not associated with *PER3* VNTR genotype in the RUNSA ($p$=0.371) or RUNNL ($p$=0.416) groups (**Figure 3.10A**); neither was the PB half-marathon race time associated with *PER3* VNTR genotype in the RUNSA ($p$=0.210) or RUNNL ($p$=0.314) groups (**Figure 3.10B**). Similarly, no association was found between the *PER3* VNTR genotype and recent marathon race time in the RUNSA ($p$=0.195) and RUNNL ($p$=0.848) groups (**Figure 3.10C**), or between *PER3* VNTR genotype and recent half-marathon race time in the RUNSA ($p$=0.140) and RUNNL ($p$=0.319) groups (**Figure 3.10D**).

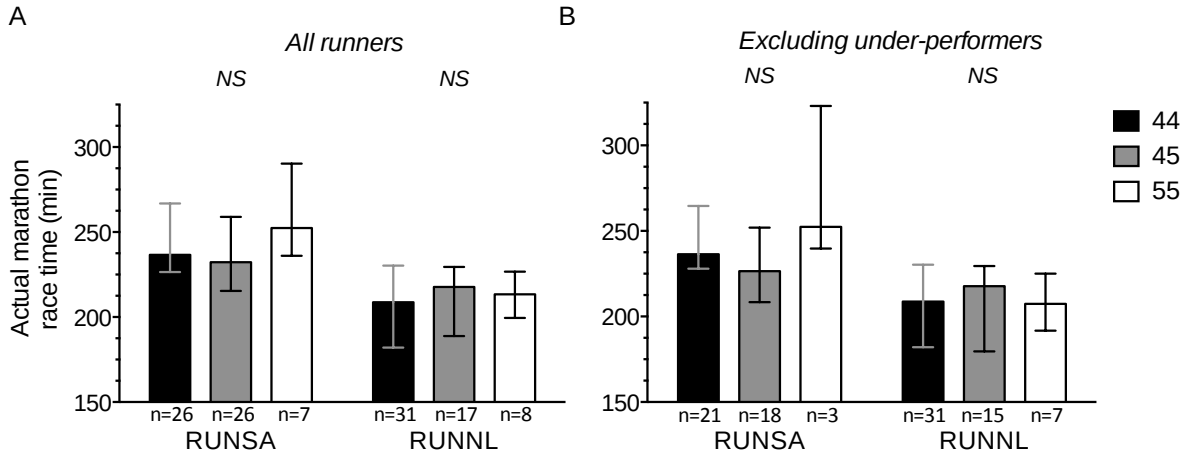


**Figure 3.9 –** ***Actual marathon race times of the RUNSA and RUNNL participants grouped by PER3 VNTR genotypes. A)*** *All participants.* ***B)*** *Only those runners that did not report any reasons to underperform. RUNSA: runners South Africa, RUNNL: runners the Netherlands, 44: PER3$^{4/4}$, 45: PER3$^{4/5}$, 55: PER3$^{5/5}$. Data are presented as median with the 25$^{th}$ and 75$^{th}$ percentiles. A Kruskal-Wallis one-way ANOVA was used to determine the level of significance. 'NS' denotes not-significant p-values (p>0.05).*

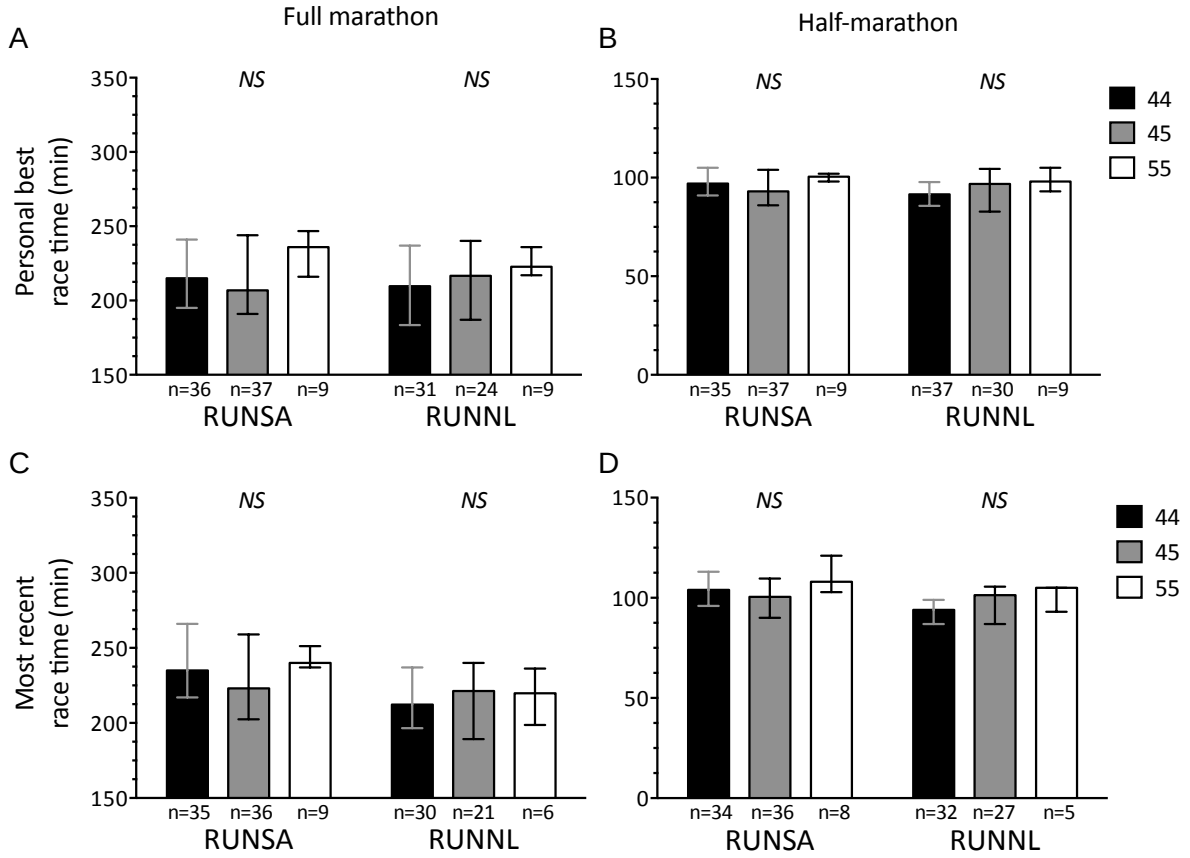


**Figure 3.10 –** ***The personal best and most recent race times for the full and half-marathon distances, stratified by PER3 VNTR genotype for each of the two running groups. A)*** *Personal best (PB) full marathon race times.* ***B)*** *PB half-marathon race times.* ***C)*** *Most recent full marathon race times.* ***D)*** *Most recent half-marathon race times. RUNSA: runners South Africa, RUNNL: runners the Netherlands, 44: PER3$^{4/4}$, 45: PER3$^{4/5}$,*

*55: PER3$^{5/5}$. Data are presented as the median with the 25$^{th}$ and 75$^{th}$ percentiles. The levels of significance were determined using the Kruskal-Wallis one-way ANOVA. 'NS' denotes not-significant p-values (p>0.05).*

### 3.5 Actual training time-of-day and marathon race time correlations

In this analysis, mean training time-of-day for each individual combines his actual midweek and weekend training data. Correlation graphs of race times versus HÖ-MEQ scores and Spearman's Rho (ρ) are shown in **Figure 3.11**. In each of the runners groups, for both PB and most recent full and half-marathon race times, no statistically significant correlation was found. Each of the linear regression lines is very close to horizontal, confirming the absence of an association.

