THE EFFECTS OF CHRONOTYPE ON MEASURES OF PHYSICAL PERFORMANCE AND STRESS REACTIVITY

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ABSTRACT

Research has indicated that an individual's chronotype can have significant effects on peak performance and stress reactivity across the day. However, there is limited research that investigates multiple measures of performance in conjunction with stress inducing tasks and their cortisol responses. Participants comprised of eighteen healthy individuals identified as early (n=10) and late chronotypes (n=8) from their responses to two chronometric questionnaires. The experimental trial consisted of three experimental sessions (08:00 hrs, 14:00 hrs and 20:00 hrs) each separated by a week's rest. Each session included two physical tasks (Maximal Isometric Grip Strength Manoeuvres and an Incremental Test to Volitional Exhaustion), one acute stress inducing task (Paced Auditory Serial Addition Task) and three salivary cortisol samples. Salivary cortisol samples following awakening were collected from each participant on the morning of the 14:00 hrs session. We found significant differences between chronotypes in both handgrip strength and VO_2 max. PASAT performance was significantly greater when both chronotypes performed at their optimal time of day. Salivary cortisol increased following both a PASAT test and following high intensity exercise, irrespective of chronotype. Finally, cortisol awakening response occurred significantly earlier and was more pronounced in early chronotypes. Our results provide evidence to support the notion that chronotype a significant impact on peak performance times between chronotypes, however the difference in response to stressful tasks is minimal. Cortisol awakening response may be a better indicator of peak performance than the individual's response to stress.

This thesis is dedicated to Dr George Balanos.

Thank you for your continued and unwavering support throughout my time at University.

I will always be grateful.

It is also dedicated to my family.

For all of your support and belief in me.

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BACKGROUND

All living beings are exposed to 24-hour light-dark and temperature cycles owing to the rotation of the earth around its own axis (Bollinger and Schibler, 2014) (Czeisler et al, 1999). When kept under constant conditions (constant darkness and temperature), organisms will display cycles of behaviour and physiology that will oscillate in a period slightly longer than 24-hours (Bollinger and Schibler, 2014). Rhythm periods of 20-28 hours are termed 'circadian' (Atkinson and Reilly, 1996). Kleitman (1963) was the first to uncover the circadian rhythm within humans when removing periodic environmental cues (zeitgebers) (Czeisler and Gooley, 2007). The research studied two subjects living within Kentucky's Mammoth Cave on a 'non-24-hour sleep/wake light/dark schedule' (Czeisler and Gooley, 2007). It was found that despite the introduction of a 28-hour activity and rest schedule, participants displayed a core temperature cycle that fluctuated around a near 24-hour clock. It was therefore concluded that not only can circadian rhythms oscillate within physiological processes (in this case core temperature) in the absence of periodic synchronising cues from the environment, these rhythms are also present when behavioural rhythms are disturbed (Czeisler and Gooley, 2007). It is clear that circadian rhythms are endogenously driven and persist under constant environmental conditions (Oster et al, 2016) (Czeisler et al, 1999). The research pioneered by Kleitman (1963) generated significant interest in the field and lead to the evolution of multiple studies. Czeisler et al (1999) studied 24 participants over a period of 29-38 days, using a forced desynchrony protocol, and implementing a 28-hour day length, outside of the range of entrainment for the human circadian pacemaker. The resultant data solidified the stability of the human circadian rhythm as an endogenous driver in multiple physiological processes.

Human circadian rhythms are observed at all levels of cellular organisation. For example, physiological parameters such as digestion, body temperature, immune response and

sleep-wake cycles will all undergo cyclical changes throughout the ≈24-hour period (Edery, 2000). It is therefore important to review how and where circadian oscillations originate (Fonken and Nelson, 2014).

The Suprachiasmatic Nucleus (SCN) is situated in the anterior hypothalamus, directly above the optic chiasm, and is referred to as the brains "Master Clock' (Hayes, Bickerstaff and Baker, 2010) (Gillette and Tischkau, 1999). The SCN is not the sole clock, but serves as the primary biological clock, coordinating numerous molecular clocks across the entire body (Hastings, Brancaccio and Maywood, 2014) (Saper, 2013) (Roenneberg et al, 2007). This synchronisation of the secondary oscillators in peripheral tissues is essential for coordinating cyclic physiology, maintaining internal synchronicity, and sustaining body rhythms (Gachon et al, 2004). The SCN is comprised of populations of cells which are able to generate circadian oscillations (Herzog, Takahashi and Block, 1998). Electrophysiological studies have revealed that these oscillations are generated at the level of the individual neurone (Yamaguchi et al, 2003). Studies have shown that when the SCN is isolated or its' neurones are cultured in-vitro, electrical activity followed a circadian rhythm (Dibner, Schibler and Albrecht, 2010). Earlier studies have also revealed an impairment in circadian function following damage to the hypothalamus (Gillette and Tischkau, 1999). Furthermore, it has been uncovered that metabolic activity in the SCN, expression of proteins and clock genes were all dependant on time (Dibner, Schibler and Albrecht, 2010). Such research concludes that the SCN is the key source of circadian rhythms at both molecular and physiological levels (Saper, 2013). The SCN is characterised simply as an autoregulatory transcription/translation feedback loop of clock genes (Yamaguchi et al, 2003) (Shearman et al, 2000). This assembly of cellular clocks in the SCN allows for intercommunication among the pacemaker neurones that are positioned in a topographic order (Yamaguchi et al, 2003). Na+ action potentials across the neurones are vital

for their communication and for maintaining the synchronised circadian oscillation within the cell (Yamaguchi et al, 2003) (Weaver, 1998).

At an organismal level, daily timing orchestrated by feedback loops works around the negative regulation of the Period (*Per*) and Cryptochrome (*Cry*) genes and their protein products (Hastings, Brancaccio and Maywood, 2014). Hastings, Brancaccio and Maywood (2014) suggest that the stability of these proteins is highly impactful on setting the correct circadian cycle. In the morning, CLOCK and BMAL 1 transcription factors activate the *Per* and *Cry* genes. Levels of *Per* and *Cry* accumulate in SCN neurones and their subsequent proteins are produced. These proteins interfere and inhibit the SCN neurones and reach a critical concentration in the cell nucleus (Gachon et al, 2004). This critical protein concentration within the nucleus interacts with the CLOCK-BMAL 1 and impedes the activation of these transcription factors (Gachon et al, 2004). As night progresses, the transcription of the *Per* and *Cry* genes declines and the proteins which are produced degrade, forming a clear molecular circadian day/night feedback loop (Hastings, Brancaccio and Maywood, 2014). From this, a new cycle of *Per* and *Cry* transcription can begin (Gachon et al, 2004). This process is present in all molecular clocks which are found in virtually all cells in the body (Schroder and Esser, 2013). This circadian timing system is only able to measure the day length approximately, therefore phase entrainment (the relationship between external and internal time) using photopic environmental information is key to maintaining synchronicity with the solar day (Roenneberg et al, 2007).

The photoperiod is the key synchroniser of circadian clocks, correcting a person's internal circadian timing system to stay in sync with external environmental time (Gachon et al, 2004). The SCN receives crucial photopic information through Intrinsically Photosensitive

Melanopsin-containing Retinal Ganglion Cells (ipRGCs), located in the retina. These rare type of ganglion cells are different from traditional rods and cones, in that light depolarises ipRGCs but hyperpolarises rods and cones (Berson, 2003). ipRGSs are not deemed 'classical' photoreceptors, as they are not required for visual function. However, photoexcitation of the melanopsin contained within the cells is extremely sensitive to light (Perez-Leon, 2006). Evidence for the importance of these photoreceptors in phase entrainment can be shown in the visually impaired population (Markwell, Feigl and Zele, 2010). Circadian rhythmicity is still apparent in those who have non-functioning visual receptors (rods and cones) through stimulation of ipRGCs (Klerman et al, 2002). However, when both eyes are removed, or in absence of photic information (constituting a distinct form of blindness) circadian rhythmicity is diminished and will result in complete desynchronization of the Master Clock (Czeisler et al, 1995) (Skene and Arendt, 2007). As ipRGSs give rise to 75-90% of projections to the SCN, their significance in entraining the circadian system is paramount (Markwell, Feigl and Zele, 2010). It is important to note that non-photic cues, such as exercise and time of feeding can entrain clocks at a muscular and peripheral level, however the SCN is highly adaptable to light (Shibata and Tahara, 2014).

Aside from an individual's physiological disposition, there is a consistent and prevailing preference for day-time or night-time activity that must be considered when looking at human circadian rhythms (Teo, Newton, and McGuigan, 2011). An early study by Kleitman (1939) highlighted that some individuals favour morning activity and other favour evening activity. Those who showed a preference for morning activity were labelled 'Larks' and those who preferred evening activity 'Owls' (Randler, Faßl and Kalb, 2017). More recently, chronotypes have been separated into early types (ECTs) and late types (LCTs), allowing margins for both intermediate and extreme types along the continuum (Roenneberg, Wirz-

Justice and Merrow, 2003) (Preckel et al, 2011). Differences between chronotypes are apparent in multiple physiological rhythms including core temperature (Lack et al, 2009), sleep-wake patterns (Taillard et al, 2003), cognitive performance (Beşoluk, Önder and Deveci, 2011) and food intake rhythms (Maukonen et al, 2017) (Teo, Newton, and McGuigan, 2011) (Randler, Faßl and Kalb, 2017). Chronotype is currently uncovered using self-reporting questionnaires such as the Morningness-Eveningness Questionnaire (Horne and Östberg, 1976) and the more recent Munich Chronotype Questionnaire (Roenneberg, Wirz-Justice and Merrow, 2003). It is therefore reasonable to hypothesise from this that individual chronotype differences may have an effect on physical performance and measures of stress reactivity.

REVIEW OF LITERATURE

Diurnal Rhythm and Physical Performance Measures

The effect of time of day on physical performance has been widely researched. As performance is a multifactorial parameter, the literature surrounding this topic is broad and incorporates a multitude of factors. Variations in athletic performance have exhibited distinctive peaks in performance times (Cappaert, 1999). It is evident that the type of exercise is extremely important when looking at performance rhythmicity and peak timings (Thun et al, 2015).

Muscular Strength and Power

There are multiple studies that have been undertaken in order to analyse the daily fluctuations in muscular strength and power in humans (Zhang, Dube and Esser, 2009). Bambaeichi et al (2005) designed a study to assess the interaction of time of day and muscle strength in females using maximal isometric and isokinetic knee strength contractions. Eight sedentary females were tested at 06:00 hrs and 18:00 hrs and undertook three attempts of each contraction mode using an isokinetic dynamometer. The results showed that there was a diurnal variation present in peak torque of the knee flexors at 18:00 hrs, 5.9% higher than at 06:00 hrs. There were no significant diurnal interactions seen within maximal contractility. Similarly, Giacomoni, Edwards and Bambaeichi (2005) found a significant diurnal rhythm in both male and female participants when performing peak isokinetic torques. Males showed an acrophase which peaked at 17:00 hrs, whereas females acrophased at 15:35 hrs. The only maximal strength condition that showed any significant diurnal curve was in the male demographic, whose maximal voluntary contraction (MVC) peaked at 16:17 hrs, however this

only existed when electrical twitches were imposed. Bernard et al's (1997) early research also showed that maximal jump power increased throughout the day. The tests took place at 09:00 hrs, 14:00 hrs and 18:00 hrs respectively. In order to determine maximum jump power, 23 males were made to stand on a contact platform that triggered a timer by the release of the participant's feet. The time that participants were in the air was recorded and from this, the jump power was calculated. Results showed that both jump power and flight time increased significantly from 09:00 hrs to 14:00 hrs, as well as from 09:00 hrs to 18:00 hrs. There was no significant increase from 14:00 hrs- 18:00 hrs, suggesting that the peak jump power occurred within these two time points. Vertical jump and standing jump can be employed as an index of explosive power, and are therefore simple yet important tests to adopt when looking at diurnal variations in human power. Reilly et al (2007) found that over four testing times in the day (08:00 hr, 12:00 hr, 16:00 hrs and 20:00 hrs) both vertical and broad jump values were at their highest and almost equal at 16:00 hrs and 20:00 hrs. This solidifies Bernard et al's (1997) early research; that there is a window of time in the afternoon from which peak values can be attained. As the majority of jumping records are broken by only a few centimetres, it should be taken into account that time of day is evidently a significant factor that may influence record breaking attempts (Atkinson and Reilly, 1996).

The rhythm in isometric handgrip strength tends to peak between 14:00 hrs and 19:00 hrs, which has been consistently identified in numerous studies (Atkinson and Reilly, 1996) (Gifford, 1987) (Ilmarinen et al, 1980). Souissi et al (2013) found that handgrip strength and MVC values of the elbow flexors are significantly higher in the afternoon. Souissi et al (2013) also commented that these diurnal variations disappeared within the participants when they were exposed to partial sleep deprivation, this however has been contested in other comparable research (Jasper et al, 2009). Jasper et al (2009) analysed the difference between handwriting speed, fluidity (fine skill) and handgrip strength (gross skill) in 10 healthy male volunteers.

Grip strength was at a minimum at 06:00 hrs and followed a clear diurnal rhythm throughout the day. Both grip strength and handwriting speed/fluidity displayed the lowest levels of performance at least three hours apart, highlighting the disparity between the two tasks.

The majority of maximal strength and power measures previously discussed tend to acrophase during early to late afternoon. Core body temperature also follows this circadian rhythm (Manfredini et al, 1998). It has been estimated that approximately 25% of observed changes in human circadian rhythms can be attributed to changes in core body temperature (Waterhouse et al, 2005) and it is imperative to understand how this affects human performance. Knowing that core body temperature is naturally at its highest in late afternoon (≈18:00 hr) (Manfredini et al, 1998), Racinais et al's (2005) study into external passive heating demonstrates the advantageous effects of increased core temperature on strength performance. After completing an MVC of the knee extensors, results showed that power was greater in the afternoon session compared to the morning. This is consistent with the core body temperature acrophase. It is important to note that this study incorporated passive heating of the leg muscle in both the morning and afternoon sessions. This external passive heating was highly effective in increasing the morning MVC performance and we can therefore assume that mimicking the effects of a higher core body temperature in the hours where it is naturally low (morning), can be exploited as a method of performance enhancement.

Following this study, Taylor et al (2011) demonstrated that implementing an extended warm-up in the morning can improve explosive neuromuscular performance at this time by up to 4-6%. From these experiments, it is clear that a relationship is present between the circadian rhythmicity of core body temperature and physical performance capabilities (Teo, Newton, and McGuigan, 2011).

It has been postulated that as body temperature increases, the body utilises carbohydrate over fat as a fuel source. This in turn can facilitate actin-myosin cross bridging mechanisms

within the skeletal muscle unit, which will exhibit performance enhancing advantages (Teo, Newton, and McGuigan, 2011). Therefore, there is evidence to suggest that a human's core temperature is phased such that the highest temperature and performance is achieved between 14:00 hrs and 20:00 hrs (Waterhouse et al, 2005). It has been found that scheduling training sessions in the evening can improve muscular strength performance by up to 20% more than those training in the morning (Manfredini et al, 1998). Therefore, understanding the muscular strength and core body temperature rhythms can provide beneficial information for performance enhancement.

