The power of chronotype – an investigation into the genetic bases of diurnal preference and mental health

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Master of Science by Research Thesis

September 2022

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Abstract

Chronotype is a continuum of diurnal (time-of-day) preferences based on the underlying circadian rhythm. It stems from genetic differences and is modulated by the environment, especially light cues. Those on the late (evening) end of the spectrum often suffer from social jet-lag and can be more prone to mental health issues. This study confirms this pattern and provides further proof that although evening-type individuals often struggle, they also perform better on fluid intelligence tests than their morning-type peers. Here, we screened a population of university students aged 18-41 (n=100) using online questionnaires and investigated the genetic changes in the *CRY1* gene of those who could attend a face-to-face meeting (n=32). Within this group, we uncovered a significant association of chronotype with age, depression, IQ, and positive affect. We also found three distinct SNPs (rs8192440, rs1056560, and rs8192441), out of which the rs8192440 polymorphism was the most frequent one, having been found in 76.7% of the studied population. None of these SNPs were found to be significantly associated with any of the studied variables. Despite this, we suggest further study of the rs8192440 and rs8192441 polymorphisms as they show promise regarding associations with mental health, chronotype, and sleep quality. Moreover, we show that the COVID-19-related lockdowns had a negative effect on the well-being, sleep quality, and self-perceived academic performance of students, while simultaneously allowing for a delay in chronotype and prolonged sleep duration. Overall, our data supports the idea that chronotype-based interventions such as delayed starting times at schools and workplaces for those with evening chronotype should be implemented to reduce the effects of social jet-leg. However, we suggest that this should be done in a structured fashion to avoid possible negative effects of delayed sleep phase such as a decrease in sleep quality and mental well-being, that could be observed during the COVID-19 pandemic.

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Acknowledgments

First, I would like to acknowledge and thank my supervisors Dr. Alpar Lazar and Prof Tamas Dalmay for their ongoing support and mentorship. Second, Dr. David Prince for his invaluable guidance in the lab, help with DNA amplification issues, and sending my samples off for sequencing when I could no longer do it myself. Third, every member of the Lazar research group - you taught me more about sleep than I ever imagined I would know as a molecular biologist. Fourth, all of my friends and family who supported me throughout this degree – I could not do it without you. And finally, to all of the participants of this study – this thesis would not exist without you and I will be forever grateful for your contribution.

Introduction

Circadian rhythms and chronophysiology

Being an evening or morning person represents an individual's underlying circadian rhythm. This rhythm, with its approximately 24 h period, manifests itself through a range of behavioural and biological functions, from socialization and productivity, through sleep-wake patterns, hormone secretion, and body temperature to gene expression (Adan *et al.*, 2012). Modern research in the field of circadian rhythmicity traces back to the work of Colin Pittendrigh and Jurgen Aschoff, who defined the basis of circadian entrainment. Pittendrigh (1960) proved that deviation from the 24 h cycle provides a mechanism for alignment of the internal time-keeping system, allowing the rhythm to be "reset" where necessary. This discovery allowed us to understand how some mammals can re-establish their clock after a period of living in extreme conditions. For example, the free-living Arctic ground squirrels do not express circadian rhythmicity during hibernation in winter but do show robust daily circadian body temperature oscillations over 24 h during their active season (Williams, Barnes and Buck, 2012; Williams *et al.*, 2012; Williams *et al.*, 2017). The same ability to reset and adjust circadian rhythmicity can be seen in humans when we travel to a different time zone or work shifts with different start points throughout the month. The environmental cues that allow for this shift in daily rhythmicity to happen, are called zeitgebers and include the time of feeding, time of exercise/activity, and probably most importantly, light cues (Schroeder *et al.*, 2012; Jilge, 1992; Stephan, 2002; LeGates, Fernandez and Hattar, 2014). It is the exposure to light that can have the effect of delaying or advancing the timing of the molecular clock. It is known that during the middle of the subjective day, when light is expected, it has no effect on the circadian phase. However, a light pulse administered around subjective dusk causes a phase delay, whereas a light pulse near subjective dawn causes a phase advance (Panda, Hogenesch and Kay, 2002). This is what enables organisms to synchronize their physiology with changing seasons and for humans specifically, to adjust to new time zones and working conditions. This relationship is often described by the Phase Response Curve (PRC) (Figure 1), which can

be used to determine when to administer melatonin and/or light in order to advance or delay the circadian phase (Arendt, 2018; Murray *et al.*, 2021). Interestingly, it has also been found that all mammalian species capable of chromatic vision do not only react to light itself but also possess a sensory mechanism for telling the time of day by changes in the spectral (colour) composition of light, which occur during dawn and dusk (Walmsley *et al.*, 2015).