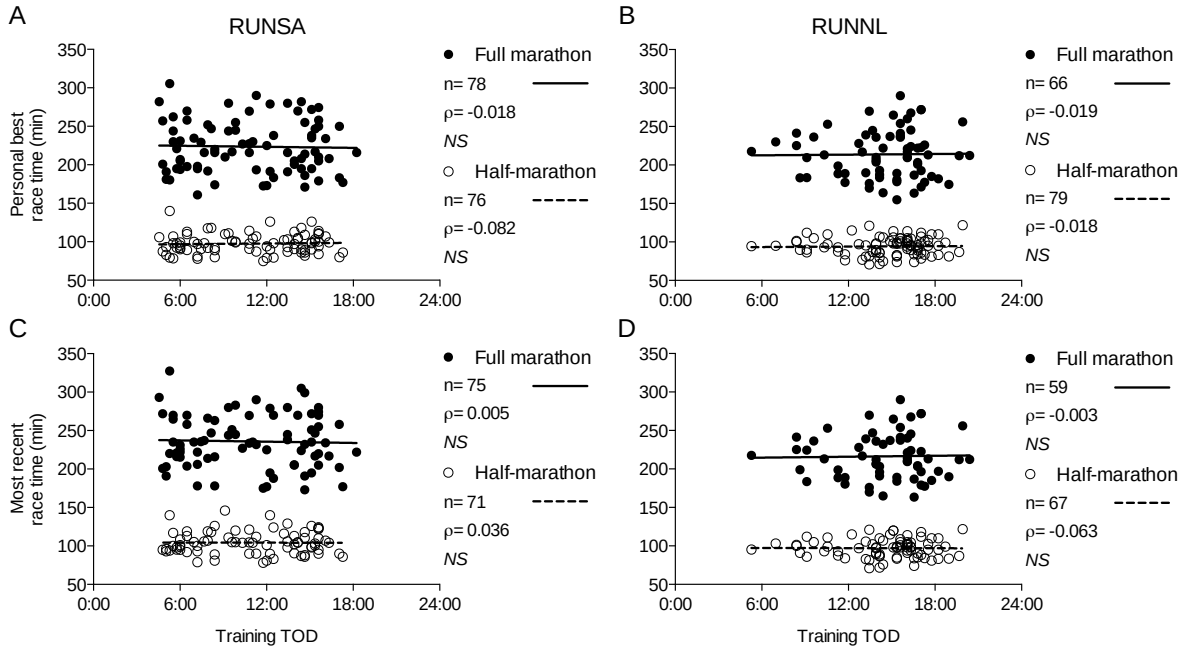


**Figure 3.11 –** ***Correlation between training time-of-day and recent and personal best full and half-marathon race times in the RUNSA and RUNNL groups. A)*** *Personal best (PB) race times of the South African runners group (RUNSA).* ***B)*** *PB race times of the Dutch runners (RUNNL).* ***C)*** *Most recent race times of the RUNSA group.* ***D)*** *Most recent race times of the RUNNL group. Correlations are presented as Spearman's Rho (ρ). 'NS' denotes not-significant p-values (p>0.05).*

### 3.6 Training time-of-day and chronotype correlations

The relationships between actual (midweek and weekend) and preferred training time-of-day (ToD) and HÖ-MEQ score in each of the four groups are presented in the tetrahedrons in **Figure 3.12**. The tetrahedron for the RUNSA group is presented in **Figure 3.12A**. The strongest correlations are between preferred training time-of-day and HÖ-MEQ score (ρ=-0.609, $p$<0.001), and preferred training time-of-day and midweek training time-of-day (ρ=0.568, $p$<0.001). Correlations were also observed between preferred training time and actual training time (weekend, ρ=0.469, $p$<0.001).

There was also a correlation between HÖ-MEQ score and actual training time (midweek, ρ=-0.371, $p$<0.001 and weeked, ρ=-0.244, $p$=0.036). Thus, the more morning-orientated the runner, the more likely he is to prefer training in the morning, and the more likely he is to actually train in the mornings during midweek days and on weekends.

The CONSA tetrahedron is presented in Figure **3.12B**. Here, the strongest correlations are those between preferred training time and actual training time (midweek, ρ=0.658, $p$<0.001 and weekend, ρ=0.628, $p$<0.001). The next strongest correlation is that between preferred training time-of-day and HÖ-MEQ score (ρ=-0.536, $p$<0.001). The correlation between training time-of-day during the midweek and on the weekend is also statistically significant (ρ=0.429, $p$=0.002). Finally, the correlations between chronotype and actual training time-of-day are the weakest, but still significant (midweek, ρ=-0.397, $p$<0.001 and weekend, ρ=-0.389, $p$=0.005). These correlations show that the more morning-orientated a South African control is, the earlier his preferred training time-of-day and the earlier he actually trains during midweek days and on the weekend. The earlier a South African control prefers to train, the earlier he actually trains during midweek days and on the weekend.

**Figure 3.12C** displays the tetrahedron of RUNNL group. The correlation between actual training ToD on the weekend and chronotype (ρ=-0.642, $p$<0.001) is very similar to that between chronotype and preferred training time-of-day (ρ=0.673, $p$<0.001). The next strongest correlation is that between preferred training time-of-day and actual training time-of-day on the weekend (ρ=0.426, $p$<0.001). The correlations between HÖ-MEQ score and actual training time-of-day are also statistically significant (weekend, ρ=-0.455, $p$<0.001 and midweek, ρ=-0.319, $p$=0.003). Thus, the more morning-orientated a Dutch runner is, the more likely he is to prefer training at an earlier time-of-day, and the more likely he is actually train at an earlier time-of-day during midweek days and on the weekend. A Dutch runner that prefers to train at an earlier time-of-day actually trains at an earlier time-of-day during midweek days and on the weekend.

Finally, the correlation-tetrahedron for the CONNL group is shown in **Figure 3.12D**. Chronotype is strongly correlated with the preferred training time-of-day (ρ=-0.542, $p$<0.001). Preferred training time-of-day also correlates strongly with training time-of-day on the weekend (ρ=0.455, $p$<0.001) and midweek (ρ=0.387, $p$<0.001). Similar to the CONSA group, while training time-of-day on the weekend correlates with training time-of-day in the midweek (ρ=0.370, $p$=0.016), neither correlate with chronotype. A more morning-orientated Dutch control is more likely to prefer to train at an earlier time-of-day. The earlier the time-of-day he prefers to train, the earlier he actually trains on the weekends and during midweek days.

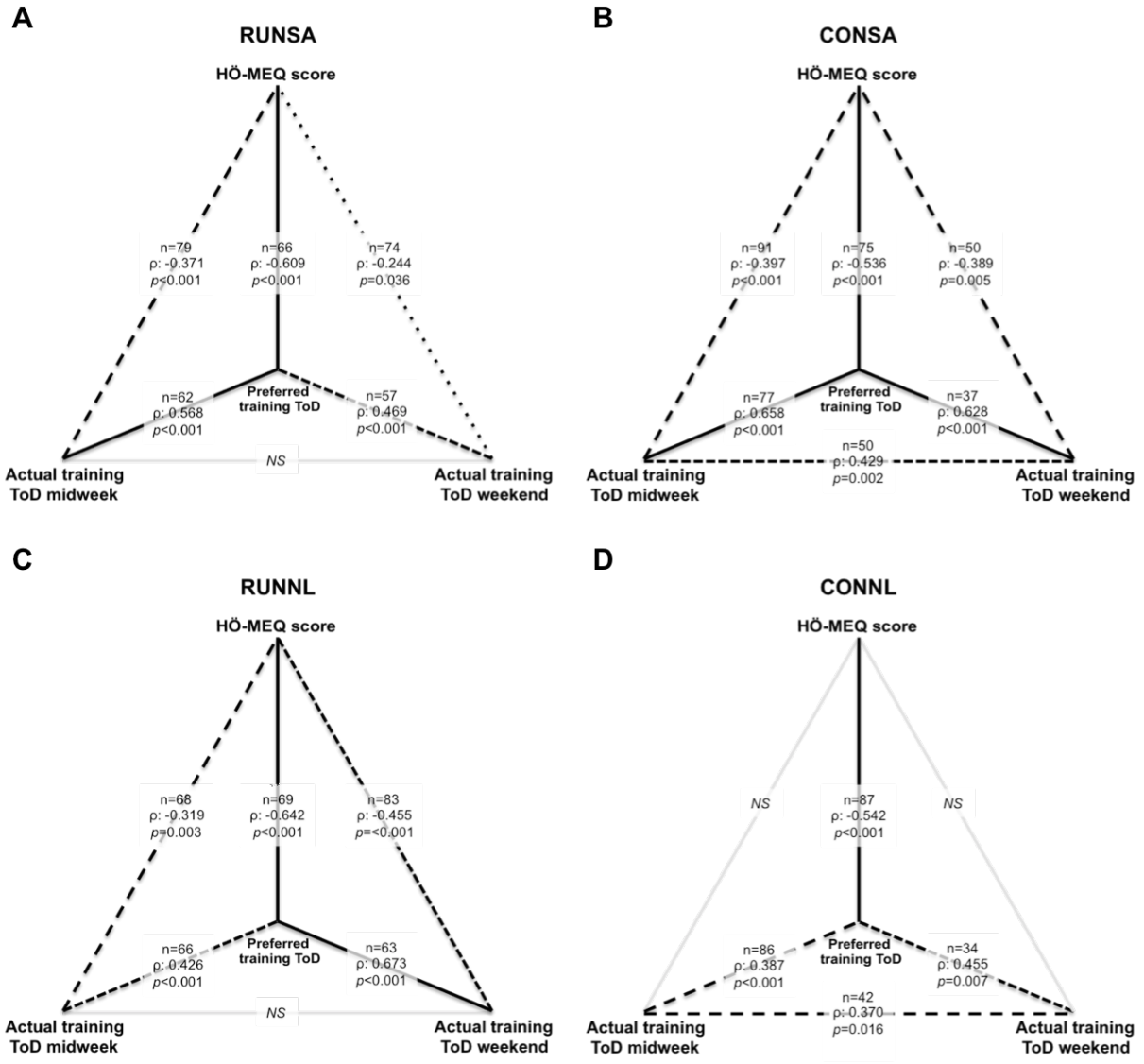


**Figure 3.12 – *Training time-of-day (ToD) and chronotype correlation models for the RUNSA (A), CONSA (B), RUNNL (C) and CONNL (D) groups.*** *The vertices of each tetrahedron represent either the HÖ-MEQ score (top) or actual and preferred training ToD (bottom). Each edge represents the Spearman's rank correlation (Rho, ρ) between the two vertices it connects. The density of the lines indicate how strong the correlation is. Stronger correlations have a higher density line, weaker correlations have a lower density line and not-significant correlations have a solid grey line denoted with "NS". RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands, CONNL: controls the Netherlands, HÖ-MEQ: Horne-Östberg morningness-eveningness personality questionnaire.*