Aerobic Performance

Aerobic fitness can be defined as the 'ability to deliver oxygen to the muscle and utilise it to generate energy during exercise' (Armstrong and Welsman, 2007). Armstrong and Welsman (2007) highlight that performance in this respect depends upon the components of oxygen delivery and the oxidative mechanisms of the working muscle. The cardiovascular, ventilator and metabolic parameters of aerobic performance all present different circadian rhythms. For example, metabolic and respiratory rhythms are minimised when exercise becomes stressful, however the core body temperature rhythm remains unaffected (Shiotani et al, 2009) (Atkinson and Reilly, 1996). It is reasonable to assume that there will also be a circadian rhythmicity present in aerobic performance measures (Atkinson and Reilly, 1996). Within the scientific community, there has been much debate as to whether aerobic performance presents a circadian rhythm. Some researchers state that the contribution of the aerobic system is increased in the evenings compared to the mornings (Fernandes et al, 2014), and others suggest that time of day has no effect (Rowland et al, 2011) (Racinais et al, 2005). In Atan et al's (2017) study, 38 male students performed an exhaustive 20-metre shuttle run across three time points (morning: 09:00 hrs, afternoon: 14:00 hrs and evening: 19:00 hrs),

each separated by two days. It was found that the mean number of shuttles measured in the afternoon was significantly higher than the amount recorded in the morning session. There were no significant differences from the afternoon to the evening session. These results correspond with the results from Hill's (1996) study, which consisted of an exhaustive high-intensity cycle ergometer test at 08:00 hrs and 16:00 hrs respectively. They found that the aerobic system responded 6% faster, the time to exhaustion was 9% greater and the peak VO_2 achieved was 7% higher in the 16:00 hrs session compared to the 08:00 hrs session. Similarly, Reilly and Baxter (1983) found that time to exhaustion in a progressive cycle ergometer test was 68% longer at 22:00 hrs than 06:00 hrs. Additionally, the total work done was 41% greater in the evening.

It is interesting to note that during aerobic exercise, a participant's perception to the intensity of the work has been seen to fluctuate due to the time of day. Hill, Cureton and Collins (1989) found that both VE and VO_2 during a maximal graded exercise test was higher within their participant cohort in the afternoon compared to the morning, consistent with previous literature. Moreover, during these tests, participants were instructed to rate their level of exertion according to a 15-point Borg scale. The scale is a continuum of 6 (very-very light) to 20 (very-very hard). Results showed that when the participants were working above their ventilatory threshold, the exercise was perceived as less strenuous in the morning than the afternoon. When the participants were working under their ventilatory threshold, their rating on the Borg scale was not affected by the time of day. This is contested by Chtourou et al (2013), who found that subjective fatigue ratings following a 5-minute judo combat session were not statistically significant between the morning and evening session. Therefore, the effect of time of day on RPE ratings is yet to be determined.

Despite the above findings, it is evident that there are contradictory findings regarding the existence of diurnal variation in VO_2 max and sub-maximal exercise (Hill, Cureton and

Collins, 1989). Rowland et al (2011) found no diurnal variability between a morning and afternoon session when undertaking a progressive cycle exercise test. No significant time of day differences were observed in pre-exercise, sub-maximal, or maximal values of oxygen uptake, or cardiovascular markers such as heart rate. The researchers debated that if diurnal rhythms were present in their population, the magnitude of such fluctuations were minimal and could not be measured accurately. This research is reinforced by Martin, Doggart and Whyte's (2001) findings, which note a lack of significant diurnal variations in their participant's 30-minute run variables between the morning and evening sessions. Thun et al (2015) suggest a variety of possible explanations for this large disparity within the research; small sample sizes, the subject's routines of training in the morning and a lowered morning core body temperature (Thun et al, 2015).

The above studies detailing the effects of time of day on aerobic performance are inconclusive and further investigation needs to be undertaken.

Anaerobic Performance

The effects of time-of-day on short-term-anaerobic performance is well documented, with the majority of research identifying a consistent peak in maximal anaerobic performance in the late afternoon (Racinais, Hue and Blonc, 2004) (Chtourou et al, 2011) (Chtourou et al, 2012). Similar to that of muscular power, it has been debated that this late afternoon peak may be linked to peak in core body temperature (Bernard et al, 1997). The exact mechanisms are unknown, however it has been proposed that for anaerobic performance the increase in muscular temperature could increase the speed of chemical processes in the muscle and may exert a beneficial passive warm-up effect (Souissi et al, 2010) (Racinais et al, 2005). When muscles are subjected to a range of temperatures (30.0ºC to 39.0 ºC in this case) and undertake maximal anaerobic power tasks, power declines by 5% for every 1ºC decrease in muscle

temperature (Souissi et al, 2010) (Bergh and Ekblom, 1979). Additionally, an increased muscle temperature can enhance metabolic reactions, increase the excitability of connective tissue and increase the velocity of action potentials (Souissi et al, 2004). This relationship, is not understood to be a causal link, but must be considered, as numerous studies take core temperature rhythms into account when understanding the basis of their anaerobic results. Racinais et al's (2005) study, using 9 male students, looked to uncover the effect that time of day had on maximal sprint power and repeated sprint ability. The researchers considered the repetitive sprint element of the methodology to be imperative, as it posed high levels of applicability to real-life sporting situation. They found that maximal sprint power was increased in the evening session (17:00 hrs-19:00 hrs) compared to the morning (07:00 hrs-09:00 hrs). There were no time of day effects on the repeated sprints, concluding that the beneficial effect of time of day may only be limited to a single expression of power before the participant becomes fatigued. Bernard et al's (1997) similar study used maximal cycling performance, multi-jump tests and dash runs to investigate maximal anaerobic rhythms at 09:00 hrs, 14:00 hrs and 18:00 hrs on separate days. Both cycling sprints and maximal jump power increased significantly from the morning to the afternoon session. The amplitude of the time of day effect seemed to be dependent on the type of exercise, with the jump condition presenting a larger increase in the afternoon than the cycle sprint. The researchers proposed that the requirement of different muscle groups for each of these tests, and their individual responses to increasing temperatures, could be accountable for the disparity between the measures.

The Wingate Anaerobic Test is a maximal intensity cycling ergometer test lasting 30-seconds (Beneke et al, 2002). It is a method frequently used within studies in order to test three indices of anaerobic performance: peak power output, mean power output and the fatigue index (Souissi et al, 2004) (Driss and Vandewalle, 2013). Melhim (1993) aimed to establish whether

diurnal rhythms were present in peak power output and mean power output using this test. Participants undertook the Wingate test at 03:00 hrs, 09:00 hrs, 15:00 hrs and 21:00 hrs respectively. They found significant diurnal rhythms in both of the anaerobic variables. The peak power output was found to be 7% higher at 15:00 hrs than at 03:00hrs. The researchers also observed that the mean power output was 16% greater at 15:00 hrs and 21:00 hrs than it was at 03:00 hrs. This finding was replicated in Souissi et al's (2010) study, who discovered similar time-of-day variations in their participant cohort. In order to embrace the entire solar day and pinpoint more specific acrophases in anaerobic performance, Souissi et al (2004) implemented a protocol that meant participants tested more frequently. They completed six Wingate testing sessions across six days. These testing sessions were conducted at the following times: 02:00 hrs, 06:00 hrs, 10:00 hrs, 14:00 hrs, 18:00 hrs, 22:00 hrs. A recovery period of at least 28 hours was allowed between each test. Results showed that peak power output reached an acrophase at 17:24 hrs and mean power output displayed a peak at 18:00 hrs, consistent with the participants mean oral temperature acrophase at 18:22 hrs. This research is consistent with that of Melhim's (1993), displaying similar diurnal profiles in aerobic performance when testing participants as frequent time points.

Despite the evidence that anaerobic exercise measures tend to peak in the late afternoon, there is research that contradicts this. When conducting the Wingate test on 12 male subjects at 6 time points throughout the day, Reilly and Down (1992) found that their results for peak power and mean power output did not display any significant diurnal rhythm profiles. The researchers concluded that tests such as broad jumps and stair runs were more sensitive to diurnal rhythmicity than the Wingate test (Reilly and Down, 1992). It is evident that additional research is required to uncover the basis for this juxtaposition in current literature.

The impact of motivational factors should be considered when deciphering the stability of the results of tests that require maximum effort from their participants (Souissi et al, 2004). Reilly

and Baxter (1983) found that participants displayed a better work tolerance in the evenings when working at 95% of their VO_2 max. This suggests that the significant acrophases uncovered in the late afternoon could be psychologically and motivationally lead, however the physiological basis for this theory is yet to be explored (Bernard et al, 1997). Many researchers who produce significant diurnal results have argued that due to the limited time span of anaerobic tests, motivation cannot be considered a confounding variable that will influence the results. (Souissi et al, 2004).

Sport Specific Rhythms

Understanding the impact that diurnal rhythmicity has in sport is key to providing information to coaches and athletes alike (Drust et al, 2005). Unlike the laboratory studies previously discussed, this applied research can provide important sport-specific information that can help with training schedules, motivational climate, event scheduling and the short-term or long-term success of an athlete or team (Drust et al, 2005).

When applied to swimming, an early study by Reilly and Baxter (1983) found that performance in both the 100m and 400m crawl peaked at an average of 22:00 hr, slightly later than the core body temperate acrophase of 19:00 hrs and 20:00 hrs. Similarly, Kline et al's (2007) participants displayed an average peak performance time at 23:00 hrs when completing a 200m swim test. Swimming performance was impaired the most from 02:00 hrs to 08:00 hrs, more so than any other time points in the day. Research by Rae, Stephenson and Roden (2015) corroborated these findings. When analysing technical swimming performance, Deschodt and Arsac (2004) uncovered a 16% increase from 08:00 hrs to 18:00 hrs in the forward movement of the hand, as well as a 6% increase in the maximal depth of the stroke. These improvements will produce a substantial enhancement in underwater patterns. The researcher suggested that

the improvements in swimming technique that occur across the day will result in an increased swimming velocity (Deschodt and Arsac, 2004).

With regards to football, Reilly et al (2007) aimed to isolate football-specific skills within the study of 8 male football players. Using a multitude of football drills, the researchers found that the tasks requiring a larger degree of motor control tended to peak earlier in the day, 16:00 hrs (juggling and chipping tasks), whereas those that involved gross skills such as dribbling speeds, tended to peak later, 20:00 hrs. These results, however, may not necessarily relate to competitive contexts, as these skills will be performed at irregular times and intervals. Therefore, this research cannot be applied to competitive football. Rahnama et al (2009) also highlighted multiple football skills that followed a diurnal rhythm profile. Dribbling speed, number of wall volleys, number of chips and the Yeagley Soccer Test all produced significantly improved performance in the afternoon sessions compared to the morning. There were no time of day effects on penalty kick performance.

In relation to racket sports, (Atkinson and Speirs, 1998) measured the serve velocity and accuracy on first and second tennis serves at three time points across the day (09:00 hrs, 14:00 hrs and 18:00 hrs). The 6 competitive tennis players that participated in the study showed that their first serves were impacted by the time of day. The velocity of the first serve is significantly higher at 18:00 hrs than any other time point, however the placement and accuracy of the serve is compromised as it is significantly lower at this time point. It is therefore difficult to ascertain which component of this study is deemed more beneficial to overall performance. If the velocity is increased, it will be harder for the receiver to return the ball. Conversely, as the accuracy is shown to be minimised in the afternoon, this may lead to errors in performance. Time of day produced no significant effects on the second serve velocity or accuracy. López-Samanes et al's (2017) similar study found that tennis serve velocity was 4.2% higher during the session at 16:30 hrs compared to the session at 09:00 hrs. Edwards, Lindsay and

Waterhouse's (2005) study into the effect of time of day on badminton serves showed no diurnal rhythmicity in the consistency of serves. They did however find that the participants performed more shots with greater accuracy in a 1 m^2 grid at 14:00 hrs than 08:00 hrs and 20:00 hrs. Factors such as the build-up of fatigue (Van Dongen and Dinges, 2000), manual dexterity acrophases (Carrier and Monk, 2000) and optimal levels of arousal (Folkard, 1990) are all consider possible reasons for varying results within similar tasks.

Despite the prevalence of diurnal rhythm research, a recognisable flaw with the studies discussed is that they have failed to measure or account for chronotype, however tend to group participants without taking individual differences into consideration. Taking into account time of day and chronotype would provide a more comprehensive understanding of an individual's peak performance.

Chronotype and Physical Performance Measures

Importance of chronotype research for athletes

More recently, separating participants into individual chronotypes and observing their performance outcomes has been explored. The background of morningness-eveningness and the expression of an individual's circadian rhythmicity is complex, however research has alluded to the fact that individual performance differences are consistent with their chronotype (Vitale and Weydahl, 2017). Understanding this individuality is imperative for coaches, athletes and support staff alike, as at the highest levels of competition, the margins for athletic success are exceedingly narrow (Facer-Childs, Boiling and Balanos, 2018). Therefore providing performance research that can allow an athlete to maximise performance through his or her training regime is essential (Roden, Rudner and Rae, 2017).

Rosa et al (2016) reviewed the impact that event scheduling could have on athletic achievement in the Rio 2016 Olympic Games. The researchers concluded that with the preliminary and final competitions scheduled at midnight, the performance of athletes and their teams may be negatively affected, compromising decision making abilities, attention span, as well as multiple psychological and physiological factors (Rosa et al, 2016). When separating athletes into chronotypes, similar obstacles were faced by the Brazilian Paralympic athletes prior to the Beijing 2008 Paralympic Games (Silva et al, 2012). Following chronotype determination in 27 of the Paralympic athletes, the authors noted that 71.4% were classified as ECTs. The predominance of morningness within the team was hypothesised to be problematic with arranging training schedules, as subject to event scheduling, the athletes were unable to train at this time. Furthermore, as the team had to travel across several time-zones to reach the host city, the effects of jet lag, adaptation and sleep efficiency also had to be taken into account (Rodrigues et al, 2015). Positively for the Brazilian Paralympic team, that consisted predominantly of ECTs, evidence shows that ECTs have less difficulty in phase advancing to new time-zones than their LCT counterparts (Waterhouse et al, 2002).

The prevalence of morningness is consistent across multiple athletic populations, with a National School Sport Athlete Survey indicating that over 85% of athletes are mid-range to morning types (Samuels, 2008). Similarly, the Canadian Bobsleigh Skeleton team displayed an overriding preference for morning activity, with more than 85% of their athletes between the mid-range to extreme ECT category, and only 14% showing moderate LCT tendencies (Samuels, 2008). This trend was only evident in high performing athletes. It was suggested that this may be due to the fact that higher performers spend more time training, therefore influencing their habitual activity phases, thus conditioning them to specific time of day training (Roden, Rudner and Rae, 2017) (Samuels, 2008). This phenomenon is clearly displayed in Rossi, Zani and Mecacci's (1983) research, as top-level athletes who practiced

sports in the morning (golf and shooting) tended to exhibit morningness, whereas those who practiced sports in the evening (water polo) tended to display eveningness. Contrastingly, those who were less proficient displayed no clear preferences for morning or evening activity (Rossi, Zani and Mecacci, 1983) (Brown, Neft and LaJambe, 2008). Taking an athletes chronotype into consideration when implementing strategies to maximise performance is clearly advantageous in creating the best environment for an athlete to thrive (Lastella et al, 2016) (Rosa et al, 2016).

It has recently been postulated that elite athletes tend to gravitate towards sports that suit their chronotype, with 72% of ECTs in an elite multi-sport cohort, choosing to be involved in 'morning sports' (Lastella et al, 2016). It is beneficial for a coach to consider an athlete's chronotype during the selection process, as an athlete is more likely to remain within the discipline and excel a sport that suits their chronotype (Lastella et al, 2016). It is clear that continued chronotype research is key to the success of high-performance athletes.

Muscular Strength and Power

The effect that chronotype has on muscular strength and power is currently non-conclusive, and the literature surrounding the topic is limited. Of the few studies which incorporate chronotype into the diurnal patterns of muscle strength and power, the results are contradictory. Brown, Neft and LaJambe (2008) measured chronotype differences between the early morning (05:00 hrs-07:00 hrs) and late afternoon (16:30 hrs-18:00 hrs) in rowing and standing broad jump performance. Although the researchers found a significant increase in ECT ergometer rowing performance in the morning, no such difference was found in standing broad jump performance. Although the results would allude to a chronotype effect on standing broad jump performance, with ECTs averaging a greater distance in the morning and LCTs averaging a greater distance in the evening, none of these results were significant. Conflicting

evidence would argue that this finding is not consistent across all measures of power and strength, therefore displaying disparity between studies. Tamm et al (2009) investigated the difference in strength performance between chronotypes using an MVC protocol. When looking at the torque produced during plantar-flexion MVCs, they found that only the LCT participants displayed a significant diurnal rhythm in force production. LCTs generated 13% more torque at 21:00 hrs and 8% more torque at 17:00 hrs than at 09:00 hrs. There were no differences found within ECTs or ICTs. A recently study by Facer-Childs, Boiling and Balanos (2018) aimed to provide a more holistic approach to the impact of chronotype on performance by encompassing multiple physical measures in their methodology. One of the tests that the researchers used was isometric grip strength test to measure MVC, a simple measure of muscular strength. ECTs performed their best MVC at 14:00 hrs, and LCTs performed best at 20:00 hrs, consistent with their chronotype. In the morning session at 08:00 hrs, ECTs MVC performance was 7.4% better than LCTs. As 20:00 hrs, LCTs performed 3.7% better than ECTs. This is consistent with the popular finding that a person's chronotype will be indicative of their athletic performance capabilities (Vitale and Weydahl, 2017). It is clear that research is limited in this area, with the majority of the research into time of day and muscular strength/power failing to consider or control for the effects of chronotype.