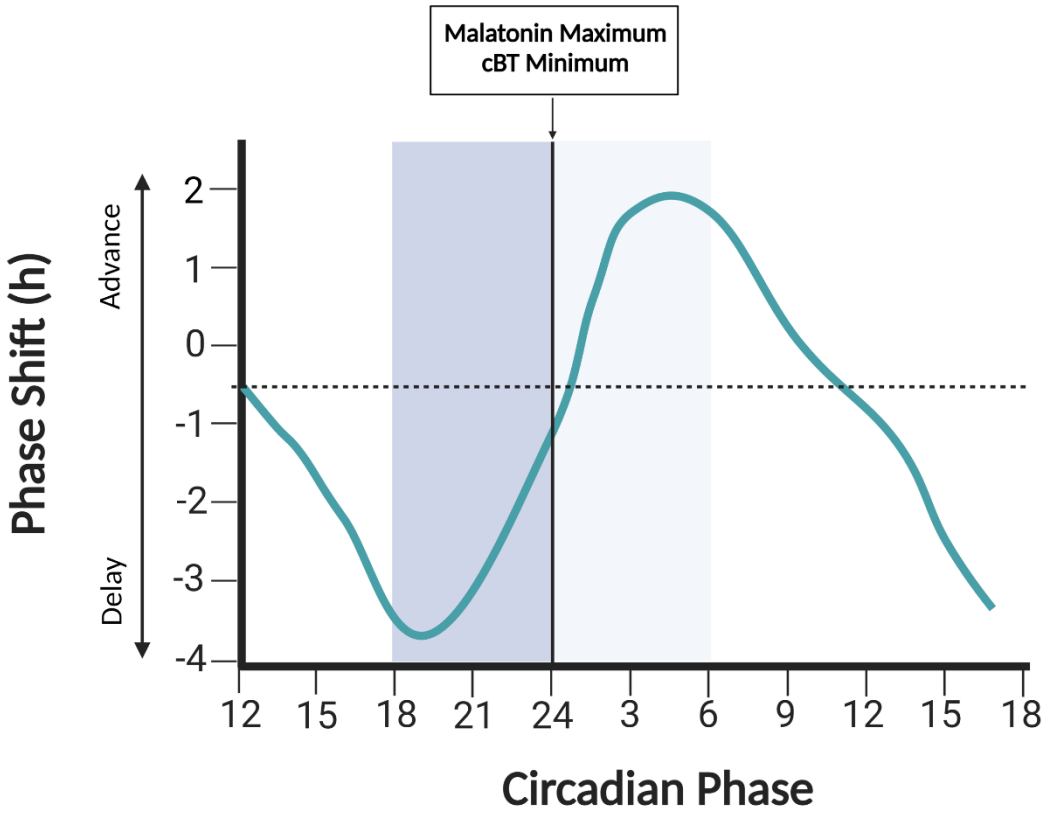

Figure 1. Exemplary Phase Response Curve (PRC) for light. Positive values on the y-axis indicate a phase advance and negative values indicate a phase delay. Light exposure prior to the melatonin maximum and cBT (core body temperature) minimum delays circadian phase and after this point advances circadian phase. Biological night is indicated by the blue/grey bar where the darker shade encompasses the circadian time at which light will induce a maximal phase delay and the lighter shade encompasses the time at which light will induce a maximal phase advance. Adapted from Murray *et al*. (2021)

Furthermore, recent research shows that circadian rhythmicity affects virtually all cells and systems in the body. A prime example is how changes in the light environment can result in important changes in endocrine function, modulating the release of several, if not all, endocrine signals according to time of the day (Czeisler *et al.*, 1995; Cajochen *et al.*, 2010; Paul and Brown, 2019). Moreover, a study by Hoyle *et al*. (2017)

showed that the circadian clock has a profound effect on the wound healing response in mice and humans. The authors observed that the cellular clock modulates the efficiency of actin-dependent processes such as cell migration and adhesion, which ultimately affect the efficacy of wound healing. Accordingly, the experimental results showed an increased fibroblast invasion for skin wounds incurred during a mouse's active phase, and a 60% faster healing of daytime wounds than night-time wounds in humans (Hoyle *et al.*, 2017). Another example can be skeletal muscle performance, which is known to rely on the time of the day. Numerous studies have shown that muscle strength is higher in the late afternoon, compared to the morning (Sedliak *et al.*, 2009; Zhang *et al.*, 2012). It has also been proven that individual chronotype plays an important role when it comes to optimal physical performance. For example, Facer-Childs and Brandstaetter (2015) have found that sports teams with a large proportion of late types were disadvantaged in morning competitions, while teams with a large proportion of early or intermediate types were disadvantaged in evening competitions.