### 3.7 Training quantity and chronotype correlation

Spearman's correlation coefficients (rho, ρ) between chronotype and training days per week, hours per week and kilometers per week (running groups only) were calculated and are shown in **Table 3.5**. When all four groups were combined, correlations were observed between HÖ-MEQ score and training days per week (ρ=0.292, $p$<0.001), hours per week (ρ=0.308, $p$<0.001) and kilometers per week (ρ=0.168 and $p$=0.023). Thus, the more morning-orientated the participants were, the more

likely they were to train more often, for longer and for greater distances. Additionally, the same correlations were analyzed for each separate group. In the RUNSA and RUNNL groups, none of the three training-quantity parameters were correlated with chronotype. However, in both the control groups, correlations between chronotype and training days per week and training hours per week were found. The higher the HÖ-MEQ score of each participant in the CONSA and CONNL groups, the more days per week that person trained (ρ=0.237, *p*=0.022 and ρ=0.244, *p*=0.016 respectively). Similarly, the higher the scores on the HÖ-MEQ, the more hours a participant from CONSA and CONNL groups trains each week (ρ= 0.239, *p*=0.021 and ρ=0.217, *p*=0.033 respectively).

**Table 3.5 – Correlations between HÖ-MEQ score and training quantity in each of the four groups.**

| | RUNSA | CONSA | RUNNL | CONNL | ALL |
|---|---|---|---|---|---|
| Training ($d \cdot wk^{-1}$) | n=92 | n=93 | n=90 | n=98 | n=373 |
| | ρ= 0.110 | ρ= 0.237 | ρ= 0.148 | ρ= 0.244 | ρ= 0.292 |
| | *NS* | *p*= 0.022 | *NS* | *p*= 0.016 | *p<0.001* |
| Training ($h \cdot wk^{-1}$) | n=90 | n=93 | n=89 | n=97 | n=369 |
| | ρ= 0.180 | ρ= 0.239 | ρ= 0.158 | ρ= 0.217 | ρ= 0.308 |
| | *NS* | *p*= 0.021 | *NS* | *p*= 0.033 | *p<0.001* |
| Training ($km \cdot wk^{-1}$) | n=92 | | n=89 | | n=182 |
| | ρ= 0.190 | - | ρ= 0.048 | - | ρ= 0.168 |
| | *NS* | | *NS* | | *p*= 0.023 |

*Data are presented as Spearman's correlation coefficient (ρ), p-value and sample size (n). RUNSA: runners South Africa, CONSA: controls South Africa, RUNNL: runners the Netherlands, CONNL: controls the Netherlands. ALL: all four groups combined. 'NS' denotes a not-significant probability (p>0.05).*

### 3.8 Daylight saving time

The CONNL participants were recruited from the 1st of October to the 12th of November 2013. Dayight Saving Time (DST) ended on the 27th of October, such that 59 (60%) participants were recruited before DST began and 40 (40%) participants were recruited afterwards. Sixteen (40%) of these 40 were recruited within the first week after the start of DST. To ensure that this change in clock time did not interfere with chronotype scoring, a one-way ANOVA was used to compare the HÖ-MEQ scores of participants recruited before and after DST. This data is presented in **Table 3.6**. The data for the "*after DST*" group is split into two further subgroups: those that were recruited within the first seven days after DST and those that were recruited after the first seven days after DST. HÖ-MEQ score was not different between the "*Before DST*" and "*After DST*" groups.

**Table 3.6 – The mean HÖ-MEQ scores of the CONNL participants recruited before and after daylight saving time (DST).**

| | HÖ-MEQ score | | | *p*-value | | |
|---|---|---|---|---|---|---|
| | | | | Before DST | After DST | 7d after DST |
| Before DST | **53.4** | ±12.2 | *59* | - | - | - |
| After DST | **55.9** | ±8.5 | *40* | *NS* | - | - |
| └ Within 7d of DST | **54.2** | ±8.7 | *16* | *NS* | *group within group* | - |
| └ After DST-7d | **57.1** | ±8.3 | *24* | *NS* | *group within group* | *NS* |

*Data are presented as **mean** ±standard deviation (SD) sample size (n). 'Before DST': controls Netherlands (CONNL) participants recruited before the end of daylight saving time (DST). 'After DST': CONNL participants recruited after the end of DST. This latter group is split into two subgroups: 'Within 7d of DST': participants recruited within the first seven days after the end of DST and 'After DST-7d': participants recruited eight or more days after the end of DST. HÖ-MEQ: Horne-Östberg morningess-eveningness personality questionnaire. P-values were calculated with a one-way ANOVA and show the probability of a statistically significanct difference between the group in the first row of the table and the corresponding group underneath the 'p-value' heading.*

# Chapter 4: Discussion

In a previous study, it was shown that South African individual-sports athletes had a bias towards the morningness chronotype that was greater than had typically been observed in populations from the United States of America, the United Kingdom and Europe (104). Therefore, this study set out to determine whether this bias could be explained by geography, genetics, or even the timing of the competitive events in South Africa for these athletes as proposed by Kunorozva *et al.* (2012). The prevalence of morning- and evening-types in a South African running group and a South African active, but non-competitive control group was compared to that of a Dutch running group and Dutch active, but non-competitive control group.

**4.1 Chronotype**

The first finding of this study was that the chronotype category distributions of the South African and Dutch control groups were similar, namely 42% and 39% morning-types (MTs) and 12% and 13% evening-types (ETs) respectively. This finding is in line with was has been found in a group of French participants (40% MTs and 11% ETs) (106). However, the results from many other studies including those on German (9% MTs and 29% ETs) (108), Italian (18% MTs and 22% ETs) (92), Spanish (12% MTs and 28% ETs) (92), American (12% MTs and 27% ETs) (71) and Lebanese participants (7% MTs and 25% ETs) (109) reported a much lower prevalence of morning-types and a higher prevalence of evening-types. Given that it is thought that the chronotype category distribution of a given population should follow a bell-shaped curve, with the majority of the population being neither-types, and fewer extreme chronotypes, the chronotype distribution found in both control groups of this study is not typical, since there is a higher representation of morning-types and fewer evening-types. Kunorozva *et al.* (2012) also found a high prevalence of morning-types and a relatively low prevalence of evening-types in an active but non-competitive South African control group (104), similar to the chronotype distribution from this study. Since the South African control group from this study, and the control group from in the Kunorozva *et al.* (2012) study were recruited using similar inclusion and exclusion criteria, and from similar populations, it is expected to see similar chronotype distributions.

What was unexpected, however, was that the Dutch control group would have a similar chronotype category distribution to the South African control group. In fact, we hypothesised that the Dutch controls would display a lower prevalence of morning-types as a result of the geographical location of the Netherlands and the environmental factors that are associated with that. A study which determined chronotype in students from six countries around the world (United Kingdom, the

Netherlands, Spain, Columbia, United States of America and India) using the composite scale of morningness (CSM) revealed that the students in the countries with temperate climates, such as England, the Netherlands and the United States of America, were least oriented towards morningness, the Netherlands scoring the lowest of all (79). Similarly, another study found that the average HÖ-MEQ score of Dutch students (n=2,481) was 48.9 (82), which falls into the category of neither-types. Both studies showed that students from the Netherlands were more likely to be neither-types or evening-types than morning-types. This is at odds with the chronotype distribution findings from this study, although it needs to be recognized that the Dutch controls in this study were older than the participants in the other studies.

Therefore, the remarkable finding here is the fact that the Dutch controls has a similar chronotype category distribution to the South African controls, which is higher than previously reported in an European population. We therefore propose that it is the fact that these two groups specifically comprise physically active individuals that accounts for the bias towards morningness. Similar to the South African control participants described by Kunozorva *et al.* (2012), the controls in this study are physically active, exercising at least twice per week for an hour, and have done so for the past two years, albeit it a non-competitive manner. To the best of our knowledge, there are two studies on adolescents which hint at a relationship between being physically active and having a higher morningness score (93,123). This study builds on these findings since a positive correlation between training volume and HÖ-MEQ score was observed (see **section 3.10**). This correlation may suggest two explanations for our findings: first, people who are more orientated towards morningness may choose to be more physically active and second, the chronotype of people who are more physically active may shifts over time so that they become more morning orientated.