Aerobic and Anaerobic Performance

With the time of day effect in aerobic and anaerobic performance displaying varied results across multiple studies (as highlighted previously), it is interesting to consider whether the effect chronotype may have on performance will display a more stable outcome.

With regards to aerobic measures, Sugawara et al (2001) separated 6 ECTs and 8 LCTs based on their responses to the Morningness-Eveningness Questionnaire. All the participants VO_2 max values were obtained using a VO_2 max test in order to set the workload for the subsequent

experimental trial. Participants performed a constant load cycle ergometer test at 07:00 hrs-08:00 hrs and 17:00 hrs-18:00 hrs. Various measures were taken throughout this test including heart rate and VO_2 values. The resultant data showed that there was neither a significant time of day effect nor a chronotype effect in resting heart rate, exercising heart rate or exercise VO_2 across all of the participants. This data therefore suggests that chronotype has no influence on aerobic performance. Similar findings were displayed in Kunorozva, Roden and Rae's (2014) study, whereby 20 trained male cyclists, all categorised as ECTs, completed a 17-minute submaximal cycling test at 60%, 80% and 90% of their maximum heart rate. Participants repeated these tests at 5 time points, spanning the solar day. The researchers found no significant differences between the time of day, chronotype, power output, speed and cadence results. However, ECTs did report higher ratings of perceived exertion in the evening sessions (18:00 hrs and 22:00 hrs), across all intensity levels, a finding consistent with Rae, Stephenson and Roden's (2015) work. It is important to note that this study did not incorporate a LCT or control group, therefore we are unable to ascertain whether chronotype is responsible for the significant and non-significant results (Kunorozva, Roden and Rae, 2014).

The relationship between anaerobic performance and chronotype has been explored in Hill et al's (1988) study. Within a cohort of 24 participants, 14 were classified as ECTs and 11 as LCTs. The participants undertook a maximal cycling ergometer test at two time points in the day, 06:00 hrs-08:30 hrs and 15:30 hrs-18:00hrs. The results showed that those who were identified as LCTs had a higher VO_2 max in the evening session, which was not associated with a longer performance time. Both chronotypes displayed a HR_{max} that was consistent with their chronotype, however these results were not significant.

A study published by Facer-Childs and Brandstaetter (2015) produced unique findings to support the hypothesis that chronotype has an effect on aerobic performance differences over 24 hours. An RU-BU chronometric questionnaire was used to identify early, intermediate and

late ‘circadian phenotypes’ (chronotypes). Following this they used a BLEEP test, a progressive aerobic endurance test, to evaluate performance across 6 time points in the day. The researchers uncovered that irrespective of circadian phenotype, all athletes tended to perform better in the evening, consistent with multiple circadian studies (Fernandes et al, 2014) (Atan et al, 2017) (Hill, 1996). When taking chronotype into account, they found that ECTs performance peaked, on average, around midday. ICTs performance peaked in the early afternoon, and LCTs peaked in the evening. The authors suggested that the most important predictor of peak performance time was the ‘time since entrained awakening’, or the length of time since the participants woke up. The researcher’s follow-up study in team sports found that the distribution of circadian phenotypes within a team is highly predictive of team performance, with LCTs displaying the strongest influence on peak team performance throughout the day (Facer-Childs and Brandstaetter, 2015). Teams with a large proportion of ECTs will be at a disadvantage in the evenings, and those with a large proportion of LCTs will be disadvantaged in the mornings ((Facer-Childs and Brandstaetter, 2015). The results from these two studies have revealed that evaluation of a person’s chronotype can have major performance advantages, not just individually, but also in a team setting. It is evident that there is conflicting research regarding the importance of chronotype in relation to athletic performance, and further exploration is required to uncover if the 26% variation in performance found in Facer-Childs and Brandstaetter’s (2015) study can be replicated in other sporting situations.

Circadian Rhythm and Stress Reactivity

Cortisol Circadian Rhythm and Awakening Response

Cortisol is a major contributing steroid hormone in the human stress system, and is derived as an end product from the hypothalamic-pituitary-adrenal (HPA) axis (Simons et al,

2015). The adrenal hormone displays a baseline circadian pattern of secretion, and additionally exhibits displays of secretory behaviour in response to stress (Bailey and Heitkemper, 2001). There is evidence to suggest that the circadian rhythmicity of this cortisol secretion is closely related to patterns of sleep and wakefulness (Edwards et al, 2001). The circadian rhythm of cortisol secretion is driven by the SCN, the brain's master clock (Edwards et al, 2001). Individuals with functioning a HPA axis will have extremely low levels of cortisol concentration that will build through the night and peak in the mornings (Chan and Debono, 2010). Thereafter, levels of cortisol will decline slowly through the day (Edwards et al, 2001). The rhythmic increase in cortisol within the first 20-30 minutes of awakening is a phenomenon entitled the 'Cortisol Awakening Response'(CAR) (Fries, Dettenborn and Kirschbaum, 2009). The CAR has been comprehensively studied, using salivary sampling techniques, a robust measurement of free cortisol levels (Wust et al, 2000). Within 30 minutes of awakening mean cortisol levels will increase by 50-100% to achieve the morning acrophase and will remain elevated for the following hour (Edwards et al, 2001) (Edwards et al, 2001). This CAR is a robust physiological pattern, seemingly unaffected by various factors including age, weight, duration of sleep and time since awakening (Edwards et al, 2001). The stability of the CAR however, does seem to be reduced by chronic stress and burnout (Schmidt-Reinwald et al, 1999). It is debated whether the CAR will impact the circadian rhythmicity of cortisol secretion throughout the remainder of the day (Schmidt-Reinwald et al, 1999), as some research has found that early risers secrete more cortisol in the first 45 minutes of awakening, and thereafter throughout the day (Edwards et al, 2001). It is imperative to consider the associations between cortisol circadian rhythm and cortisol stress response, as this may indicate a person's ability to cope with stressors (Simons, Cillessen and de Weerth, 2017). Cortisol not only plays a role in psychological stress responses, but can also help to allow multiple physiological systems to

function effectively (mobilising energy resources, inducing vasoconstriction, increasing heart rate) (Dickerson and Kemeny, 2004).

Time of day and Cortisol Response to Stressors

It is accepted that there are various acute stressors that can significantly increase circulating levels of cortisol in saliva (Bonato et al, 2017). These include cognitive tasks, public speaking and noise exposure (Dickerson and Kemeny, 2004). When looking at the influence of time of day on this cortisol inducing stress response, Lovallo, Farag and Vincent (2010) have found that the salivary cortisol response to a speech task, memory task and mental arithmetic task is more pronounced in the morning (09:00 hrs) than in the afternoon (13:00 hrs) when comparing them to rest days. When negating the rest day, the difference between the morning and afternoon responses is minimal. In a study involving short-distance bus drivers, the researchers aimed to evaluate whether there was a difference in the cortisol stress response throughout the day when comparing and morning and afternoon shifts (Diez et al, 2011). It was found that in both morning and afternoon shifts, the prevalence of psychological stress was high in all workers. However, the salivary cortisol levels were more pronounced in the afternoon workers. New research published by Yamanaka, Motoshima and Uchida (2019) has found that when comparing two randomly assigned participant groups (irrespective of chronotype), salivary cortisol responses to the Trier Social Stress Test (TSST) were more prominent in the morning group, with significant increases immediately after and 10-minutes following the test. No significant increases were found in the afternoon group, and their cortisol response as a group was significantly lower than that of the participants in morning. Literature surrounding time of day cortisol responses to stress tasks is very limited, and needs further exploration to understand at which time of day, if any, salivary cortisol responses are increased across the population.

It is understood that circulating cortisol is increased by high intensity exercise that acts as a stressor (Del Corral et al, 2016) (Hill et al, 2008). Jacks et al (2002) has indicated that salivary cortisol concentration is linearly related to exercise intensity, and therefore most research regarding exercise and cortisol will focus on intensities of 60%> of maximal oxygen consumption. This is the minimum level that is seen to provoke a substantial cortisol response (Tremblay, Copeland and Van Helder, 2005). Del Corral et al (2016) studied whether the time of day had an effect on the magnitude of the cortisol response to maximal exercise. Their study found that salivary cortisol was consistently higher in the morning than it was in the evening, at baseline, 5-minutes post graded exercise test and 20-minutes post exercise test. In the morning session there was an increase of 86% in salivary cortisol between the baseline measure and 20-minutes post exercise. These findings suggest that there is a pronounced cortisol reaction to exercise earlier in the morning than there is in the evening. This research is supported by Kanaley et al's (2001) work on 10 males serum cortisol responses to a submaximal graded treadmill test. They repeated the test on three separate occasions: 07:00 hrs, 19:00 hrs and 24:00 hrs. These times were chosen specifically to encompass the diurnal rhythm of cortisol secretion. Peak cortisol concentrations occurred at 07:00 hrs, followed by 24:00 hrs and 19:00 hrs. The researchers concluded that the diurnal rhythm in cortisol secretion will, as a consequence, influence cortisol's response to exercise.

The effects of diurnal rhythmicity in the cortisol response seem to differ when applied to sport-specific situations. Dimitriou, Sharp and Doherty (2002) conducted a study, in which 14 competitive male swimmers were required to produce a baseline saliva sample, complete a 5x400m swimming test, and produce one final saliva sample in order to measure their cortisol levels. The swimmers replicated this protocol at 06:00 hrs and at 18:00 hrs. The results showed that exercise induced a significant increase in salivary cortisol at 18:00 hrs (76.1%), however there was no clear cortisol rhythm profile evident throughout the day. This studies practical

methodology, focusing on actual sporting endeavours was unique in its design. It is clear that there is a difference between the results in the laboratory-based exercise studies and this more practical research. Further exploration of cortisol's response to exercise as a stressor in applied sporting situations is therefore required.

Chronotype and Stress Reactivity

Chronotype and Cortisol Awakening Response

Numerous studies have uncovered a link between a person's chronotype and its effect on the time at which their cortisol awakening profile acrophases (Bailey and Heitkemper, 1991). Bailey and Heitkemper's (1991) early study was the first to discover a significant difference between ECTs and LCTs cortisol awakening response. They reported that LCTs demonstrated a delay in their morning peak of salivary cortisol in comparison to their ECT counterparts. Only 60% of the LCTs reached a peak within the 55-minute sampling period compared to 80% of ECTs. The amplitude of the morning acrophase was also higher in ETCs than LCTs. The researchers attributed this later acrophase in LCTs to the lower levels of arousal that they experience in the morning, regardless of whether they had reported themselves as being 'well rested'. Similarly, Axelsson et al (2003) found that in 42 shift workers, those who displayed a 'morning personality type' produced higher serum cortisol levels in a blood sample taken in the morning (07:00 hr-09:00 hr) compared to those who displayed 'evening personality types'. These results were further supported in Kudielka et al's (2006) cohort of 38 male ECTs and LCTs. Population and intervening factors such as sex, age, day of the week and individual sleep duration were consistently controlled. They observed a significantly higher level of salivary cortisol in ETCs in the first hour after awakening.

The overriding view of the current literature is that that chronotype is closely associated with cortisol profiles after awakening, with ECTs displaying peaks that are more pronounced and occur earlier than their LCT counterparts.

Chronotype and Cortisol Response to Stress Tests

Research has shown that stress tasks can induce cortisol responses in humans (Buske-Kirschbaum et al, 1997) (Schmidt-Reinwald et al, 1999) (Kirschbaum, Pirke and Hellhammer, 1993). It can therefore be postulated that different chronotypes may vary in their cortisol responses to psychological stress. Marvel-Coen, Nickels and Maestripieri (2018) was the first study to investigate cortisol response to an acute stress task whilst separating chronotypes. They used the TSST as a method to induce mild psychological stress in their participants. Following the TSST, a saliva sample was immediately taken and subsequently assayed to expose levels of free cortisol. Whilst levels of salivary cortisol in both ECTs and LCTs were significantly increased following the TSST, the cortisol response in ECTs was significantly higher than their LCT counterparts. However, there is minimal research in this area and it is inconsistent.

An alternative study by de Punder, Heim and Entringer (2019) recently adopted a similar protocol, recruiting 28 participants, comprising of 5 ECTs, 19 ICTs and 4 LCTs. The aim of the study was to investigate whether there was a relationship present between chronotype and the cortisol response to stress reactivity. The findings showed that following exposure to the TSST, those who were identified as LCTs demonstrated the highest salivary cortisol response to the stress test. The researchers suggest that this finding could be attributed to the higher levels of psychological stress that LCTs reported on the PSS scale than either ECTs and ICTs (Kantermann et al, 2012). Such personality differences between chronotypes (i.e stress perception, extraversion, introversion, competitiveness) may play a key role in

predicting their physiological responses to stressors (Marvel-Coen, Nickels and Maestripieri, 2018) (Pruessner et al, 1997). However, this research fails to provide evidence of peak timings for these responses, and reveals the opportunity exploration.

Chronotype and Cortisol Response to Exercise Stress

Currently only one new publication exists that evaluates the chronotype differences between salivary cortisol levels in response to high intensity exercise. Bonato et al (2017) suggested that generally, individuals are required to adapt to working schedules where they must either exercise early in the morning or late in the afternoon, which often may not be aligned with their chronotype. They aimed to evaluate the influence of chronotype on cortisol measures when exercising at 08:00 hrs and 20:00 hrs. All participants completed a Yo-Yo intermittent recovery test in order to calculate the working percentages that must be achieved in the high-intensity interval exercise (HIIE) that followed. The HIIE was comprised of 4 bouts of 4-minute exercise at 90-95% of $HR_{peak,}$ and 3 minutes of active recovery at 50-60% HR_{peak}. Analysis showed that across the entire participant cohort and regardless of chronotype, salivary cortisol was significantly higher at 08:00 hrs than 20:00 hrs. When factoring in chronotype, LCTs displayed significantly higher cortisol responses to HIIE than ECTs in the morning session (08:00 hrs). LCTs continued to produce higher levels of salivary cortisol than ECTs following the cessation of exercise at 08:00 hrs. The LCTs also displayed levels of cortisol that were higher than pre-exercise values at 08:00 hrs, 60 minutes following the termination of the HIIE test. This indicates that LCTs could possess slower recovery kinetics when performing high intensity exercise outside of their 'evening preference'. As this is the first publication to present such findings, continued research is required to offer viable comparisons and determine its validity.

Aims and Hypothesis

The following investigation aims to answer the following research questions:

1. Is an individual's chronotype significantly indicative of their peak performance over three time points during the day?
2. Is an individual's cortisol awakening (CAR) and salivary cortisol response to multiple stressors across three time points during the day influenced by their chronotype?
3. Can an individual's chronotype influence the relationship between stress reactivity and performance?

We hypothesise that an individual's chronotype will be significantly indicative of their peak performance time over three time points across all measures of performance. It is expected that the timing of an individual's CAR will be closely related to their chronotype, with those identified as ECTs experiencing an earlier and more pronounced peak in salivary cortisol, and those identified as LCTs displaying a later and smaller peak. We also hypothesise that the intensity of an individual's salivary cortisol response to both a stress test and exercise tests will be affected by the chronotype that they identify with. Finally, we hypothesise that chronotype will have a significant effect on the relationship between the individual's stress reactivity and performance across three time points during the day.