It must be kept in mind that the active control groups from this study and the Kunorozva *et al.* (2012) study are older than the other populations in the literature that have been assessed for their chronotype. The South African and the Dutch active control groups both had a median age of 36 ±15 years, the active controls in Kunorozva *et al.* (2012) had a mean age of 32.9 ±8.1 years (104) and the French and New Zealand participants 17-81 (106) and ±40 years (89) respectively, while the German, Italian, Spanish, American and Lebanese participants had ages between 19 and 25 years (71,92,108,109). Morningness has been associated with an older age (69) (see **section 1.4.2.3**), and, besides physical activity, the older age may thus be an alternative explanation for the greater tendency to morningness in the active control groups in this study and that of Kunorozva *et al.* (2012).

The second finding of this study was that the Dutch runners scored significantly higher than the Dutch controls on the HÖ-MEQ, albeit it with a small effect size (0.149). A key difference between the Dutch runners and control groups was that the runners had a higher training volume (both days and hours per week) compared to the Dutch control group. Therefore, since the Dutch runners also had a higher HÖ-MEQ score, we interpret this as more evidence for the hypothesis that being more physically active is associated with a bias towards a preference for mornings. Indeed, a correlation between training days per week and training hours per week and HÖ-MEQ score has been found in the combined groups, however, no correlation was found between training volume and HÖ-MEQ score when looking at the Dutch runners group individually. One explanation may be the homogeneity of this group; that is, each participant in the running group is already very active and is already orientated towards morningness such that the effect of greater levels of physical activity on chronotype is diminished. The Dutch control group, however, are not as active as the runners, and thus a higher level of activity may correspond to a greater tendency to morningness.

The third finding of this study was that the South African runners group had significantly more morning-types and fewer evening-types and scored higher on the HÖ-MEQ than the South African control group did, just as the Dutch running group was more morning-orientated than the Dutch control group. Interestingly, the South African runners had been training for more days per week and more hours per week than the South African active control group had. This again suggests that the degree to which each group is physically active, may explain the difference in chronotype between the two groups, as it did between the Dutch runners and controls. However, the effect size between the South African running and control groups is larger (ES: 0.281) than between the two Dutch groups. And thus, being more physically active does not entirely explain why the South African runners are more morning orientated than the South African active controls.

Lastella *et al.* (2010) found that out of 23 Australian elite triathletes, twelve were neither-types, eleven were morning-types and none were evening-types (121). A higher prevalence of morning-types in athletes was also found by Kunorozva *et al.* (2012) who reported a similar finding in 119 Two-Oceans ultra-marathon (56km) runners, in which 67% were morning-types and only 3% were evening-types, compared to 41% morning-types and 7% evening-types in an active but non-competitive control group (104). Both studies proposed that evening-types may not select sports which require early rising, or that the athletes' chronobehaviour may adapt to the early morning event start times. The average start time of thirty Western Province Athletics (WPA, South Africa) races that cover at least 21.1 km in 2013 was 06:24 (range 05:15 to 08:00). These early start times

may make participation unfavourable for people who do not prefer rising early and be active in the morning. In contrast, the average start time of the twenty-one North Brabant province (NB, the Netherlands) races that cover at least 21.1 km in 2013 was 11:41 (range 09:00 to 16:00), which one might argue is favourable for all chronotypes. The difference in marathon start-time between the South African and Dutch marathons may be an additional explanation for the observation that the difference in morningness between the South African runners and controls are larger than the differences in morningness between the Dutch runners and controls.

The fourth finding of this study is that the South African runners were more morning-orientated than the Dutch runners. On one hand, this observation may be explained by the greater training volume reported in the South African runners group compared to the Dutch runners group. This is in line with the earlier mentioned explanation that the greater bias towards morning preference between the two Dutch groups may be a cause or a result of the amount of physical activity undertaken. However, the above mentioned theory that the early marathon start times in South Africa may explain the higher prevalence of morning-types in the South African runners group compared to the South African control group, may also explain why the South African runners are more morning-orientated than the Dutch runners.

Another contribution towards the South African runners being more morning-orientated than the Dutch runners may be the geographical location of South Africa. Chronotype appears to be associated with climate zones, with individuals in the tropics (closer to the equator) being more orientated towards mornings than individuals from subtropical climate zones (further from the equator) (139). It has also been suggested that people in warmer climates may be more morning-orientated than people in colder climates (79). The circadian clock may play an important role in this association, since bright is more efficient for entraining purposes then dim light and this effect is largest on late chronotypes, so that eveningness tendencies are reduced (133). Additionally, longer photoperiods and higher light intensity are more effective for circadian rhythm entrainment than shorter photoperiods and lower light intensities (132). South Africa has an annual amount of direct sunlight of 2993 hours (out of a possible 4383 hours), while the Netherlands only has 1568 hours of direct sunlight each year (202). Although photoperiod may not be determined by the amount of direct sunlight, light intensity is. Therefore it may be reasonable to consider that the geographical differences between the two countries may affect chronotype. However, since this difference in chronotype between the two countries was not observed in the South African and Dutch active control groups, either geographical location does not play a considerable role in affecting chronotype, or the other factors mentioned above such as physical activity are over-riding this effect.

Additionally, the South African runners actually trained at an earlier time-of-day than the Dutch runners, which may either be a cause or reason for being more morning-orientated. When athletes get up early in the morning to train, they will be exposed to more sunlight than those that remain indoors (albeit in bed or not). Light exposure is considered to be the most important zeitgeber of the master oscillator in the suprachiasmatic nucleus (29), and may thus affect chronotype. Additionally, runners train primarily outside in natural light, while most active but non-competitive controls train in indoor gyms and may thus be exposed to artificial light. Natural daylight may be brighter in nature, which is more affective to circadian entrainment than less bright light (132). Additionally, natural daylight contains more light from the blue spectrum, which is the kind of light that acts on the melanopsin photopigment in the eyes' retina's to entrain the circadian clock (63).

Apart from activity level, the events' start times, and to a lesser extent geographical location, confounding factors may be involved in the finding of a higher prevalence of morning-types in the South African and Dutch running populations. For example, in this study, age correlates positively with HÖ-MEQ score, such that the older the runner, the higher the HÖ-MEQ scores. However, all sample groups have a similar age and age is thus unlikely a confounding factor. Likewise, ethnicity and sex are unlikely confounding factors because all participants are white males. Since the running and control samples within each country reside on the same latitude, this is unlikely to influence the difference observed between the running and control groups. Neither can night time shift-work explain the difference in chronotype between these groups, since participants that were currently or had been involved in such labour were not included to this study.

To conclude, both running groups are more morning-orientated than their respective control groups, which is as hypothesized and may be explained by the higher level of physical activity of the runners. The South African runners group was found to be more morning-orientated than the Dutch running group, and the explanation may lie in the selection of morning-types, or the habituation to morningness, caused by the early marathon start times of South Africa. Factors that are associated with the difference in geographical location such as light intensity and climate are unlikely to play an important role in this finding, since the control groups from both countries had similar chronotype category distributions.

**4.2 The *PERIOD3* VNTR polymorphism**

In all four groups, similar numbers of participants were genotyped as *PER3*$^{4/4}$ and *PER3*$^{4/5}$, while very few were genotyped as *PER3*$^{5/5}$. Likewise, the *PER3* VNTR allele distribution was similar amongst all four groups, with the *PER3*$^{5}$ allele being least prevalent (±33%). These distributions are in line with what other studies have reported in European American (34% *PER3*$^{5}$), Ghanian (36% *PER3*$^{5}$), Iranian (30% *PER3*$^{5}$), Wolof Senegalese (31% *PER3*$^{5}$), Ukrainian (31% *PER3*$^{5}$), British (32% *PER3*$^{5}$), Finish (33% *PER3*$^{5}$), Nigerian (34% *PER3*$^{5}$), Norwegian (34% *PER3*$^{5}$), Afghan (34% *PER3*$^{5}$), Banthu (35% *PER3*$^{5}$), Malawian (35% *PER3*$^{5}$), Sudanese (36% *PER3*$^{5}$), Ladin-speaking Tyrolean (36% *PER3*$^{5}$), African American (36% *PER3*$^{5}$), Anatolian (37% *PER3*$^{5}$), Indian (32% *PER3*$^{5}$) and European cohorts (33% *PER3*$^{5}$) (90,137,166). Thus our findings seem similar to what has been published in the literature for many other populations.

In only a few other populations, has the *PER3*$^{5}$ allele been found to have a lower prevalence, including Han Chinese (19% *PER3*$^{5}$), Mongolian (11% *PER3*$^{5}$), Sichuan Chinese (18% *PER3*$^{5}$), Japanese (19% *PER3*$^{5}$) and Chad (25% *PER3*$^{5}$) populations (137,166). Rather, other studies have reported higher frequencies of *PER3*$^{5}$ allele carriers in some populations, including a second African American population (41% *PER3*$^{5}$), two Papuan New Guinean populations (59% and 81% *PER3*$^{5}$ respectively) and Indian (42% *PER3*$^{5}$), Italian-speaking Tyrolean (42% *PER3*$^{5}$), Caucasian (43% *PER3*$^{5}$), North Sudanese (46% *PER3*$^{5}$), Ethiopian (52% *PER3*$^{5}$), Yemenis (52% *PER3*$^{5}$), and a Black African (42% *PER3*$^{5}$) population (90,137,166).

Based on the findings from this study, there was no pattern that suggests a geography-based association with the *PER3* VNTR distribution for any of the four groups, which is in agreement with what has been proposed before (137). Likewise, there does not appear to be any association between the *PER3*$^{5}$ allele frequency and ethnicity or ancestry. Thus, the genetic variation may have occurred randomly via sexual selection rather than survival-based natural selection. Ciarleglio *et al*. (2008) proposed that genetic drift, and not natural selection based on latitudinal clines is the cause of differences in *PER3* VNTR genotype distributions among worldwide populations (166).

A somewhat surprising finding was that the allele distribution observed in the South African runners in this study is different to that published previously describing South African Ironman triathletes, ultra (56km) marathon runners and cyclists, in whom the *PER3*$^{5}$ allele was dominant (56, 58 and 61% respectively) (104). Instead we found that the runners from this study had a similar *PER3* VNTR distribution to the previously reported control group (38%) (104). This observation is interesting since both groups consisted of Caucasian South African male individual-sport endurance

athletes of similar age. Kunorozva *et al.* (2012) proposed that the athletes in their study had to cope with early-morning competitive event participation, which may have caused a selection based on clock-gene genotypes that drive a preference for mornings, such as the *PER3*$^{5}$ allele (104). Although this theory seems plausible in the context of their study, it doesn't seem to fit in with the results from this study. The runners from this study also had to cope with early morning marathon start times, and yet did not have a higher frequency of the *PER3*$^{5}$ allele compared to our active controls. However, the runners in this present study were recruited at a standard marathon (42.2 km), while those recruited by Kunorozva *et al.* (2012) were participating in a 56 km ultra-marathon (104). Although the two events had similar start times, one may speculate that running a longer distance requires more training. Since our data suggests that many South African runners train in the morning, before leaving for work, the only option to train more and get to work at the same time is to get up earlier in the morning. This requires the runners to be able to cope with rising very early in the morning. At this point, if a person does not have the right genetics to cope with early-morning training, such as the *PER3* VNTR genotype and possibly other clock-related genes, that person may choose not to participate in sports that require training at such times. Thus leaving a group of ultra marathon runners, with a higher frequency of the *PER3*$^{5}$ allele [associated with mornings (135,160)], compared to a group of classic marathon runners.

**4.3 Association between chronotype and the *PER3* VNTR polymorphism**

Interestingly, this study did not find any association between the *PER3* VNTR genotype and chronotype when the data for all four groups were pooled, or when the groups were examined individually. Although various studies have not found evidence of an association between the *PER3* VNTR genotype and chronotype either (203-206), other studies have (104,135,160). Kunorozva *et al.* (2012) for example, found a strong association between the *PER3* VNTR genotype and HÖ-MEQ score in South African individual-sport endurance athletes and active controls (104). Based on the Kunorozva *et al.* (2012) study, and given the higher prevalence of the *PER3*$^{4}$ *allele in the* South African runners in the present study, a higher number of evening-types and neither-types would be expected. Instead, we found that the South African runners from this study contained more morning-types, and as such there was no relationship between chronotype and the *PER3* VNTR genotype. Therefore, whereas Kunorozva *et al.* (2012) may well argue that "genetically predisposed" morning-types "chose" ultra-endurance events, it is not possible to make that same argument based on the findings of this study. Instead, since all four groups in the present study are active and there is a mismatch between chronotype and the *PER3* VNTR genotype, perhaps the effect of habituation on

chronotype can explain these observations. That is, the participants will "get used" to early-morning race start times, such that their chronotype shifts to a more morningness orientation, despite their *PER3* VNTR genotype. As a result, this habituation may mismatch their chronotype with their *PER3*-based genetic predisposition, thus diminishing any association between their *PER3* genotype and chronotype. This may not have happened in the Kunorozva *et al.* (2012) study, since those runners may have had to cope with earlier wake-up times to such extent that a *PER3*$^{5}$ allele is necessary.

In conclusion, it is hypothesized that the *PER3* VNTR genotype did not affect chronotype to such extent that it can explain the large differences in chronotype between the groups. This finding increases the plausibility of the argument that habituation may be the cause of the higher frequency of morning-types in the South African and Dutch groups and that chronotype-based selection may play an additional role in the higher prevalence of morning-types in the South African runners group.

**4.4 Chronotype and training time-of-day**

HÖ-MEQ score is strongly negatively correlated with preferred training time-of-day in all groups, which means that the participants that are more morning-orientated (higher HÖ-MEQ score) prefer to train at an earlier time-of-day. Or perhaps, the participants prefer to train at a time-of-day that reflects their chronotype. It can be speculated that the participants who are able to freely choose the time-of-day at which they prefer to train, without the restrictions of work and family obligations, are likely to choose the time at which they feel physically at their best. In most cases, this will depend on their innate circadian clock from which chronotype is determined indirectly. To the best of our knowledge, this study is the first study to describe the correlation between preferred training time-of-day and chronotype.

HÖ-MEQ score is also negatively correlated with the actual training time-of-day in the South African runners and control groups and the Dutch runners group, but not in the Dutch control groups. However, the correlation between HÖ-MEQ score and actual training time-of-day during midweek days and on the weekend are weaker than the correlation between HÖ-MEQ score and preferred training time-of-day. This suggests that in most cases, a person cannot aways train at their preferred time-of-day, or in other words, a person cannot always train at a time-of-day that suits their chronotype. This effect seems largest in the Dutch control group, since no correlation between chronotype and actual training time-of-day was found. However, the actual training times of the Dutch control group are dependent on the opening times of the gyms at which the participants were

recruited and may thus not reflect their chronotype. Unlike the correlation between HÖ-MEQ score and preferred training time-of-day, the correlation between HÖ-MEQ score and actual training time-of-day can go either way. On one hand, participants that have a more morning-orientated chronotype may often train in the early morning. On the other hand, it could mean that people who habitually train in the early mornings, become more morning-orientated, and thus have an earlier preferred training time-of-day (i.e. habituation). For example, participants that wake up early to train may be exposed to sunlight earlier than participants who stay in bed longer. As mentioned before, the earth's dark-light cycle is considered to be the most important zeitgeber in humans (29). Exposure to light may thus be very important in human entrainment, and may therefore also affect a person's chronotype.

Finally, a correlation between preferred training time-of-day and actual training time-of-day was found in each group. Suggesting that in most cases, people actually train at the time-of-day they prefer to train. Most likely, a person's actual training time-of-day depends on that person's preferred training time-of-day. Additionally, this relationship may go the other way, such that people that train at a specific time-of-day may get used to that training time-of-day and so it may become their preferred training time-of-day. Participants that were not able to train at their preferred training time-of-day were asked what kept them from training at that specific time. The most-mentioned reason was work obligations, while family obligations, club training time-of-day and the schedule of training buddies were also mentioned. This again suggests that a person's preferred training time-of-day may influence his/her actual training time-of-day, rather than the other way around. Since in this study (i) a person's preferred training time-of-day has such a strong correlation with his chronotype, and (ii) preferred training time-of-day is correlated with actual training time-of-day and (iii) chronotype is more weakly correlated with actual training time-of-day than to preferred training time-of-day, it is likely that a person actually trains at the training time-of-day at which they prefer to train, which depends on their chronotype. However, this does not exclude the possibility that a person's actual training time-of-day influences his/her chronotype through habituation. Rather, the evidence from this study suggests that it is less unlikely.

A substantial number of studies have described the effect of training time-of-day on athletic performance. Chtourou and Souissi (2012) combined many of those studies in a review (186). Their main conclusion is that "adaptations to training are greater at the time of day at which training is regularly performed compared to other times" (186). To the best of our knowledge, there are no studies that describe the effect of training time-of-day taking into account behavioural aspects such as chronotype. Based on the results from this study, we suggest that in addition to age, night-time

shift work, exercise and geographical location, training time-of-day may very well be an additional non-genetic factor that can influence a person's chronotype, and that this could be at least one of the links between a higher prevalence of morning-types in individual endurance sports such as marathon running.

**4.5 Association between chronotype and marathon race time**

The morning-types in the South African running group performed better in the Country Estate Weskus marathon (Langebaan, South Africa), and had statistically better personal best full and half-marathon performances and better most recent full marathon times than the evening-types did. In comparison, the performance of the Dutch running group in the Lage Landen marathon (Eindhoven, the Netherlands) as well as their reported personal best and most recent full and half-marathon race times were independent of chronotype.