METHODS

Participants

18 (11 male, 7 female) healthy volunteers participated in the study (Table 1). All participants were categorised as either ECTs or LCTs through self-reported questionnaires (Horne and Östberg, 1976) (Roenneberg, Wirz-Justice and Merrow, 2003). Experimental procedures were approved by the University of Birmingham School Ethics/Health and Safety Committee and all participants provided written consent prior to participation.

Participants were excluded from this study if they were identified as a smoker, suffered with depression, possessed any prior or existing sleep related or neurological disorders (i.e. insomnia), were taking any medications that may affect their sleep (i.e. melatonin) or suffered with any cardiovascular or respiratory disease. Participants that did not identify with any of the exclusion criteria, were aged between 18-40, and were identified as ETCs or LCTs, were eligible to participate.

Table 1. Participant characteristics.

	All (N=18)	ECTs (N=10)	LCTs (N=8)
Age (y)	24±4	24±4	24±4
Height (cm)	169.8±7.6	169±8.7	171±5.8
Mass (kg)	64.7±6.6	64.8±7.3	64.1±6.3
MSF_{sc} (hh:mm)	04:50±02:25	02:47±00:38	07:25±00:19
Wake-up time free days (hh:mm)	08:50±02:13	06:58±00:43	11:09±00:33

Table 1. (Continued).

	All (N=18)	ECTs (N=10)	LCTs (N=8)
Sleep duration (hh:mm)	07:24±00:47	07:49±00:43*	06:54±00:33*
MEQ Score	48.7±15.7	60.9±9.1	33.5±3.5

Values are mean ± standard deviation (SD). * denotes statistical significant between chronotypes (p=.008). Abbreviations: MSF_{sc}= corrected mid-sleep time for free days, MEQ Score= Morningness-Eveningness Questionnaire score.

Study Design

The present study adopted a single-blinded, randomised, cross-over design across 3 experimental trials. Prior to the first laboratory visit, all potential participants completed the Morningness-Eveningness Questionnaire (MEQ) (Horne and Östberg, 1976) and the Munich Chronotype Questionnaire (MCTQ) (Roenneberg, Wirz-Justice and Merrow, 2003) in order to identify both ECTs and LCTs within the cohort. Those individuals identified as either ECTs (MSF_{sc} less than 03:30 h) or LCTs (MSF_{sc} greater than 07:00 h) were invited to participate in the study.

Before testing commenced, participants completed a general health questionnaire, DASS21 questionnaire, Epworth Sleepiness Scale questionnaire and Pittsburgh Sleep Quality Index to ensure all inclusion criteria were met. All experimental procedures were thoroughly explained and written informed consent was obtained from every participant before testing began.

The trials consisted of 2 physical tests, as well as saliva sampling at 3 intervals. The timing order of the 3 experimental trials (08:00 hrs, 14:00 hrs and 20:00 hrs) were randomised,

and all participants were blinded to their chronotype. Each participant independently collected 4 awakening saliva samples on the morning of the 14:00 hrs trial, and transported them to the testing session.

Preliminary Testing

357 potential participants were asked to complete two chronometric questionnaires in order to identify their chronotype. The two questionnaires were either sent to participants via email or given a hard copy. Initially participants completed the MEQ, which resulted in a score ranging from 16-86. The scores on the MEQ related to the participant's levels of morningness or eveningness. Secondly, the participants completed the MCTQ. ECTs and LCTs were identified from this using mid-sleep time calculations (MSF_{sc}), and comparing the MSF_{sc} to a matched population in a British chronotype database. Although ICT individuals are the most commonly identified within the general population (Martynhak et al, 2010), in order to be clear about the influence of chronotype and minimise the effects confounding factors, it was important that we separated the chronotypes as much as possible. Therefore, in order to answer our research question, we isolated both ECTs and LCTs and excluded ICTs. From the 357 individuals that completed the questionnaires, 55 were eligible for participation (34 ECTs and 21 LCTs). 18 individuals agreed to participate in the study (10 ECTs and 8 LCTs).

Experimental Trial

The experimental trial consisted of three identical sessions across three weeks. These three sessions took place at 08:00 hrs, 14:00 hrs and 20:00 hrs respectively. Participants completed all three sessions in a randomised order. The 3 experimental sessions had precisely

a week's rest period between each. Participants were asked not to consume alcohol within the 24-hour period before the testing day. They were also advised not to consume any caffeinated drinks on the day of testing. No sleep restrictions were imposed on the participants as it was important for them to maintain normal sleeping and dietary habits in between the experimental sessions. Participants arrived at the School of Sport, Exercise and Rehabilitation Sciences (University of Birmingham, Y18) approximately 10 minutes prior to the start of the trial. On the first laboratory visit, the height (Model 220, Seca, GER) and body mass (Champ II Scale, OHAUS) of each participant were recorded. Participants were then required to read a cortisol sampling protocol sheet and produce an initial baseline saliva sample into a sterile 15 mL centrifuge tube (Greiner Bio, CELLSTAR). This stimulated sample was at least 2 mL in volume. The sampling protocol sheet ensured that participants were aware of the correct technique that they had to implement when providing all subsequent saliva samples across the experimental trial (Adam and Kumari, 2009). All samples were immediately placed on ice until the end of the collection (Reid et al, 1992).

Following collection of the saliva sample, a blood pressure cuff (Omron Basic M2 Blood Pressure Measuring Device) was applied to the participants left arm, above the elbow in order to assess heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) (Mathias, Stanford and Houston, 2004). A baseline measurement was recorded. Participants then completed the Paced Auditory Serial Addition Test (PASAT) (6-minute version). Instructions, alongside a 30-second practice were given before every PASAT attempt. Blood pressure and heart rate measurements were taken at 2, 4 and 6 minutes after the PASAT commenced. The PASAT was scored out of 183 responses.

Following this, participants rested passively for 10 minutes, and were instructed that they were not allowed access to phones or laptops. Following this 10-minute rest, a further 2 mL saliva sample was produced. Participants were then asked to complete 3 Maximal Isometric Grip Strength manoeuvres with 2-minute rest periods and the highest of these 3 results was recorded.

Finally, participants completed an incremental test to volitional exhaustion on a cycle ergometer (Excalibur Sport, 925909 Ergometer Exercise Bike). Following a 5-minute warm-up at 60 W, participants cycled at 90 W, increasing by 30 W every 2 minutes. Heart rate and RPE values were obtained every 2 minutes. The test was completed when participants were unable to maintain a rate higher than 50 rpm or voluntarily stopped. VO_2max values were calculated from the analyser output (JAEGER® Vyntus CPX, CareFusion, GER). When the test concluded, a final saliva sample was taken. Participants were able to leave the laboratory following the final sample whenever they felt fit to do so.

The experimental methodology was consistent across all 3 trials. Throughout the 3-week testing period, participants were required to follow their normal living habits, however they were required to abstain from naps. All sleeping habits were documented in a self-reported daily sleep diary across a 2-week period, including 2 weekends.

Measurements and Techniques

Questionnaires. The MEQ is comprised of 19 questions that are used to determine the time at which the respondent's 'maximum propensity for activity lies' within the day (Zavada et al, 2005). The questionnaire uses 4-choice questions that will correspond with a definite morning-type, moderate morning-type, moderate evening-type and definite-evening type, depending on a score ranging from 16-86 (Taillard et al, 2004). Lower scores depict a later

chronotype and larger scores, an earlier chronotype. The questions are largely subjective, evaluating wake up times, preferred times for activity, as well as subjective alertness (Levandovski et al, 2013). The MEQ is a highly regarded questionnaire within the scientific community when considering its psychometric properties (Jankowski, 2015). According to multiple studies, the MEQ presents high validity and test- retest reliability and is therefore used as a primary tool in most chronophysiological research (Tonetti, 2007) (Osonoi et al, 2014). Participants also completed the MCTQ in conjunction with the MEQ. The MCTQ provides measurements based on sleep behaviours rather than sleep preferences, assessing a person's sleep times on both working days and free days (Levandovski et al, 2013). As a relatively new measure, there are few studies that have assessed the psychometric properties of the MCTQ (Di Milia et al, 2013). However, it has been suggested that as the MCTQ is the only available chronometric questionnaire to measure the cost of adaptation to social rhythms, and has been validated against 'more than 600 sleep diaries, actigraphy and hormonal markers' (Di Milia et al, 2013). It is highly regarded in the literature as a stable measure of chronotype (Di Milia et al, 2013). All of the questions on the MCTQ were relative to the present circumstances of the respondent. From this questionnaire, a mid- sleep time (MSF_{sc}) was calculated. To identify the individuals chronotype from the MCTQ, data from a gender and age matched population sample was analysed. From this matched population, the boundaries for ECTs was set at an MSF_{sc} less than 03:30 h. LCTs were restricted to those with an MSF_{sc} greater than 07:00 h. Kantermann, Sung and Burgess (2015) suggest that the MSF_{sc} is a better indicator of endogenous circadian timing than the scores accumulated from the MEQ, therefore the MEQ scores in this study were used only to solidify the chronotype outcome from the MCTQ (Zavada et al, 2005). If the MCTQ and the MEQ chronotype outcomes were substantially different, we excluded the participant. Completion of the two questionnaires took on average 15 minutes. Potential participants were made aware within a week of the questionnaire completion whether

they were eligible to participate in the study. All participants were blinded to the chronotype they were assigned to.

Paced Auditory Serial Addition Task. The Paced Auditory Serial Addition Test is known as a valid technique in order to evoke acute psychological stress (Lejuez, Kahler and Brown, 2003). The PASAT used within this study was administered in an auditory format, most consistent with clinical research (Tombaugh, 2006). In the task, participants are continuously presented with random numerals ranging from 1 to 9 for 6 minutes. Participants are required to sum the last two presented numerals and answer verbally as soon as possible (Tanosoto et al, 2015). As the PASAT becomes faster, the number of incorrect responses increase, as do the levels of self-reported stress (Tanosoto et al, 2015) (Tombaugh, 2006.). The performance within the test is not significantly correlated with the participant's general intelligence or arithmetic abilities (Gronwall and Wrightson, 1981). Supplementary methods of stress induction were adopted throughout the PASAT test. A loud buzzer was administered following any incorrect response, stutter, mumble or hesitation. Additionally, the participants were asked to watch themselves on a screen throughout the test, and were told that their body language was being assessed in order to measure their levels of anxiety. Blood pressure and heart rate measurements were taken at baseline, 2 minutes, 4 minutes and 6 minutes of the PASAT. The maximum score that could be achieved on the test was 183 correct responses.

Handgrip Strength. We used a simple maximal isometric handgrip strength test as a measure of muscle strength (Bohannon, 2001). The participants grip strength was measured using an electronic hand dynamometer (Takei Hand Grip Dynamometer). Participants were instructed to hold the dynamometer in their dominant hand with their elbow fully extended to their side. Following a 'go' signal, the participants applied maximum grip pressure and

squeezed the dynamometer for 6 seconds. This process was repeated three times with two-minute rest periods between each. Each attempt was accompanied by a consistent motivational script, encouraging participants to achieve maximum effort. The highest value of the three attempts was recorded (kg).

Incremental Test to Volitional Exhaustion (VO_2 max Test). The incremental test to volitional exhaustion was used in this study as a method to measure participant's maximal oxygen uptake (VO_2max) at different time points (Mauger and Sculthorpe, 2012). Prior to the test, the participants mass and height measurements were inputted, and they were fitted to the stationary bike ergometer (Excalibur Sport, 925909 Ergometer Exercise Bike). The individual's settings were recorded in order to ensure that the cycling position for each participant was consistent across all trials. Participants were also fitted to a facemask. The gas analyser system (JAEGER® Vyntus CPX, CareFusion, GER) was calibrated prior to the test commencing, and the masks were checked for leakage (Lobben et al, 2018). Each participant completed a warm up period of 5 minutes cycling at 60 W which preceded the exercise test. Participants commenced the test by pedalling at a workload of 90 W, which was increased by 30 W every 2 minutes. The test continued until participants were unable to maintain a rate higher than 50 rpm or they voluntarily stopped the test due to exhaustion. All participants were verbally encouraged by the researches in order to ensure that they reached their maximum capacity. Throughout the test, rates of oxygen production (VO_2) and carbon dioxide consumption (VCO_2) were measured by breath-by-breath indirect calorimetry using an automatic analyser system (JAEGER® Vyntus CPX, CareFusion, GER). Following volitional exhaustion, the data was reduced to 10 second averages, and the VO_2max was considered as the highest 10 second VO_2 value recorded during the incremental test (Souza et al, 2015). Heart rate (Polar H7 Bluetooth 4.0 Heart Rate Sensor) and RPE, a measure of physical effort on a

scale of 6 (very light) to 20 (very hard) were obtained every two minutes and at exhaustion (Borg, 1970).

Salivary Cortisol Measures

Collection of saliva samples. Salivary cortisol collection is regarded as a valid measure of unbound cortisol concentration as it accurately reflects the amount of free cortisol in plasma (Heinrichs et al, 2003) (Vining and McGinley, 1987). Therefore saliva collection provides a reliable and non-invasive measure of the biologically active cortisol present within the participant (Randler and Schaal, 2010). Four awakening saliva samples and three saliva samples per experimental session were obtained from each participant throughout the duration of the study, as per the manufacturer's instructions (Tecan UK Ltd). Participants were instructed not to eat or consume caffeine 60 minutes prior to collection. Moreover, they were instructed to avoid alcohol for 12-hours and not to brush their teeth 45 minutes prior to sampling. Any deviations to the instructions could be recorded by the participants on a sample collection record sheet. Saliva samples were collected in sterile 15 mL centrifuge tubes (Greiner Bio, CELLSTAR) using a widely accepted 'spitting' method (Gomar-Vercher et al, 2018) (Nagler and Hershkovich, 2005). Participants were instructed to allow saliva to pool in their mouths before spitting into the sterile tubes. No time limit was given on sample collection; however, 2 mL was the minimum volume accepted (not including foam).

Morning samples. Cortisol awakening response samples were collected by the participant on the morning of their 14:00 hr session. This day was selected to ensure that both ECTs and LCTs could wake naturally without the constrains of an alarm. Saliva samples were collected at four time points: (M1) immediately after awakening, (M2) 15 minutes after waking

up, (M3) 30 minutes after waking up, (M4) 60 minutes after waking up. Participants recorded the exact times of their sampling on a sampling collection record sheet provided to them. This sheet also allowed space for recording any issues that may have affected the measurements in the sampling period. All samples were refrigerated until being transported to the laboratory in cool bags. Samples were placed on ice immediately as they arrived at the laboratory.

Experimental samples. Saliva samples were collected before the PASAT, 10 minutes after the PASAT, and following the VO_2 max test. All samples were iced immediately. The timings and method of collection were identical over the 3 experimental sessions (08:00 hr, 14:00 hr, 20:00 hr).

Handling of Samples. Immediately after all specimen collections were completed, the saliva samples were centrifuged (Thermo Fisher Scientific, Multifuge X1R) at 1500 rpm for 10 minutes at 4°C. Centrifuging the samples prior to freezing removed any remaining bacteria, exfoliated epithelial cells and debris (Rudney, Staikov and Johnson, 2009). Following centrifuging, the remaining supernatant was drawn with a pipette (Eppendorph Research Plus, 20-200µL) into 1.5 mL Eppendorf tubes (Eppendorf Tubes® 3810X) and stored in the freezer (New Brunswick Scientific U725-G) at -71°C until they were assayed. Prior to assaying, selected samples were thawed at room temperature and centrifuged at 1500 rpm for 10 minutes at 4°C to any remove solids (Westermann, Demir and Herbst, 2004). Following this, all samples were vortexed (IKA Vortex 2 shaker) ready for the assay.

Analysis of Salivary Samples. Samples were analysed using IBL International Cortisol Saliva ELISA kits (Tecan UK Ltd). Analysis was followed as per the manufacturer's instructions (IBL International Hamburg GmbH, Hamburg, Germany). All saliva samples

were assayed in duplicate. Any samples with signs of blood or containing red pigmentation were discarded, as this could have had a significant effect on assay results.

Reagents: All reagents used in the ELISA kit were provided by the manufacturer (IBL International Hamburg GmbH, Hamburg, Germany).