While this seems to suggest that morning-types the South African runners group outperform evening-types, a weakness in the statistics behind this finding is the low sample size of the evening-type groups in both the South African and Dutch runners groups. Only two South African runners and a maximum of four Dutch runners were categorized as evening-types, leaving a rather small and perhaps unrepresentative sample of the whole South African and Dutch marathon running evening-types population. For this reason, Spearman's correlations were performed between HÖ-MEQ score and race times. In the South African runners, personal best and most recent half-marathon performance was negatively correlated with HÖ-MEQ score, suggesting a better race performance for those runners with a preference for mornings. This correlation was not found to be significant for the personal best and most recent full marathon times, likely because of the larger spread of marathon race times in the full marathon compared to the half-marathon. Similarly, no correlation between personal best and most recent full and half-marathon performance and HÖ-MEQ score was found in the Dutch running group. Thus, the factor that influences chronotype-dependent marathon performance in the South African running group is not present in the Dutch running group.

The South African morning-type runners did not train for more days, hours or kilometres per week than the neither-types or evening-types, thus although marathon performance was associated with training volume, this factor seems to be an unlikely explanation for the better marathon performance observed in the South African morning-type runners in this study. Edwards *et al.* (2005) found that training at the same time-of-day at which a performance test is scheduled positively

affects cycling performance in a time-trial (195). Likewise, Chtourou *et al.* (2012) found that adaptations to strength training are greater at the time-of-day at which training was scheduled compared to other times (186). Therefore, it is possible that the morning-types in this study, who trained in the morning, were at an advantage since marathon races in South Africa start in the early morning. Additionally, Kunorozva *et al.* (2014) found that the perception of effort in morning-type cyclists is lower when exercising in the morning compared to when exercising in the evening (207). A lower perception of effort my be beneficial to prolonged physical performance in which a pacing strategy is important (208), such as marathon running.

In a 2000 m rowing time-trial, morning-types who trained in the morning performed significantly better in the morning than in the evening (193), compared to evening-types who also trained in the morning. It was suggested that the timing of best basic performance is partly determined by naturally occurring morningness or eveningness predispositions (193), and may play a role in the better marathon performance of the morning-types in the South African running group. Since the South African marathons start so early in the morning, the morning-types who train in the morning have better chance of shifting peak performance to the morning compared to the evening-types. These '*naturally occurring morningness or eveningness predispositions*' may be the same predisposition that makes a person choose to participate in a specific competitive sport, such as marathon running. Especially when it appears that a morningness predisposition is required as is the case in the South African running scene where race start times are early.

One of these possible predispositions, the *PER3* VNTR genotype, was not associated with marathon performance in this study. Since the *PER3* VNTR genotype was also not associated with chronotype, the absence of a correlation between the *PER3* VNTR genotype and marathon performance was expected. However, although Kunorozva *et al.* (2012) found a correlation between chronotype and the *PER3* VNTR genotype, a correlation between personal best and most recent marathon race times and the *PER3* VNTR genotype was not found (104). It is important to state that in that study no association was found between personal best and most recent marathon race times either. Future research may investigate other clock gene polymorphisms that may potentially influence marathon performance, especially those associated with physiological differences.

**4.6 Strengths and weaknesses**

One of the strengths of this study is the specificity and homogeneity of the participant groups. Since chronotype is affected by age (89,209), only participants with ages between 25 and 50 years were recruited, resulting in four groups with similar ages and truly representative of adults, avoiding adolescent and older age chronotype changes. Similarly, only white males were recruited since the *PER3* VNTR polymorphism is known to vary between ethnic groups (137), and there may be a sex-effect on chronotype (92-94). Care was also taken to match the season and day length of the chosen marathons and the time of recruiting of participants in each country, as both may affect chronotype (132).

In this study, the daylight saving time ended during the two-month recruitment period in October and November in the Netherlands. While little research has been done on the effect of daylight saving time transition on chronotype, it is conceivable that this may have an effect. Kantermann *et al.* (2007) found that adjustments to daylight saving time are chronotype-specific. Morning-types seem to adapt easier to the start of daylight saving time (210,211) and are less sleepy for up to three weeks after the start of daylight saving time than evening-types (212). Although morning-types advanced their centre of activity (i.e. the time at which the midpoint of the beginning and end of their activity [non-sleep] occurs) by only 40 min, evening-types did not adjust their times of activity to the start of daylight saving time (210). The 16 participants that were recruited during the first seven days after the end of daylight saving time did not have a different HÖ-MEQ to those recruited before the beginning of daylight saving time. Similarly, the 24 participants that were recruited after the first seven days after the end of daylight saving time had a similar average HÖ-MEQ score as the participants that were recruited before the end of daylight saving time. These results agree with the findings of Monk *et al*. (1976), proposing that no changes occur within five days after the end of the daylight saving time period (213). Thus, since the HÖ-MEQ score of the participants recruited 'before' and 'after' daylight saving time did not significantly differ from each other, it is unlikely that recruiting around the daylight saving time end period had an effect on chronotype in this cohort.

Apart from strengths, there are also limitations to this study. One such limitation is the unequal distribution of the chronotype categories in each group whilst comparing the marathon race time. Since only a small proportion of runners were evening-types, the statistics may not be as robust as they would have been if the groups were of larger and similar sizes. It was chosen to recruit 100 participants for each group because the main aim was to compare the chronotype and genotype distributions between the South African and Dutch groups. If the main aim had been to investigate

the association between marathon performance and chronotype, equal numbers of morning-types, neither-types and evening-types would have been recruited, if it was possible to anticipate the low frequency of evening-types in these groups at all. For future directions it may be worth considering increasing the sample size. Additionally, including other countries may increase the validity of this study and may create a better understanding of how the marathon start time affects chronotype around the world. Finally, instead of, or in addition to using the HÖ-MEQ to assess the participants' chronotype, the MCTQ could have been used. This could potentially have resulted in a more even chronotype distribution.

# Chapter 5: Summary and Conclusions

The purpose of this study was to investigate whether the observation that South African individual-sport endurance athletes were primarily morning-orientated (104) can be explained by geography, the *PER3* VNTR polymorphism genotype, or the timing of the competitive events in South Africa for these athletes.

To the best of our knowledge, this is the first study to compare the chronotype distribution between South African and Dutch runners and active control groups. Since no difference in chronotype between the South African and Dutch active control groups was found, we hypothesize that either geographical location does not play a considerable role in affecting chronotype in these South African and Dutch participants, or other factors are over-riding this effect. We therefore conclude that geographical location, and therefore in this case latitude and sunlight exposure, is unable to satisfactorily explain the observation that South African individual-sport endurance athletes are primarily morning-orientated.

We also suggest that the higher prevalence of morning-orientated participants in this study cannot be explained solely by the *PER3* VNTR genotype, since the *PER3* VNTR genotype distribution was similar between the groups. Additionally, the *PER3* VNTR genotype was not associated with chronotype in the South African and Dutch groups. Our findings were different to those Kunorozva *et al.* (2012), who found that the South African ultra-marathon runners, which were also more morning-orientated, had a higher prevalence of the $PER3^5$ VNTR genotype, and the $PER3^5$ VNTR genotype was associated with chronotype. Thus, we propose that in this study, the early-morning marathon start times may have shifted the participant's chronotype to such extent that it may dissociate chronotype with the *PER3* VNTR polymorphism genotype. The ultra-marathon runners from the study of Kunorozva *et al.* (2012) may have to get up earlier in the morning than the classic marathon runners from this study, for the simple reason that running longer distances may require more training and, as our data suggests, South African runners often train in the early morning. For that reason, employed South African ultra-marathon runners may have to get up earlier in the morning than South African classic marathon runners and thus, people that have the required morningness-predispositions such as the $PER3^5$ VNTR allele, are more likely to participate in such ultra-marathon events than people that carry the $PER3^4$ VNTR allele. Although we propose that the *PER3* VNTR genotype cannot solely explain the higher prevalence of morning-types in the South African runners in this study, it is very likely that the *PER3* VNTR genotype does play an important role in the chronotype distributions found in the study of Kunorozva *et al.* (2012).

This may explain the mismatch between the *PER3* VNTR genotype and chronotype in the South African control group and the Dutch runners and control groups. However, we did not find an explanation for the *PER3* VNTR genotype and chronotype association in the South African control group from Kunorozva *et al.* (2012). To conclude, both early start time of South African endurance events and physical activity may explain the higher prevalence of morning-orientated people in South African individual-sport endurance athletes.

Finally, we propose that the ability of morning-orientated runners to cope with early-morning racing may explain the faster marathon race-times in these runners, or rather, the disability of runners that are not morning-types to cope with early-morning racing. Marathons in the Netherlands do not start early in the morning, potentially explaining why the marathon performance of the Dutch runners is not related to chronotype. The *PER3* VNTR genotype was not associated with marathon race time in the South African or Dutch running group. Since no association between the *PER3* VNTR genotype and chronotype was found, the absence of an association between the *PER3* VNTR genotype and marathon race time was to be expected.

In conclusion, this study provides preliminary evidence to suggest that (i) chronotype may be modified by being physically active and (ii) habituation to physical activity at a given time-of-day may condition chronotype. Therefore, future studies making use of a prospective design would be useful to confirm these findings. In addition it would be interesting to study the effects of matching training time-of-day to racing time-of-day in neither-type and evening-type runners in South Africa to see whether their marathon performance can be improved.

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# Appendix 1

**CIRCADIAN RHYTHMS AND SPORT:**

**THE *PERIOD3* VARIABLE NUMBER TANDEM REPEAT POLYMORPHISM, CHRONOTYPE AND MARATHON RACE TIME IN SOUTH AFRICAN AND DUTCH RUNNERS**

**PARTICIPANT INFORMATION SHEET AND CONSENT FORM**

Dear Volunteer,

Thank you for volunteering to participate in the University of Cape Town's study on **Circadian Rhythms and Sport**. This study will be conducted by researchers from the UCT/MRC Research Unit for Exercise Science and Sports Medicine, Department of Human Biology, Faculty of Health Sciences and UCT's Department of Molecular and Cell Biology, Faculty of Science.

**Background**

We are interested in researching the "circadian rhythms" (i.e. 24-hour or daily rhythms) of your body. These rhythms are found in almost all living creatures, are driven by your "internal body clock", and play a role in all kinds of processes within the body. In humans, they even influence behaviour, such as the time of day you wake up or get sleepy. A person's preference for activity at different times of day is known as their 'chronotype'. People generally fall into one of three main chronotype groups: morning-types, neither-types and evening-types. Morning-types tend to wake up and go to bed earlier than neither-types or evening-types, and are generally more productive in the earlier parts of the day. You could say that their internal body clocks run faster than those of evening-types or neither-types. In contrast, evening-types usually find it more difficult to wake up early, are more productive in the later afternoon or evening, and often go to sleep after midnight. A person's chronotype is measured using the Horne-Östberg morningness-eveningness personality questionnaire (HÖ-MEQ).

Chronotype is influenced by different factors including gender, age, and season of the year. But importantly, your genes, which are your inherited genetic material, are also partially responsible for your unique circadian rhythm and chronotype. A group of genes, known as the "clock" genes, are involved in setting your biological clock to a 24-hour rhythm. A number of research studies have shown that small variations in these genes influence chronotype. For example, a particular variant within one of the clock genes, known as the *PERIOD3* gene, has been associated with people being either morning-types or evening-types.

Our research team has shown that well-trained competitive Caucasian male runners, cyclists and Ironman triathletes in South Africa are more likely to be morning-types compared to active but non-competitive Caucasian males. This may be partly explained by the fact that most competitive events for individual endurance athletes in South Africa have early morning start times (06h00 – 07h00), perhaps better suiting morning-types. In the Netherlands, these races generally start around 10h00,

and as such may not be exclusively beneficial for morning-types. Therefore, in this study we will compare the chronotypes of South African and Dutch runners. We expect to find fewer morning-types in the Dutch running population. We are also interested in comparing the variants within certain clock genes between the South African and Dutch runners, as well as in non-runners from these two countries. We expect that the South African runners will have a higher proportion of morning-types than the South African non-runners, as well as both groups from the Netherlands.

Finally, since it is believed that circadian rhythms influence sport performance, it is likely that chronotype may also play a role in performance. Answering questions on this topic may be of interest for coaches and trainers of individual athletes or teams, as well as for recreational sports participants wishing to improve performance. As such, it will be useful to compare the marathon race times of endurance runners with different chronotypes and different versions of the *PER3* gene, to see if a relationship exists between running performance, chronotype, and versions of the *PER3* gene.

**What are the aims of this study?**

1. To determine and compare the chronotype and *PER3* VNTR genotype make-up of Dutch Caucasian male marathon runners and a control population of active but non-competitive Dutch Caucasian males.
2. To determine and compare the chronotype and *PER3* VNTR genotype make-up of South African Caucasian male marathon runners to a control population of active but non-competitive South African Caucasian males.
3. To compare the chronotype and *PER3* VNTR genotype data of the Dutch and South African populations.
4. To investigate the relationship between *PER3* VNTR genotype and chronotype in the Dutch and South African populations.
5. To determine whether marathon race time is related to chronotype or *PER3* VNTR genotype in the Dutch and South African marathon runners.
6. To determine whether oral temperature (a maker of innate circadian rhythm) is related to chronotype or *PER3* VNTR genotype in the Dutch and South African runner and control populations.

**What kind of participants are we looking for?**

**Runners:**

- Caucasian males
- Between 25 to 50 years of age
- Have been running for at least two years (participating in recreational races)
- Have participated in at least one marathon in the past year
- Preferably have entered the **Weskus Marathon** at Langebaan on the 20th of April 2013 **(not mandatory)**

**Non-runners:**

- Caucasian males
- Between 25 to 50 years of age

- Exercise at least twice a week (past two years)
- Do **not** participate in running training or racing (past three years)
- Do **not** participate in any other competitive sporting activity (past three years)

**What do we want you to do?**

We will either ask you to come to the Sports Science Institute of South Africa in Newlands, or we will meet you at your running club (runners) or a venue convenient to you. During this 45-minute visit we will explain the study, including all of the risks and benefits to you. We will also answer any questions you might have regarding the study. If you decide to participate, you will than sign an informed consent form. Next, we will weigh you and measure your height; this data will be used to calculate your body mass index (BMI) – a ratio of your height to weight, often used as an indirect measure of obesity. We will then ask you to fill in a questionnaire relating to your personal information, running training and racing history (runners only), exercise history (non-runners) and the HÖ-MEQ questionnaire, specifically designed to determine your chronotype (i.e. preference for mornings or evenings). Lastly, an appropriately trained staff member will take a 5ml (approximately one teaspoon) blood sample from a vein in the crook of your arm. Should you prefer not to donate a blood sample, we will swab the inside of your cheek with a sterile cotton swab to obtain a cheek cell sample. This procedure is quick and painless. We will use the blood or cheek cells to extract your genetic material (DNA) to assess which versions of the clo*ck* genes you carry.

Before you leave, we will give you a digital oral thermometer and a logbook so that you can record your oral temperature every hour while you are awake over three consecutive days. These measurements will give us an indication of the timing of your unique circadian rhythm.

**What are the benefits of participating?**

You will receive information regarding your personal chronotype. Once this study has been completed in its entirety, we will share the outcome with you! You will not be remunerated for participating in this study.

**What are the risks of participating?**

The potential risks to you during blood collection are minimal and are related to the blood sample collection technique from the veins on your arm (ante-cubital veins). The risks are: infection, delayed healing, blood clot (haematoma), physical pain, mental discomfort and injury to a nerve or a vessel. These risks are small and will be minimized by the use of staff trained to take blood samples (trained phlebotomists), use of sterile techniques and the use of disposable, single-use materials. There are no risks related to donating a cheek cell sample for subsequent DNA analysis.

**What are the ethical considerations?**

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

Participants will not be included unless they have signed the consent form, after the investigator has explained this study and its potential risks. You are invited to ask the investigator any questions you

may have relating to the tests and the procedures throughout the study (contact information below). Participation in this 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.

All records and results generated from this study will be stored in a password-protected computer database to ensure your confidentiality and your information will not be passed on to any other party(ies). Your DNA sample will only be used for the purposes explained to you, namely to determine your genotype for the clock genes, and will be destroyed on completion of this study. You may request that your blood, cheek cell and DNA sample be destroyed before the completion of the study. You will remain anonymous in any publication resulting from this study.

Finally, the University of Cape Town (UCT) undertakes that in the event of you suffering any significant deterioration in health or well-being, or from any unexpected sensitivity or toxicity, that is caused by your participation in the study, it will provide immediate medical care. UCT has appropriate insurance cover to provide prompt payment of compensation for any trial-related injury according to the guidelines outlined by the Association of the British Pharmaceutical Industry, ABPI 1991. Broadly-speaking, the ABPI guidelines recommend that the insured company (UCT), without legal commitment, should compensate you without you having to prove that UCT is at fault. An injury is considered trial-related if, and to the extent that, it is caused by study activities. You must notify the study doctor immediately of any side effects and/or injuries during the trial, whether they are research-related or other related complications. UCT reserves the right not to provide compensation if, and to the extent that, your injury came about because you chose not to follow the instructions that you were given while you were taking part in the study. Your right in law to claim compensation for injury where you prove negligence is not affected. Copies of these guidelines are available on request.

**Contact information**
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**CONSENT FORM**

I understand that researchers from the University of Cape Town (UCT) are conducting a study called "**Circadian rhythms and sport: the *PERIOD3* variable number tandem repeat polymorphism, chronotype and marathon race time in South African and Dutch runners**". The research team is from the UCT/MRC Research Unit for Exercise Science and Sports Medicine within the Department of Human Biology, Faculty of Health Sciences and the UCT's Department of Molecular and Cell Biology, Faculty of Science. I acknowledge that the investigator has explained all aspects of the study to me in detail.