Enzyme Conjugate: ready to use; contains chromatographically purified cortisol, conjugated to enzyme horseradish peroxidase (HRP).

Standards A-F: ready to use; contains cortisol in a buffer with <0.1% BSA and <0.1% ProClin preservatives. Standard concentrations are as follows: 0 nmol/L (Standard A), 0.41 nmol/L (Standard B), 1.10 nmol/L (Standard C), 4.69 nmol/L (Standard D), 19.3 nmol/L (Standard E) and 82.8 nmol/L (Standard F).

Controls 1+2: ready to use; contains cortisol (low and high concentration), buffer, <0.1% BSA and <0.1% ProClin preservatives.

TMB Substrate Solution: contains TMB (3,3',5,5'-tetramethylbenzidine), buffer and stabilizers.

TMB Stop Solution**:** ready to use; contains 1 M H_2SO_4 (sulfuric acid).

Wash Buffer: concentrate; phosphate buffer and Tween. Diluted wash buffer 1 to 10 with distilled water prior to use.

Microtiter Plate: 96 well plate, can be divided into 3 separate runs. Plate wells coated with anti-cortisol antibodies (rabbit).

ELISA Test Principles. The 'enzyme-linked immunosorbent assay' (ELISA) is based on the competition principle. An unknown amount of cortisol within the sample competes for the binding sites of the antigen-specific coated antibodies that cover the 96 wells (Westermann, Demir and Herbst, 2004). Following an incubation period, the reaction is stopped using a wash method. Following the wash and substrate reaction, the intensity of the colour that has accumulated within the wells is inversely proportionate to the level of antigen within the sample (Westermann, Demir and Herbst, 2004). All results can be calculated against a provided standardised curve.

ELISA Test Procedure. 50 µL of standards A-F and controls 1-2 are pipetted into their respective wells. 50 µL of each sample are pipetted into the remaining microtiter plate wells. Each standard, control and sample are duplicated. 100 µL of enzyme conjugate is added into each well. The plate is covered with adhesive foil and incubated at room temperature (18-25°C) for 2 hours on an orbital shaker (IKA, VIBRAX VXR basic) at 400 rpm. Following incubation, the solution is discarded, and all wells are washed four times with 250 µL of diluted wash buffer. 100 µL of TMB substrate solution is added to each well. The plate is covered with an adhesive foil and incubated for 30 minutes at room temperature (18-25°C) on the orbital shaker (400 rpm). Finally, 100 µL of TMB stop solution is pipetted into each well, changing the colour of the solution from blue to yellow. The optical density of the plate was read immediately after adding the stop solution with a photometer (BioTek™ ELx800™ Absorbance Microplate Reader) at 450 nm (if left for over 15 minutes readings would be compromised). The standard curves were

calculated using the KC Junior software. All assays were run with quality controls before and after samples, adhering to the rules of GLP (good laboratory practice).

Statistical Analyses

Data is expressed as mean ± standard deviation (SD) unless stated otherwise. All data was analysed using SPSS statistics software (IBM SPSS® software, v25, SPSS Inc. Chicago, IL). Statistical significance is inferred if $p \leq 0.05$. Exact p values are given to two significant figures. All participants test results (excluding cortisol concentrations, heart rate, RPE and blood pressure) were converted into percentage changes in order to make direct comparisons between the tests. Percentage changes were calculated by taking the best scores and using this value as 100%. For example, if a participant achieved their best VO_2 max value in the evening session at 50 $ml \cdot kg \cdot min^{-1}$, this was taken as 100%. If the participant then achieved a VO_2 max of 40 $ml \cdot kg \cdot min^{-1}$ in the morning testing session, this was presented as 80%. All between and within-subject time point differences (PASAT, handgrip strength, VO_2 max,) were analysed using a two-way repeated measures ANOVA (Park, Cho and Ki, 2009). Within the data, where the sphericity assumption was not met, p values were adjusted using the Greenhouse-Geisser Epsilon (< 0.75) or the Huynh-Feldt epsilon (> 0.75), in order to account for the within-subject correlation (Park, Cho and Ki, 2009) (Atkinson, 2002). Post-hoc comparisons using the Holm-Bonferroni correction were performed when statistically significant effects in the data set arose. This post-hoc correction is deemed 'more powerful' than the classic Bonferroni correction, however is still able to keep the inflation of a type 1 error under control (Abdi, 2010).

RESULTS

Chronometric data

Comprehensive analysis of the 357 individuals that competed the chronometric questionnaires identified a total of 9.5% ECTs (n=34), 84.5% ICTs (n=302) and 6% LCTs (n=21). From the 18 participants that took part in the study (10 ECTs and 8 LCTs), average wake up and sleep-onset times reported in the MCTQ differed between chronotypes. ECTs average wake up time was 06:32±00:30 hrs on work days and 06:58±00:43 hrs on free days. LCTs average wake up time was 10:01±00:33 hrs on work days and 11:09±00:33 hrs on free days. ECTs average sleep-onset was 22:38±00:43 hrs on work days and 23:03±00:31 hrs on free days. LCTs average sleep onset was 03:12±01:21 hrs on work days and 03:54±00:37 hrs on free days. Calculated corrected mid-sleep time for free days (MSF_{sc}) was 02:47±00:38 hrs for ECTs and 07:25±00:19 hrs for LCTs respectively. Mean MEQ scores were 60.9±9.1 in ECTs and 33.5±3.5 in LCTs, validating the MCTQ chronotype outcomes.

Physical Measures

Handgrip Strength. Figure 1 displays the diurnal variation between ECTs and LCTs in handgrip strength performance. All percentage changes are relative to the individual's performance. A repeated measures ANOVA revealed a significant difference between chronotypes at 20:00 hrs ($p=.004$, $\eta_p^2= .407$). LCTs elicited significantly greater handgrip strength values (98.7±0.8 %) than ECTs (92.4±1.6 %) at this time. There were no further significant differences between chronotypes at 08:00 hrs ($p>.05$) or at 14:00 hrs ($p>.05$). When looking within the ECT group, handgrip performance significantly declined ($p=.002$) by 6.8% across the day from 08:00 hr (98.8±1.3 %) to 20:00 hrs (92.0±1.6 %). This significant decline in performance was also evident in ECTs from 14:00 hrs (96.2±1.5 %) to

20:00 hrs. LCTs handgrip performance significantly increased (p=.037) by 5.5% from 08:00 hrs (93.6±2.8 %) to 20:00 hrs (98.7±0.8 %).

VO_2 max. Figure 2 illustrates the diurnal variation between ECTs and LCTs in VO_2 max values following an incremental test to volitional exhaustion. Analysis revealed a significant difference between ECTs and LCTs VO_2 max at 08:00 hrs (p=.007, η_p^2= .379). VO_2 max was 2.9% greater in ETCs (99.4±0.4 %) than LCTs (96.5±0.9 %) at this 08:00 hr time point. All within-group differences were non-significant.

VO_2 max RPE: Figure 3 presents the diurnal variation between ECTs and LCTs in VO_2 max RPE ratings. LCTs RPE ratings increased significantly (p=.048) by 4.1% from 08:00hrs (18.2±0.3) to 20:00 hrs (19.3±0.3). There were no significant differences within the ECTs RPE across the day (p>.05). No significant differences were found between chronotypes at any of the three testing points.

VO_2 max heart rate: Figure 4 illustrates the diurnal variation between ECTs and LCTs peak heart rate response during an incremental test to volitional exhaustion. Heart rate displayed a stable decrease across the day in ECTs, and a stable increase across the testing sessions in LCTs. These within group heart rate variations however, were not significant (p>.05). No between group differences were found at any of the time points.

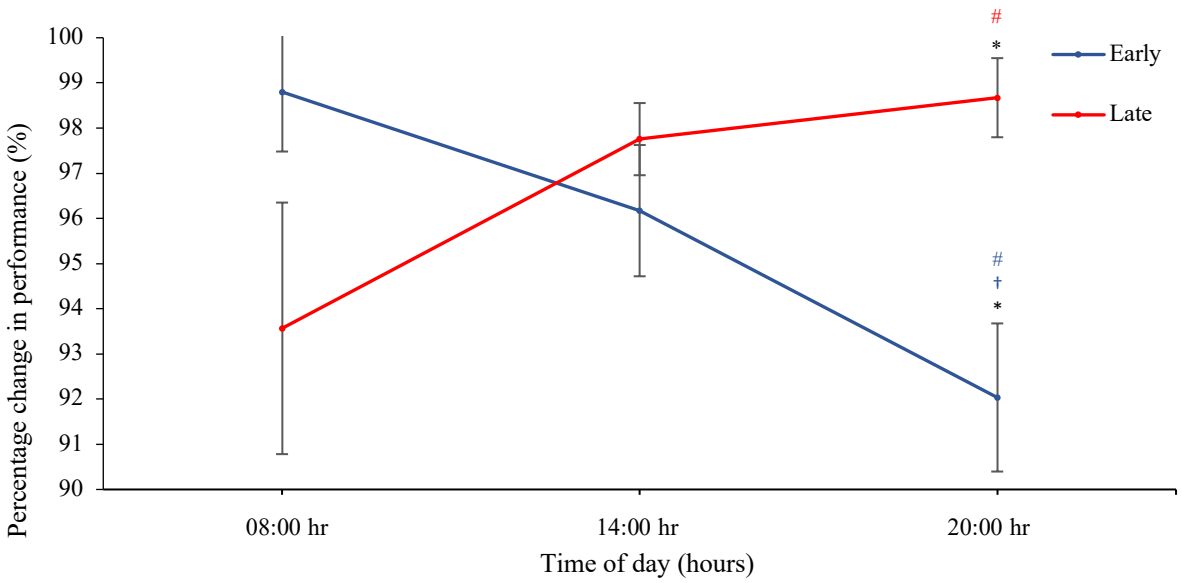

Figure 1: Diurnal variation of handgrip strength in ECTs (blue line) and LCTs (red line). (#) denotes statistical significance from 8am (<.05). (†) denotes statistical significance from 2pm (<.05). (*) denotes statistical significance between chronotypes (<.05). Colour of significance symbol is indicative of the chronotype group it relates to. All significant values were derived from a repeated-measures ANOVA. Data are means ± SE.

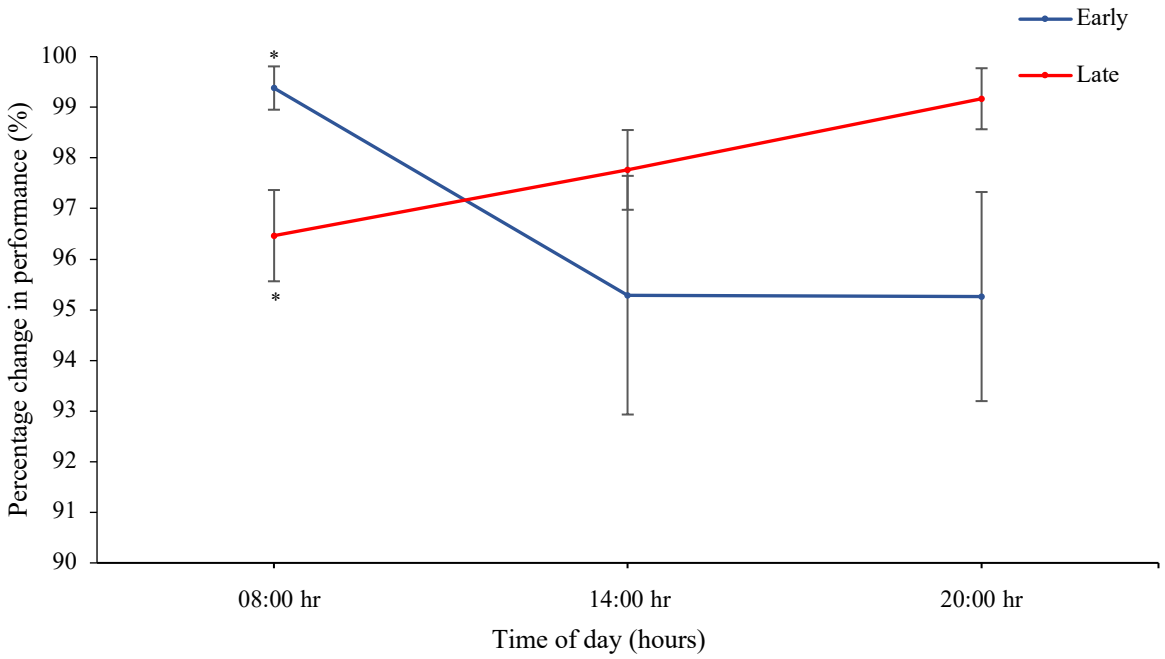

Figure 2: Diurnal variation of VO_2 max in ECTs (blue line) and LCTs (red line). (*) denotes statistical significance between chronotypes (<.05). All significant values were derived from a repeated-measures ANOVA. Data are means ± SE.

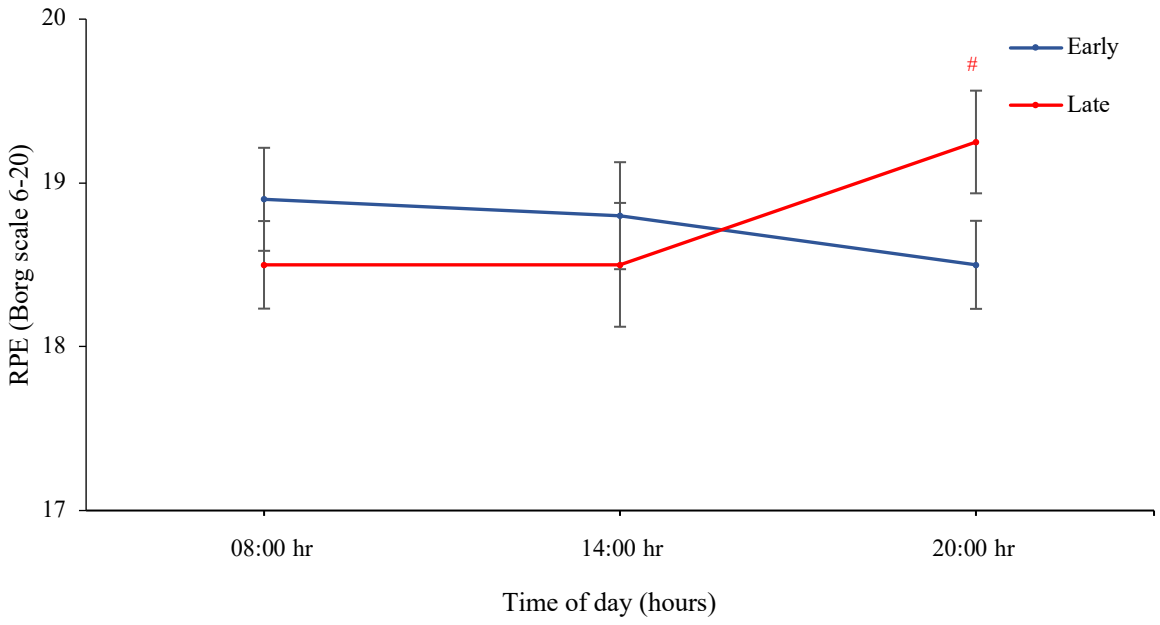

Figure 3: Diurnal variation of VO_2 max RPE in ECTs (blue line) and LCTs (red line). (#) denotes statistical significance from 8am (<.05). Colour of significance symbol is indicative of the chronotype group it relates to. All significant values were derived from a repeated-measures ANOVA. Data are means ± SE.

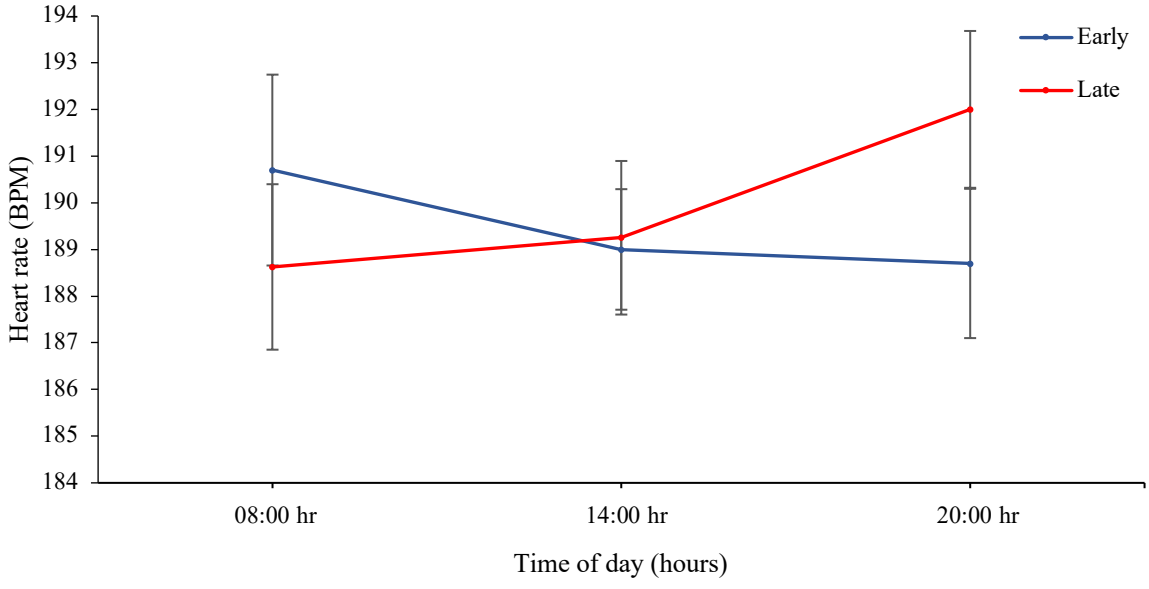

Figure 4: Diurnal variation of VO_2 max heart rate in ECTs (blue line) and LCTs (red line). All significant values were derived from a repeated-measures ANOVA. Data are means ± SE.

Stress Reactivity Measures

PASAT: Figure 5 presents the diurnal variation between ECTs and LCTs in PASAT performance. Analysis revealed a significant difference between ECTs and LCTs PASAT performance at 08:00 hrs (p=.002, η_p^2= .471) and 20:00 hrs (p=.019, η_p^2= .297). ECTs (95.9±2.7 %) performance was 20.1% better than LCTs (78.5±3.9 %) in the morning session. Conversely, LCTs (96.3±2.9 %) performance was 16.3% better in the evening session than the ECTs (81.7±4.5 %). A significant difference (p=.046) was uncovered within LCTs, who's PASAT performance linearly increased throughout the day by 22.7% from 08:00 hrs (78.5±3.9 %) to 20:00 hrs (96.3±2.9 %). This increase was also significant (p=.014) in LCTs performance from 08:00 hrs to 14:00 hrs (93.7±2.6 %). No significant differences were found within ECTs at any of the three time points.

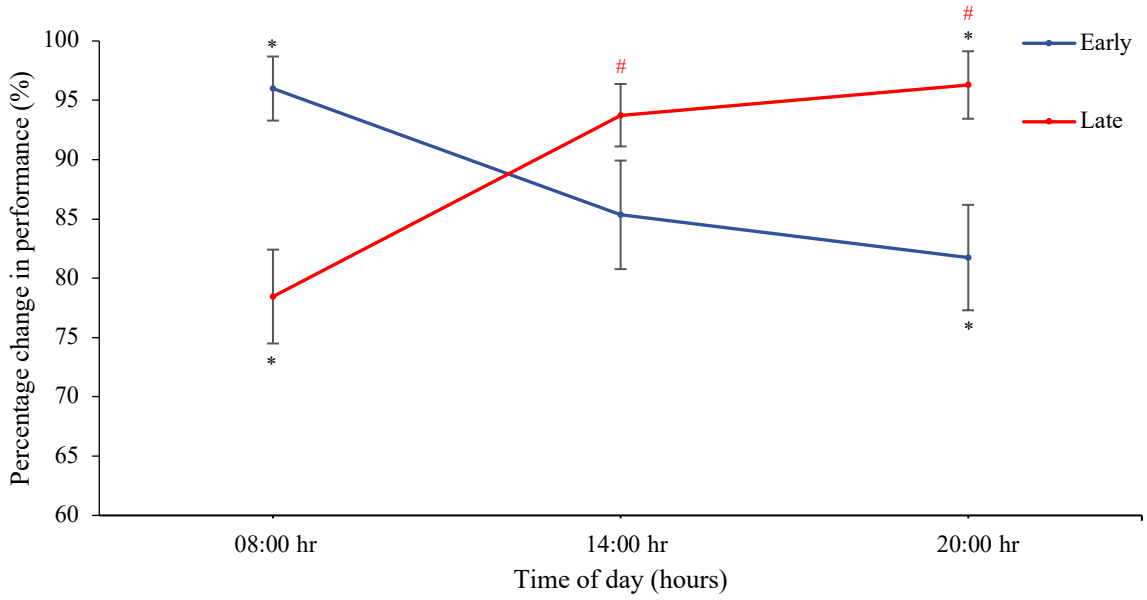

Figure 5: Diurnal variation of PASAT performance ECTs (blue line) and LCTs (red line). (#) denotes statistical significance from 8am (<.05). (*) denotes statistical significance between chronotypes (<.05). Colour of significance symbol is indicative of the chronotype group it relates to. All significant values were derived from a repeated-measures ANOVA. Data are means ± SE.

Salivary Cortisol Responses

Salivary cortisol response at 08:00 hrs: Figure 6 depicts the variation between ECTs and LCTs salivary cortisol response in the 08:00 hr experimental session. A significant increase in cortisol concentration within both ECTs and LCTs was revealed following analysis.

ECTs cortisol concentration increased linearly from the pre PASAT sample (0.41±0.02 ug/dL) to the post PASAT sample (0.59±0.02 ug/dL) by 45.8% ($p<.001$). Subsequently, concentration values significantly increased by 49.0% from post PASAT to post exercise (0.89±0.04 ug/dL). There was also a significant increase in the values from pre PASAT to post exercise ($p<.001$). Similar significant trends in cortisol increases were uncovered in the LCT cohort. Salivary cortisol concentration increased significantly ($p<.001$) from the pre PASAT sample (0.34±0.03 ug/dL) to the post PASAT sample (0.54±0.04 ug/dL). There was also a 49.1% increase ($p<.001$) from post PASAT to post exercise (0.8±0.04 ug/dL) in the LCTs. Finally, the overall increase of 134.8% from pre PASAT to post exercise was highly significant ($p<.001$).

All between group differences were non-significant.

Salivary cortisol response at 14:00 hrs: Figure 7 presents the variation between ECTs and LCTs salivary cortisol response in the 14:00 hr experimental session. There were clear significant differences between the ECTs and LCTs in the pre PASAT concentrations ($p=.031$) and the post PASAT concentrations ($p=.027$). ECTs (0.39±0.02 ug/dL) displayed significantly higher levels of salivary cortisol in the pre PASAT sample than LCTs (0.34±0.02 ug/dL). This increase was consistent in the post PASAT sample, where ECTs cortisol levels (0.59±0.02 ug/dL) were 4.6% higher than LCTs concentrations (0.57±0.02 ug/dL) at this sampling point. There were no significant differences between the chronotypes in their post exercise cortisol concentrations ($p>.05$).

Within the ECTs, all increases in cortisol concentration throughout the 14:00 hr experimental session were significant ($p<.001$). Concentrations increased from pre PASAT (0.39±0.02 ug/dL) to post PASAT (0.59±0.02 ug/dL) by 50.2%, and from post PASAT to post exercise (0.87±0.05 ug/dL) by 46.6%. The increase from first pre PASAT sample and the final post exercise sample was also significant.

The significant results within the ECTs were mirrored in the LCT participants. Increases in salivary cortisol concentration across the afternoon session in LCTs were significant at all sampling points ($p<.001$). Analysis displayed a 66.3% increase from pre PASAT (0.34±0.02 ug/dL) to post PASAT (0.57±0.02 ug/dL), and an 54.1% increase from post PASAT to post exercise (0.88±0.04 ug/dL). Concentrations also increased significantly from pre PASAT to post exercise.

Salivary cortisol response at 20:00 hrs: Figure 8 displays the variation between ECTs and LCTs salivary cortisol response in the 20:00 hr experimental session. There is a significant difference between the ECTs and LCTs cortisol response post exercise ($p=.05$). At this sampling point, LCTs (0.93±0.05 ug/dL) possess 15.2% higher levels of salivary cortisol than ECTs (0.79±0.04 ug/dL). There were no significant differences between chronotypes pre PASAT ($p>.05$) or post PASAT ($p>.05$) concentrations.

Similar to the 08:00 hrs and 14:00 hrs sessions, the 20:00 hrs session displayed significant increases in ECTs cortisol concentration across all three sampling points ($p=.000$). There was a 53.8% increase in concentration from pre PASAT (0.37±0.02 ug/dL) to post PASAT (0.57±0.02) ($p=.000$). A further marked increase of 40.8% from post PASAT to post exercise occurred (0.79±0.04 ug/dL) ($p=.000$). The overall concentration increase throughout the session from pre PASAT to post exercise was also significant ($p=.000$).

Similarly, LCTs cortisol concentration increases were all significant within the 20:00 hrs session (p=.0000. Concentration significantly increased by 75.1% from pre PASAT (0.36±0.02 ug/dL) to post PASAT (0.62±0.03 ug/dL), and by 48.9% from post PASAT to post exercise (0.93±0.04 ug/dL). The overall increase from pre PASAT to post exercise was also statistically significant (p=.000).

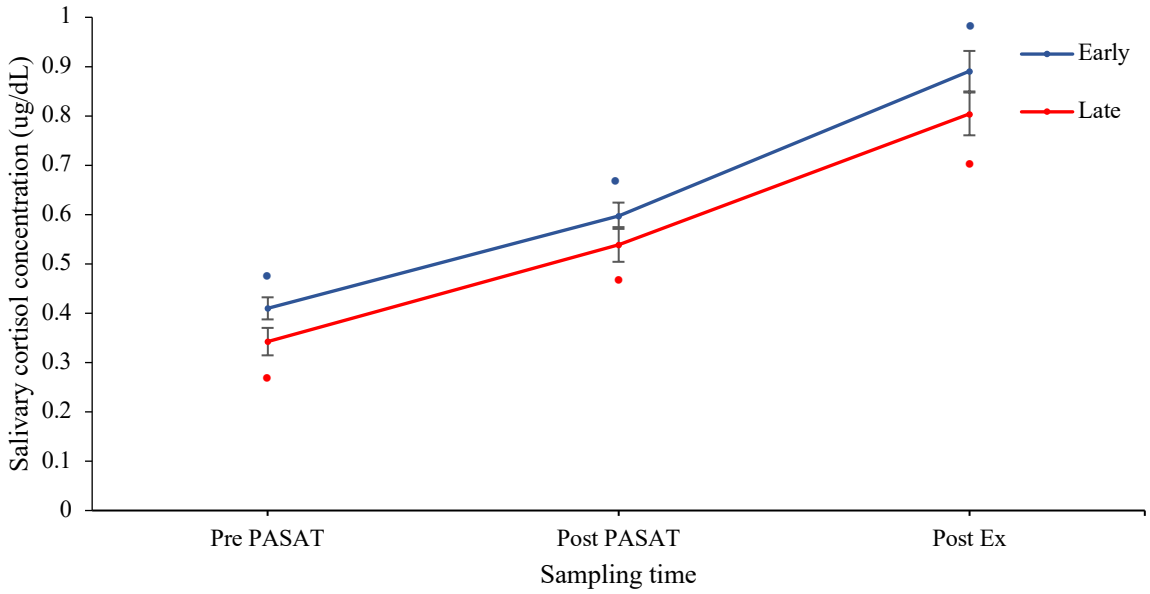

Figure 6: Salivary cortisol responses at 08:00 hrs in ECTs (blue line) and LCTs (red line). (•) denotes significance across all time points within chronotypes (<.001). Colour of significance symbol is indicative of the chronotype group it relates to. All significant values were derived from a repeated-measures ANOVA. Data are means ± SE

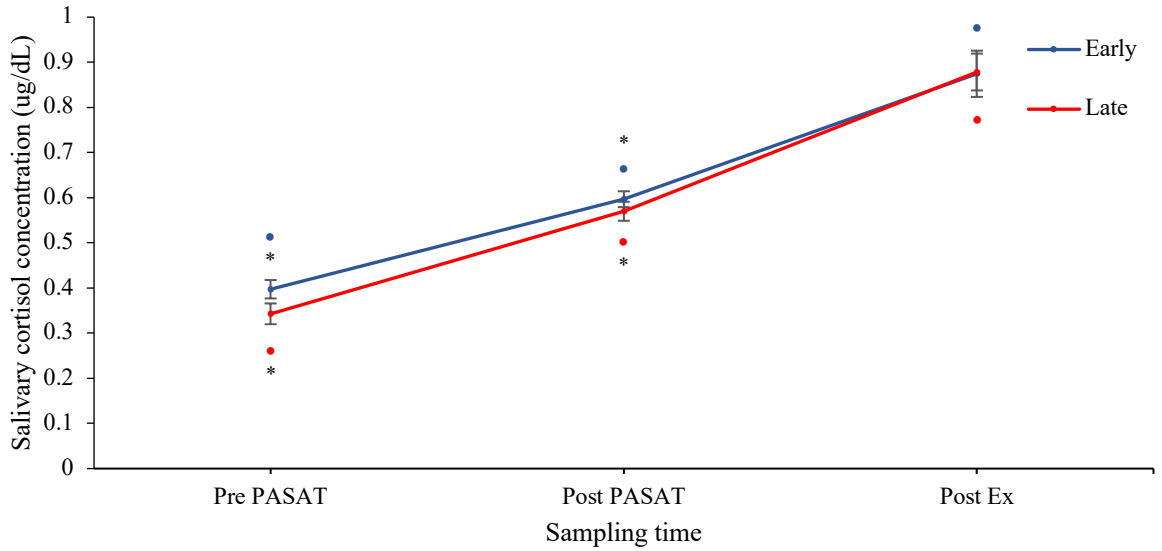

Figure 7: Salivary cortisol responses at 14:00 hrs in ECTs (blue line) and LCTs (red line). (•) indicates significance across all time points within chronotypes (<.001). (*) indicates significance between chronotypes (<.05). Colour of significance symbol is indicative of the chronotype group it relates to. All significant values were derived from a repeated-measures ANOVA. Data are means ± SE

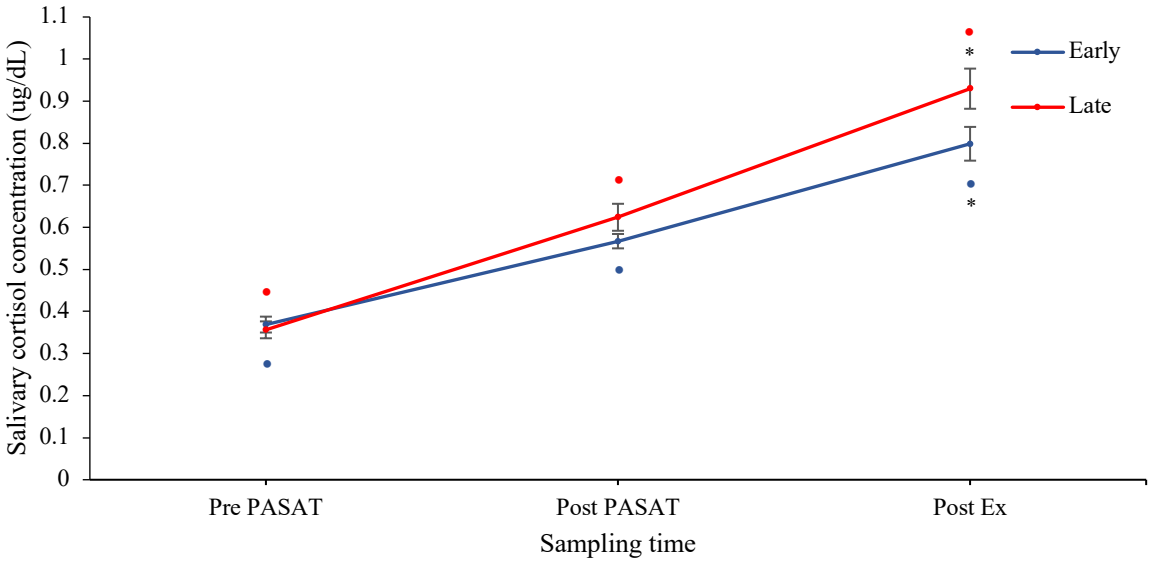

Figure 8: Salivary cortisol responses at 20:00 hrs in ECTs (blue line) and LCTs (red line). (•) indicates significance across all time points within chronotypes (<.001). (*) indicates significance between chronotypes (<.05). Colour of significance symbol is indicative of the chronotype group it relates to. All significant values were derived from a repeated-measures ANOVA. Data are means ± SE

Cortisol Awakening Response

Cortisol Awakening Response: The average waking time for ECTs on the morning of the cortisol awakening response sampling day was 06:51 ± 00:43. The average waking time that the LCTs recorded on this morning was 09:38 ± 00:41.