- I understand that I will visit the Sports Science Institute of South Africa in Newlands once. This session is described in details in the *participant information sheet* above.
- I agree that during this meeting my height and weight will be measured.
- I also agree to fill in a questionnaire disclosing my personal details, running training and racing history (runners only), exercise history (non-runners only), and the HÖ-MEQ to determine my subjective chronotype.
- I agree to donate either a 5mL blood sample or a cheek cell sample to determine my genotype for the clock genes.
- I agree to measure my hourly oral temperature during waking hours for three days with the provided digital oral thermometer and to write the readings down in the *Oral Temperature Logbook* and return these to the researchers at the end of the study.
- Runners who plan to participate in the Weskus Marathon on the 20$^{th}$ of April 2013 only: I acknowledge that I would have participated in the Marathon in the absence of this study, and do so at my own risk. I also agree that my 2013 Weskus Marathon race time data may be collected from the Weskus Marathon website after completing the race and used for this study.
- I understand that I may ask the investigator any questions about the tests and results of the study.
- I have been informed about the risks involved in participating in this study. These risks are described in detail in the accompanying *participant information sheet*.
- I understand that my personal details will be treated confidentially.
- I understand that I may withdraw from this study at any time without stating any reason.
- I also understand that the investigator may withdraw me from this study at any stage.
- I understand that any biological samples will be destroyed on completion of the study.

- I also understand that I may request that my biological samples be destroyed prior to completion of the study.
- I understand that I will receive general feedback regarding my chronotype as established by the HÖ-MEQ and the outcome of the study after completion.
- I understand that I will not be remunerated for participating in this study.

I agree to participate in the study.

_____

**(Full name of participant)**

_____ _____

(Signature of participant) (Date)

_____

**(Full name of investigator)**

_____ _____

(Signature of investigator) (Date)

If witness required:

_____

**(Full name of witness)**

_____ _____

(Signature of witness) (Date)

# Appendix 2

**CIRCADIAN RHYTHMS AND SPORT:**

**THE *PERIOD3* VARIABLE NUMBER TANDEM REPEAT POLYMORPHISM, CHRONOTYPE AND MARATHON RACE TIME IN SOUTH AFRICAN AND DUTCH RUNNERS**

## A. PERSONAL DETAILS

First name: _____

Surname: _____

Postal address: _____

_____ Code: _____

Email address: _____

Phone number: _____ Cell phone: _____

Date of birth: _____ Gender: _____

Height (cm): _____ Weight (kg): _____

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): _____

Current country of residence: _____

Time resided in country of residence: _____ (months / years)

## B. MEDICATION AND SUPPLEMENT USE

1. Do you currently take any medication?

   ☐ **No**

   ☐ **Yes**

   If yes, please provide the following information:

| Name | Purpose (e.g.. High blood pressure) | Years taken |
|---|---|---|
| | | |
| | | |
| | | |

2. Do you currently take any dietary supplements / vitamins?

   ☐ **No**

   ☐ **Yes**

   If yes, please provide the following information:

| Name | Purpose (e.g. Muscle growth) | Years taken |
|---|---|---|
| | | |
| | | |
| | | |

## C. RUNNING TRAINING AND RACE HISTORY *(runners only)*

1. For how many years have you been training for running?
   _____ **Years**

2. For how many years have you been running marathons?
   _____ **Years**

3. How many marathons have you participated in in the last 12 months?
   _____

4. Will you be participating in the Weskus Marathon in Langebaan, South Africa on the 20th of April 2013?
   - ☐ **No**
   - ☐ **Yes, my race number is:** _____

5. How many days do you train per week? (on average in the last **three months**)
   _____**Days/wk**

6. How many hours do you train per week? (on average in the last **three months**)
   _____ **Hours/wk**

7. What is your weekly training distance? (on average in the last **three months**)
   _____ **Km/wk**

8. For each given day of the week, what time of the day do you usually train (on average) and are these your preferred / chosen times of the day to train (No/Yes)?

| | **Monday** | **Tuesday** | **Wednesday** | **Thursday** | **Friday** | **Saturday** | **Sunday** |
|---|---|---|---|---|---|---|---|
| **Session 1** | | | | | | | |
| Preferred? | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y |
| **Session 2** | | | | | | | |
| Preferred? | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y |

9. ***For Q8, if 'Not preferred' for any of the sessions:*** What time of the day would you choose to train if you were entirely free to plan your schedule?
   _____ am / pm

10. ***For Q8, if 'Not preferred' for any of the sessions:*** Why are you unable to train at your chosen time?
    _____

11. What is your **personal best time** for a whole marathon and a half marathon (if applicable) and in what year was that?

| | Hours | Minutes | Seconds | In (year) |
|---|---|---|---|---|
| **Marathon** | | | | |
| **Half Marathon (21km)** | | | | |

12. What is your most **recent/current time** for a whole marathon and a half marathon (if applicable) and in what year was that?

| | Hours | Minutes | Seconds | In (year) |
|---|---|---|---|---|
| **Marathon** | | | | |
| **Half Marathon (21km)** | | | | |

13. Do you currently train for any other sport?

☐ **No**

☐ **Yes**

If yes:

What sport/s?

_____

How many days per week do you train for the other sport/s?

_____ **Days/wk**

14. Do you currently or have you in the past participated competitively in any other sport?

☐ **No**

☐ **Yes**

If yes, in which sport(s) have you participated competitively, when and for how long?

_____

_____

## D. EXERCISE HISTORY *(non-runners only)*

1. For how many years have you been exercising (i.e. recreational activities for the purpose of keeping fit)?

_____ **Years**

2. How many days do you exercise per week? (on average in the last **three months**)

_____**Days/wk**

3. How many hours do you exercise per week? (on average in the last **three months**)

_____ **Hours/wk**

4. What is the focus of your training? (Tick all that apply)

☐ **Strength** ☐ **Flexibility**

☐ **Cardio** ☐ **Cross-training**

☐ **Other:** _____

15. For each given day of the week, what time of the day do you usually train (on average) and are these your preferred / chosen times of the day to train (No/Yes)?

| | **Monday** | **Tuesday** | **Wednesday** | **Thursday** | **Friday** | **Saturday** | **Sunday** |
|---|---|---|---|---|---|---|---|
| **Session 1** | | | | | | | |
| Preferred? | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y |
| **Session 2** | | | | | | | |
| Preferred? | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y | ☐N ☐ Y |

16. ***For Q15, if 'Not preferred' for any of the sessions:*** What time of the day would you choose to train if you were entirely free to plan your schedule?

    _____ am / pm

17. ***For Q15, if 'Not preferred' for any of the sessions:*** Why are you unable to train at your chosen time?

    _____

18. Do you currently or have you in the past participated competitively in any sport?

    ☐ **No** (Skip question 10)

    ☐ **Yes**

19. In which sport(s) have you participated competitively, when and for how long?

    _____

    _____

## E. HORNE-ÖSTBERG MORNING-EVENING PERSONALITY QUESTIONNAIRE

**INSTRUCTIONS**

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

**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?

_____ am / pm

**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?

_____ am / pm

**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?

- ☐ Not at all dependent
- ☐ Slightly dependent
- ☐ Fairly dependent
- ☐ Very dependent

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

- ☐ Not at all easy
- ☐ Slightly easy
- ☐ Fairly easy
- ☐ Very easy

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

- ☐ Not at all alert
- ☐ Slightly alert
- ☐ Fairly alert
- ☐ Very alert

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

- ☐ Not at all good
- ☐ Slightly good
- ☐ Fairly good
- ☐ Very good

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

- ☐ Very tired
- ☐ Slightly tired
- ☐ Fairly refreshed
- ☐ Very refreshed

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

- ☐ Seldom or never later
- ☐ Less than one hour later
- ☐ 1-2 hours later
- ☐ More than 2 hours later

**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?

- ☐ Would be on good form
- ☐ Would be on reasonable form
- ☐ Would find it difficult
- ☐ Would find it very difficult

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

_____ am / pm

**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?

- ☐ 8.00 am – 10.00 am
- ☐ 11.00 am – 1.00 pm
- ☐ 3.00 pm – 5.00 pm
- ☐ 7.00 pm – 9.00 pm

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

- ☐ Not at all tired
- ☐ A little tired
- ☐ Fairly tired
- ☐ Very tired

**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:

- ☐ Wake up at the usual time and not go back to sleep
- ☐ Wake up at the usual time and doze
- ☐ Wake up at the usual time and go back to sleep
- ☐ Wake up later than usual

**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?

- ☐ Would NOT go to bed until 6.00 am
- ☐ Nap before 4.00 am and sleep after 6.00 am
- ☐ Sleep before 4.00 am and nap after 6.00 am
- ☐ Only sleep before 4.00 am and remain awake after 6.00 am

**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:

- ☐ 8.00 am – 10.00 am
- ☐ 11.00 am – 1.00 pm
- ☐ 3.00 pm – 5.00 pm
- ☐ 7.00 pm – 9.00 pm

**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?

- ☐ Would be on good form
- ☐ Would be on reasonable form
- ☐ Would find it difficult
- ☐ Would find it very difficult

**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?

from _____ am / pm _____ to _____ am / pm

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

_____ am / pm

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

- ☐ Morning type
- ☐ More morning than evening
- ☐ More evening than morning
- ☐ Evening type