Figure 9 displays the diurnal variation between ECTs and LCTs cortisol awakening response profile. Analysis using a repeated measures ANOVA revealed significant differences between chronotypes at 0 minutes ($p=.012$) and 15 minutes since awakening ($p=.000$). ECTs salivary cortisol concentration (0.63±0.1ug/dL) was 35.2% higher than LCTs (0.44±0.03ug/dL) immediately after awakening. Similarly, ECTs salivary concentration (1.02±0.1ug/dL) was 30.4% higher 15 minutes after awakening than LCTs (0.75±0.03ug/dL). No significant differences between chronotypes were found at 30 minutes after awakening ($p<.05$) or 60 minutes after awakening ($p>.05$).

Multiple significant differences were displayed within the ECT and LCT groups. All changes in cortisol concentration across the four sampling times were significant from 0 minutes in the ECT group. The largest cortisol increase in ECTs was displayed from 0 minutes (0.63±0.05 ug/dL) to 15 minutes (1.02±0.03 ug/dL), with an increase in concentration of 62.2% ($p<.001$). Increases from baseline were also shown at 30 minutes ($p<.001$) (0.99±0.05 ug/dL) and 60 minutes ($p<.016$) (0.77±0.03 ug/dL). With the cortisol peak at 15 minutes in the ECTs, this concentration was significantly higher than the concentration of cortisol at 0 minutes ($p<.001$) or 60 minutes ($p<.001$). The only non-significant difference within the ECT group was the concentration change between 15 and 30 minutes.

LCTs cortisol concentration at 0 minutes (0.44±0.03 ug/dL) was significantly different to the cortisol values reached at 15 minutes ($p<.001$) (0.75±0.05 ug/dL), 30 minutes ($p<.001$) (0.87±0.03 ug/dL) and 60 minutes ($p<.001$) (0.75±0.03 ug/dL). The peak achieved at 30 minutes was 97.03% higher than the cortisol concentration in the sample taken immediately

following awakening. All within group differences that occurred from the three other time points were non-significant.

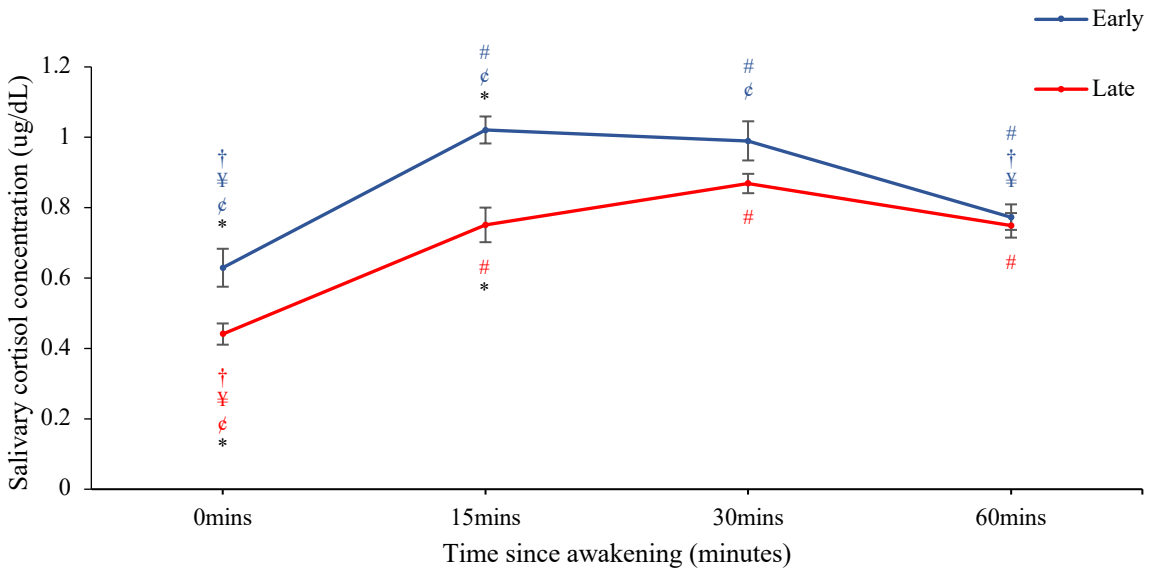

Figure 9: Cortisol awakening response in ECTs (blue line) and LCTs (red line). (#) denotes statistical significance from 0mins (<.05). (†) denotes statistical significance from 15mins (<.05). (¥) denotes statistical significance from 30mins (<.05). (¢) denotes statistical significance from 60mins (<.05) (*) denotes statistical significance between chronotypes (<.05). Colour of significance symbol is indicative of the chronotype group it relates to. All significant values were derived from a repeated-measures ANOVA. Data are means ± SE.

DISCUSSION

The present study was designed to investigate the effects of chronotype on performance, stress reactivity and cortisol responses across three time points during the day. There were multiple components to the study, and numerous results were generated in connection to the hypotheses. Arguably the most significant findings were that both measures of physical performance elicited significant differences between chronotypes. The simple performance measure, handgrip strength, was significantly greater at 20:00 hrs in LCTs than their ECT counterparts. As hypothesised, both the increase in strength from 08:00 hrs to 20:00 hrs in ECTs and the decrease in strength from 08:00 hrs to 20:00 hrs in LCTs was significant (Fig.1). Additionally, in the aerobic measure of physical performance (incremental test to volitional exhaustion), ECTs exhibited a significantly greater VO_2 max at 08:00 hrs than LCTs (Fig.2). The second key finding was that ECTs displayed a trend for superior PASAT performance in the morning and inferior performance in the evening when compared to the LCTs (Fig.5). Thirdly, with regard to cortisol responses, we reported significant increases in cortisol concentration from pre PASAT to post PASAT and to post exercise within all the testing sessions, irrespective of chronotype (Fig.6, Fig.7, Fig.8). Strikingly, the 20:00hrs session was the only one to display significant differences between chronotypes, with ECTs presenting higher cortisol concentrations pre PASAT and post PASAT than LCTs at this time. Finally, the cortisol awakening response was significantly higher in ECTs than LCTs at 0 minutes after awakening and 15 minutes after awakening (Fig. 9), a finding consistent with multiple literary reports.

Time of day, chronotype and measures of physical performance

An individual's strength characteristics are strongly associated with their levels of physical performance and athletic success (Suchomel, Nimphius and Stone, 2016). Handgrip

strength is a simple measure of performance that provides a clear representation of overall upper body and lower body strength (Cronin et al, 2017). It is well established that handgrip strength peaks in the late afternoon/early evening irrespective of chronotype (Atkinson and Reilly, 1996) (Gifford, 1987) (Ilmarinen et al, 1980), however only a few studies incorporate this factor. In our study, LCTs handgrip strength was significantly greater than ECTs in the evening. This chronotype difference is in accordance with the work of Facer-Childs, Boiling and Balanos (2018), who reported that LCTs MVC performance was 3.7% better than LCTs at 20:00 hrs, consistent with individual chronotype. We did not uncover a significant difference between the chronotypes at any other time point. One possible explanation for this finding is the association between elevated core body temperature and strength performance. As previously discussed, it has long been recognised that the acrophase in core body temperature occurs in the late afternoon, and that improvements in strength performance are coupled with this peak (Taylor et al, 2011) (Waterhouse et al, 2005) (Teo, Newton, and McGuigan, 2011). Previous research has also found that LCTs peak core body temperature occurs approximately 1.5 hrs later than that of ECTs (Foret, Benoit and Royant-Parola, 1982), and that they possess a larger amplitude in this peak (Baehr, Revelle, and Eastman Baehr, 2000). In our study, LCTs could have been advantageously influenced by this heightened peak in core temperature, with the 1.5 hr delay pushing closer to our 20:00 hrs session. This meant that LCTs were performing at their preferred time of day, and that their core body temperature at this time was likely to be at its highest. As the difference in handgrip strength in the morning between chronotypes was not significant, it could be that an increased core body temperature is key to producing significant results in strength measures. We did not measure core body temperature; however it would be interesting for future studies to replicate our methodology and consider this as a possible basis for the results. Alternatively, Tamm et al (2009) proposed that the increases in MVC torque across the day in their LCT population resulted from an increased central nervous

system drive to the muscle. This was due to increases in spinal and cortical activity. They also suggested that whilst ECTs displayed a more consistent variation in their MVC performance across the day, they were never able to achieve their 'true physiological maximum' (Tamm et al, 2009). It could therefore be postulated that the reason LCT's showed a significantly greater handgrip strength value at 20:00hrs than ECT's is due to the fact that these participants are able to achieve a true maximum at their preferred time of day. It is also important to note that ECTs performance at 08:00 hrs was not significant better than LCT's. This is due to physiological increases such as an increased CNS drive (as mentioned in this study). This however, is speculative and the physiological factors underpinning this theory are yet to be identified.

We have also shown that both ECT and LCTs grip strength follows a significant diurnal profile, whereby the ECTs performance steadily declines over the testing period (08:00 hrs, 14:00 hrs and 20:00 hrs), and LCTs strength increases throughout the day. This is a key finding in our results, as this would highlight a clear 'synchrony effect' in each chronotype, with performance improving at optimal times and declining at non-optimal times of day (Lara, Madrid and Correa, 2014). Our results support much of the current literature surrounding the performance profiles of aerobic performance (Facer-Childs and Brandstaetter, 2015), maximal exercise (Hill et al, 1988), and cognitive ability (Schmidt et al, 2007) (Preckel et al, 2011). Therefore, this finding, consistent with our hypothesis, adds considerable weight to the current body of research.

Our second finding showed that ECTs VO_2 max achieved in an incremental test to exhaustion was significantly greater than LCTs at 08:00hrs as predicted. Interestingly, we did not uncover any other notable differences between the chronotypes at any other time point, nor any within chronotype rhythms across the day, in concordance with Sugawara et al (2001) and Kunorozva, Roden and Rae (2014). One possible explanation for the finding within the ECT cohort could be attributed to their levels of intrinsic motivation. Roeser, Schlarb and Kübler (2013) indicated

that ECTs are associated with elevated levels of motivation and goal orientation, whereas a disadvantageous attitude and poor motivational stability is more pronounced in LCTs (Roeser et al, 2012). It has been suggested that in order to elicit a true VO_2 max, a high degree of motivation is imperative, as a lack of motivation may limit maximal effort (Clark, Poole-Wilson and Coats, 1994) (Rivera-Brown, Rivera and Frontera, 1992) (Grant et al, 1995). Therefore, it is fair to assume that motivation could be a contributing factor in explaining why ECTs reached a higher VO_2 max at their optimal time of day, and why no such finding was apparent in LCTs. It is also important to note that in the 08:00 hr session, LCTs would usually be sleeping for another 02:02 hrs on average. As research claims that performing tasks during a person's 'biological night' will have adverse effects on performance (Sato, Ida and Kojima, 2017) (Facer-Childs, Boiling and Balanos, 2018), our LCT participant were at a clear disadvantage throughout this testing session. There were no significant heart rate differences between chronotypes or across the testing sessions when undertaking the test, concurring with the work of (Willis, O'Connor and Smith, 2005). The only significant RPE result was measured in LCTs from 08:00 hrs to 20:00 hrs. This increase in the perception of effort across the day, was not mirrored in performance. As this is the first study to our knowledge to incorporate RPE responses to an incremental test to exhaustion with regards to chronotype, it is possible that LCTs perceive exertion as 'harder' at their optimal time of day. Alternatively, the perception of increased effort may be the result of an actual increase in physiological effort at this time, however this increase was not paralleled in their performance outcomes. The mechanisms underlying the greater RPE reported by LCTs in the evening remain unknown, and require further exploration as RPE may be associated with 'circadian phases of processes that are more cognitive or emotional in nature' (Rossi et al, 2015).

When looking at the two measures of physical performance implemented in this study, both the handgrip and incremental test to volitional exhaustion elicit opposing chronotype

significance. As previously described, LCTs handgrip strength was significantly greater than ECTs at 20:00 hrs (their optimal time). This was not the case in their VO2 max values. Similarly, ECTs VO2 max was significantly greater than LTCs at 08:00 hrs (their optimal time), however this was not the case for their handgrip strength. It is possible that the complexity and intensity required for these tasks is responsible for the differing chronotype responses. Rae, Stephenson and Roden (2015) and Vitale et al (2017) concurred that ECTs have lower symptoms of fatigue and report higher levels of vigour in the mornings in submaximal, time trial and self-paced exercise. Correspondingly, Vitale et al (2017) found that LCTs are more fatigued and show less vigour in the mornings than their ECT counterparts during a bout of high intensity exercise. It is possible that our results in both of these performance tests are driven by the intensity and length of the task, which is a consistent chronotype difference found when examining cognitive tasks (Preckel et al, 2011). As the incremental test to volitional exhaustion is comprised of stages of 3 minutes each, one stage within this test is much longer than the handgrip strength test overall. As ECTs are shown to be less fatigued in the morning when performing longer length exercise tests, this could be why our ECTs are significantly better at this time. However, as the handgrip strength test is a gross motor skill that requires maximal effort but takes less time to complete, LCTs advantageous strength measures in the evening could be more affected by body temperature (as discussed above) and extraneous variables, rather than fatigue and motivational responses that ECTs respond better to in extended maximal tasks.

It is important to discuss the potential role that sleep duration, deprivation and sleep inertia may have had on our performance results. The participants average sleep durations were obtained from their initial chronometric questionnaire data, and LCTs displayed a significantly shorter sleep duration on average than their ECT counterparts, in concurrence with Dagys et al (2012) and Roepke and Duffy (2010) research. Assuming that the sleep duration derived from

the chronometric questionnaires is true for that of the participants sleep durations over the testing period, this shorter sleep duration may be a contributing factor to the reduction in VO_2 max and PASAT performance within the LCT group in the morning session. Sleep inertia in particular is characterised as 'the state of cognitive impairment immediately upon awakening from sleep' (Ritchie et al, 2017). This phenomenon proposes an impairment in performance following awakening, that continues to improve 2-4 hours following wake up time (Selvi et al, 2007). Therefore, the inertia effect may have impacted our participants in the morning session. Ritchie et al (2017) found that the duration of sleep inertia is exacerbated in LTCs, even if their sleep history and duration is similar to that of the ECTs. Moreover, the impairment in performance that can be caused by sleep inertia is largely affected by sleep duration (Ritchie et al, 2017). It is therefore fair to assume that our LCT cohort may have been adversely affected by a longer period of sleep inertia in the morning session, and in addition to their shorter sleep duration than the ECTs, could be a contributing factor to their performance detriment at this time. Unfortunately, we did not measure sleep duration prior to each visit to the laboratory, nor were any sleep restrictions imposed, as we recommended that the participants to follow their natural sleeping patterns. Therefore, we cannot use this data conclusively within our main analysis to understand the influence of sleep duration/inertia within our cohort. Upon replication, this would be an exciting focus for further exploration.

Time of day, chronotype and measures of stress reactivity

Both chronotypes are understood to possess different responses to stress induction, with LCTs presenting elevated cardiovascular stress responses, independent of testing times (Roeser et al, 2012), and ECTs displaying a significant increase in cortisol response compared to LCTs when exposed to stress (Marvel-Coen, Nickels and Maestripieri, 2018). In our study, both ECTs and LCTs performed significantly better on a stress inducing mental arithmetic task

(PASAT) at their preferred time of day, consistent with the trends in physical performance that our participants displayed. LCTs however, were the only group to display a diurnal rhythmicity in PASAT performance, with a significant improvement throughout the day. The PASAT is not only stress inducing, but it places demands on cognitive abilities and information processing speeds (Rogers and Fox, 2012). Preckel et al's (2011) research has uncovered that eveningness is positively correlated to a person's cognitive ability. It is postulated that this increased academic ability is due to the fact that LCTs 'have difficulty adjusting to the universal schedule of academic institutions' (Preckel et al, 2011). Overcoming the inconvenience of this schedule and adapting to earlier starts and working hours, may lead to LCTs producing higher problem-solving capabilities. As the participants in our cohort were primarily university students, the LCTs could easily have been exposed to the same scheduling issues, providing them with a pronounced cognitive capability. This could be a reason for why the LCTs in this study significantly improved in their PASAT performance throughout the day, whereas no such significant trend was uncovered in our ECTs. It is fair to conclude from this result that participants tend to perform better in stress inducing tasks at their optimal time of day. Furthermore, we speculate that stress is managed better and is not negatively impactful on performance when taking chronotype into account. LCTs display clear diurnal rhythms in their PASAT performance which could be attributed to their increased level of cognition and problem-solving capabilities.

Correlations between cortisol stress response and chronotype were also significant. When looking at the cortisol responses to the PASAT, the PASAT was effective in inducing significant increases in salivary cortisol in both chronotypes at 08:00hrs, 14:00hrs and 20:00hrs. The only significant difference between the chronotypes following the PASAT test was at 14:00hrs, where ECTs cortisol concentrations were significantly higher than that of their LCT counterparts at this time, concurring with the work of Marvel-Coen, Nickels and

Maestripieri (2018) and (Maierova et al, 2016). As this is only the second study to investigate the cortisol responsiveness to an acute psychological stressor in ECTs and LCTs, the similarity in the increased responsiveness of ETCs in both our study and the work of Marvel-Coen, Nickels and Maestripieri (2018) is extremely interesting. The increase in ECTs cortisol response occurring at 14:00 hrs, a time considered as an intermediate time point for both chronotypes, is consistent with the observation in Kudielka, Bellingrath and Hellhammer's (2007) study. They presented that 'morning persons' seem to have higher levels of daytime cortisol than 'evening persons'. We can therefore speculate that daily fluctuations in cortisol between chronotypes are more impactful on the cortisol response to stress than the optimal timings are. It is important to note that although there was a significant difference between chronotypes and cortisol response to the PASAT in the 14:00 hrs session, there were no significant differences found between their performances on the task at this time. The increased cortisol level in this group does not impact the cognition and processing speeds required to perform well in this arithmetic task, concurring with the conclusions of work by Lupien et al (2002). Therefore, not only have we shown a chronotype difference in the cortisol responsiveness to a stress task that is consistent with recent research, we have also uncovered that the difference this cortisol response between chronotypes has, has no impact on PASAT performance, a finding that can considerably add to the current body of research.

Exercise is well known as a physical stressor that can cause substantial increases in cortisol secretion (Brownlee, Moore and Hackney, 2005). The physical activity-induced increases of salivary cortisol are dependent on the exercise intensity, with only high intensity exercise eliciting a cortisol response (60% or more of a person's VO_2 max) (Hill et al, 2008). Reasons for this include that higher intensity exercise demands cortisol to increase the availability of substrates for metabolism, and meet the metabolic demands of fuel homeostasis (Luger et al, 1987) (Anderson and Wideman, 2017). Here we have shown that cortisol

concentration is significantly increased from baseline, and from post PASAT values after completing the incremental test to volitional exhaustion. This increase was visible at all testing time points (08:00 hrs, 14:00 hrs and 20:00 hrs), and within both chronotype groups. The increases in cortisol following the high intensity bout displayed similar trends across all three testing sessions, concurring with the work of Thuma et al (1995) who suggested that when comparing to a pre exercise baseline, rises in salivary cortisol following a maximal oxygen consumption treadmill test were very similar in the morning and the evening. We also uncovered that LCTs had a significantly more pronounced cortisol response than ECTs to the exercise task at 20:00 hrs, their preferential time of day. However, this increase was not mirrored in their performance. Our finding therefore demonstrates that high intensity exercise can further amplify a cortisol response at all time points following a stress task. This novel result is dissimilar to that of Ponce, del Arco and Loprinzi's (2019) study, who found that acute exercise and psychological stress do not produce significantly different effects on increasing cortisol concentrations. Lovallo et al (2006) also highlighted that cortisol responses to mental stressors were much larger than the responses to exercise. However, their study only used a moderate exercise intensity protocol, which is well known not to elicit large cortisol increases, and therefore hinders its significance. It is evident that further investigation is needed to uncover whether this increase following psychological stress induction in our study occurred as a result of the methodological timing of the testing session (exercise followed immediately after PASAT test), or whether this high intensity exercise response is in fact greater than the stress induced response when performed separately.

Chronotype and Cortisol Awakening Response

The final significant finding within our study was that ECTs displayed significantly higher levels of salivary cortisol in their CAR profiles at the point of awakening and 15 minutes

after awakening. Multiple studies have solidified this finding and share the same prevailing view of our results (Kudielka, Bellingrath and Hellhammer, 2007) (Bailey and Heitkemper, 2001) (Randler and Schaal, 2010). Furthermore, our study found that cortisol concentration begins to decline in the ECTs from 15 minutes following wake up to the end of the 60-minute sampling period, whereas LCTs still showed an increase up until 30-minutes post awakening. Therefore, LCTs show a delayed and less accentuated cortisol response to waking in the mornings compared to their counterparts (Abbruzzese, Klingmann and Ehlert, 2014).

It has been proposed that a lower cortisol response to awakening in LCTs and a prolonged increased phase could explain why they tend to have difficulties when preparing for the day, and in students, tend to be disadvantaged during study and exams (Randler and Frech, 2006). Randler, Faßl and Kalb (2017) have speculated that the reasoning for this disadvantage is that the LCTs could be waking too late to elicit a maximal CAR, and reaching this maximum may be key to unlocking a person's full potential for daily activities. Lower morning values also seem to be related to fatigue and a lowered capacity to engage in tasks (Oginska et al, 2010). However, in our cohort, the LCTs performed significantly better in the stress inducing mental arithmetic task (PASAT) at their preferred time of day compared to ECTs, so clearly their lower CAR did not hinder them at this time. It would be fascinating to uncover if there was significant interaction between a lower CAR and multiple measures of cognition, performance and health, to decipher whether this does in fact have a detrimental effect on performance.

We are able to speculate on the mechanisms that link this rise in cortisol with an individual's chronotype. Kudielka et al (2006) suggest that morningness is associated with more pronounced adrenocortical activation in response to awakening, that does not remain increased throughout the course of the day (Clow et al, 2004). This would apply to our cohort, with ECTs displaying a significantly more pronounced awakening response at 0-minutes and

15-minutes post awakening. This underlying adrenocortical activity difference between chronotypes could be a key determinant in the timing and responsiveness of cortisol to awakening. As LCTs are hypothesised to be at a cognitive disadvantage because their cortisol acrophase occurs later and such a peak is smaller than ECTs, the scientific mechanism underlying this chronotype difference could provide a scientific basis for implementing methods to enhance the response and minimise the possible negative effects of a lowered CAR (Figueiro and Rea, 2012). Further research is necessary to uncover the biological differences between these chronotypes.

As our ECTs woke up at 06:58±00:43 on average on their free days, and our LCTs at 11:09±00:33, we arranged the cortisol awakening sampling to occur on the morning of the 14:00 hrs session, to minimise the possible effects of sleep deprivation in our LCT cohort. This also meant that all participants were able to wake naturally, without an alarm, minimising the effects that a forced wake-up may have on CAR (Anderson and Wideman, 2017). This is important, as constraining the timing of the cortisol sampling in the morning could produce artificial responses. In order to minimise this result, it was vital that we allowed both ETCs and LCTs to wake naturally, so that we are able to conclude that any differences in cortisol amplitude are 'tied to the individuals circadian clock, rather than the imposed 24-hour clock' (Dockray and Steptoe, 2011). Our chronotype results in regard to CAR can therefore be considered as highly reliable.

Chronotype, performance and stress reactivity

The final aim of this research was to understand whether an individual's chronotype would influence the relationship between their stress reactivity and performance. When evaluating the results, it is clear that there are multiple links between the measures. Firstly, as discussed, exercise was able to induce a significantly amplified cortisol response following the

stress test (PASAT), regardless of the time of day or chronotype. The only significant difference in this response to exercise, taking chronotypes into account, occurred at 20:00 hrs. At this time point, LCTs cortisol concentration was significantly greater than ECTs. However, this was not mirrored in the performance, as at this time, there were no significant differences in the VO_2 max performance between the chronotypes. Ponce, del Arco and Loprinzi's (2019) found similar results, whereby they concluded that salivary cortisol increases regarding chronotypes cannot be held accountable for the potentially differing effects of vigorous exercise.

Similarly, all cortisol concentrations were significantly increased from baseline following the stress test (PASAT). The only notable difference between the chronotypes cortisol responses to the PASAT occurred at 14:00hrs, where ECTs displayed a higher increase in cortisol to their LCT counterparts. This increase also did not seem to have an effect on their PASAT performance at this time, as they did not perform better than the LCTs at 14:00hrs. Also, the ETCs and LCTs PASAT performance was heightened at their optimal time of day (08:00 hrs and 20:00 hrs respectively). Therefore, we can conclude that cortisol increases are not sufficient to improve or hinder performance in in a mental arithmetic task, whether these increases are produced by stress or not, concurring with Ponce, del Arco and Loprinzi's (2019). A more likely cause for changes in performance can be related to individual chronotype, rather than cortisol responses.

When comparing the different chronotypes CAR in this study, ECTs presented a higher and earlier acrophase in cortisol concentration than LCTs. It has been proposed that the CAR is believed to act as a 'booting mechanism' in order to physiologically prepare a person for daily tasks (Anderson and Wideman, 2017). It is also evident that in athlete cohorts, exercise interventions can display an increase in CAR following training, with increases in a triathlon cohort (Gouarné et al, 2005) and football (Minetto et al, 2008) respectively. As training

interventions aim to improve performance, an adaptation such as an increased CAR as a response, would allude to its beneficial effects in athletic performance (Anderson and Wideman, 2017). Within our participant cohort, only ECTs performed significantly better in the incremental test to volitional exhaustion, reaching a greater VO2 max than the LCTs at 08:00 hrs. Similarly, ECTs performed significantly better in the PASAT test at 08:00 hrs than LCTs. It is possible that these results occurred as a response to a heightened CAR, however we did also uncover significant improvements and increases in the LCTs performance over the three testing sessions, with significantly improved performance in both the handgrip strength test and the PASAT at their preferred time of day (20:00 hrs). Therefore, it may be that CAR is an appropriate biomarker for predicting peak performance, however there is insufficient evidence in this study to provide causal conclusions.

Limitations of the present study

Although efforts were made to ensure that our procedures were fully robust, we acknowledge that there are limitations to our methodology. In comparison to that of Facer-Childs, Boiling and Balanos (2018), our sample size was small, and could mean that our results do not possess sufficient power to in order to be generalised to the overall population (Faber and Fonseca, 2014). As our participant cohort displayed an average age of 24±4, uncovering true chronotypes was difficult, as younger adults appear to be distributed more so in the intermediate category than an older population (Biss and Hasher, 2012). Therefore, replication of this study using a larger sample would be advantageous to deciphering the impact of chronotype; especially important when smaller effects and interactions are of interest (Vetter, Juda and Roenneberg, 2012).

It is possible that the self-reported and subjective nature of using questionnaires to identify chronotypes could be subject to multiple levels of response bias (Donaldson and Grant-

Vallone, 2002). The use of actigraphy watches as an objective measure of daytime activity and sleep/wake durations would provide further consolidation of the chronotype outcomes (Cochrane, Robertson and Coogan, 2012), as Goldstein et al (2007) suggested that no strong conclusions regarding chronotypes should be drawn without using objective measures. In our study, we used two effective chronometric questionnaires to minimise the chances of finding an incorrect chronotype (Zavada et al, 2005). This makes our research more reliable than many other chronotype based studies which only rely solely on one chronometric questionnaire. Therefore, correct procedures have been implemented where possible.

We also relied on the participants to produce and store the cortisol samples at home on the morning of the 14:00 hrs session. Incorrect storage and transportation of the samples prior to laboratory testing could have affected the quality of the samples. As the sensitivity of the analytic techniques are at a high degree, it is important that the samples are of a high quality (Vaught and Henderson, 2011). To minimise the detrimental effects of spoilt samples, all participants were provided with a cortisol sampling sheet, explaining the correct procedures and sampling methodology that they should adopt at home. Participants were also asked to document the exact time at which they took the sample, along with any issues that they may have encountered that might affect the sample. However, this could be subject to social desirability bias (Donaldson and Grant-Vallone, 2002). Upon replication, it would be beneficial to the reliability of this study to ensure that participants are monitored when completing their samples, perhaps scheduling a sleeping night at the laboratory the night before completing the samples.

There are multiple references in literature and throughout this paper to the effects of core body temperature on strength and maximal performance (Racinais et al, 2005) (Teo, Newton, and McGuigan, 2011) (Waterhouse et al, 2005). Incorporating core body temperature into this study could have provided a more holistic understanding of the internal rhythms and

how this exhibits its effect on our physical and stress measures of performance. We are therefore aware that our results cannot be attributed to changes in chronotype alone. Similarly, it would be interesting to look at the effects that time since entrained awakening had on our results, as Facer-Childs and Brandstaetter (2015) highlighted that this was the most important predictor of peak performance within their cohort.

It must be acknowledged that there were issues with the methodology and sampling techniques implemented within this study that may hinder its validity. However, we have employed multiple strategies to minimise the detrimental effects of these issues. Despite these limitations, our results are coherent with multiple previous findings on the physical performance responses (Facer-Childs, Boiling and Balanos, 2018), stress reactivity responses (Marvel-Coen, Nickels and Maestripieri, 2018) and cortisol awakening responses (Bailey and Heitkemper, 1991) of different chronotypes.

Conclusions

In conclusion, this study has highlighted the importance of exclusively separating participants into chronotypes when measuring performance and stress reactivity. ECTs performance in both a maximal exercise test and a stress inducing PASAT test will be significantly improved at their optimal time of the day. Similarly, LTCs handgrip strength and PASAT performance will be greater than their ECT counterparts at their optimal time. Moreover, we have found that a psychological stress test will induce increased secretion of cortisol in both chronotypes. Following this, a physiological stress test (VO_2 max) will further increase this level of circulating cortisol in both chronotypes. Our research also suggests that ECTs present a faster and increased cortisol response to awakening than LCTs, consistent with the current body of literature. From these results, we have been able to speculate that the cortisol fluctuations and responses to multiple stress tasks between chronotypes are not able to

account for changes in we see in performance. Our findings have added considerably to the current field of research, providing multiple results that will be advantageous in elite sports settings. Chronotype should be exploited in training and competition scheduling of events, as it has provided clear responses across all the physical measures used, however the intensity and length of the performance should be considered. Our research provides clear evidence that including chronotype in strategies to maximise performance will be beneficial in providing a firm basis to the athlete to enable them to thrive. These results are not only applicable to an athletic population, but can also stimulate significant interest in multiple population's e.g. shift workers, emergency services and the military (Facer-Childs, Boiling and Balanos, 2018).

Although cortisol levels following psychological stress and physiological stress testing increased in both chronotypes, there were minimal chronotype differences between the responses, and no effect on performance was apparent. It would be therefore beneficial for future studies to focus on chronotype CAR differences as a predictor of peak performance, as this is a non-invasive and easily attainable biomarker that has produced promising results in our study and others. Our research provides a firm basis for further scientific exploration.

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