Last, but not least, there is strong evidence that circadian rhythms deteriorate with age at both the behavioural and molecular levels, leading to age-associated changes in tissue physiology, including impaired stem-cell maintenance and tissue deterioration (Rogers, Hunt and Pekovic-Vaughan, 2018). It has been found that genetic and environmental disruptions to the molecular clock can affect such processes as cellular antioxidant signalling and protective mechanisms (Pekovic-Vaughan *et al.*, 2014; Stringari *et al.*, 2015; Kondratov *et al.*, 2009; Goljanek-Whysall *et al.*, 2016) as well as cellular differentiation of specific cell types including adipogenesis (Shimba *et al.*, 2005), neurogenesis (Malik *et al.*, 2015), osteogenesis (Samsa *et al.*, 2016), angiogenesis (Bhatwadekar *et al.*, 2017) and myogenesis (Chatterjee *et al.*, 2015; Andrews *et al.*, 2010). Therefore, disturbances in this temporal coordination have been implicated in a variety of pathologies including age-related chronic diseases and cancer (Zhang *et al.*, 2017; Okazaki *et al.*, 2016; Rogers, Hunt and Pekovic-Vaughan, 2018). Moreover, clock disruption in mice leads to reduced muscle and bone mass as well as increased levels of metabolic obesity (Andrews *et al.*, 2010; Samsa *et al.*, 2016; Shimba *et al.*, 2005), which are phenotypes often associated with human ageing. However, even though the molecular clock works through physiological means, circadian rhythms not only affect

physiological health and well-being but also a mental one, virtually influencing every aspect of our lives.

Chronotype and the problem with eveningness

When talking about the relationships between psychopathology, cognitive ability, and circadian rhythmicity, the focus usually falls on either, sleep and its effects on the individual, or the so-called chronotype. The latter is a term that relates to the sleep-wake patterns based on an individual's circadian rhythm, which forms a continuum of diurnal (time-of-day) preferences. There are three main chronotypes: morning, intermediate, and evening, with some extreme cases at each end of the spectrum. Most people present with intermediate chronotype, with only about 40% being classified as morning and evening types, respectively (Adan *et al.*, 2012). Morning-type individuals or the so-called 'morning larks' show a preference for waking up and working early during the day when they achieve most of their physical and mental ability. Evening-type individuals, or the so-called 'night owls' are the opposite, and prefer later wake-up and bedtimes, achieving peak productivity towards the end of the day. These preferences are based on genetic (both hereditary and random mutations in the genome) and environmental influences (e.g. amount of light exposure), and modulated by age, sex, and social activities. Men typically present a more delayed chronotype than women before the age of 40, but both sex groups present the greatest variation in chronotype between the age of 15 and 25, with a clear preference towards eveningness in adolescence and towards morningness in childhood and after the age of 50 (Fischer *et al.*, 2017).

Furthermore, it is now known that circadian preference is related to both physical and mental health (Basnet *et al.*, 2017). Eveningness has been found to be associated with lower sleep quality (Bavarsad *et al.*, 2015), higher BMI (Anothaisintawee *et al.*, 2018), increased risk of depression (Kitamura *et al.*, 2010; Merikanto *et al.*, 2015), bipolar disorder (Giglio *et al.*, 2010; Melo *et al.*, 2017), anxiety (Taylor and Hasler, 2018, Fares *et al.*, 2015), and addictive disorders including alcohol and illegal drug consumption,

compulsive internet use, and excessive engagement with computer games and social media (especially among young people) (Prat and Adan, 2011; Lin and Gau, 2013; Vollmer *et al.*, 2014). Evening-type individuals are also much more likely to develop a delayed sleep phase syndrome (DSPS), which appears as a two or more hours delay in the sleep-wake timing when compared with the socially accepted norm. Given that school attendance or other work-related duties still require an early morning start, individuals with evening chronotype and, therefore, delayed sleep timing, will end up with considerably shorter and poorer sleep during the weekdays compared to their morning type peers. This usually results in social jet-lag (discrepancy between the natural biological clock and the social norm) characterized by chronic fatigue, daytime sleepiness, low mood, and academic difficulties, perpetuating the difficulties to synchronize their internal rhythms with external requirements (Sharma and Feinsilver, 2009). On the other hand, it has also been shown that the evening chronotype is associated with higher general intelligence (IQ), emotional intelligence (EI), and cognitive ability (Kanazawa and Perina, 2009; Piffer *et al.*, 2014; Stolarski and Jankowski, 2015; Preckel *et al.*, 2011). However, these relationships tend to vary depending on the features of the studied group and it has been suggested that when it comes to mental health disorders, evening types might simply be overrepresented, while morning types are underrepresented (Kivelä, Papadopoulos and Antypa, 2018; Müller *et al.*, 2015). It has also been argued that the relationship between intelligence and evening chronotype might depend on social factors such as work timing (Ujma *et al.*, 2020) and not the internal biological clock. Overall, the evidence for a causal role of chronotype in mental health and cognitive ability is rather limited (Skarke *et al.*, 2017) and there is much room left for clarification of the strength and nature of these relationships.

So far, when it comes to the link between mental illness and chronotype, two main putative mechanisms have been suggested, namely: sleep/circadian disturbances caused by environmental factors and neural/psychological mechanisms with shared genetic factors (Taylor and Hasler, 2018). The former relies on the aforementioned idea of misalignment between the delayed sleep phase of evening-type individuals and the social expectations, which causes social jet lag, sleep loss, and disturbed sleep and therefore also lowered mood and eventually mental illness. The latter focuses on the

fact that the molecular clock is present in all cells of the body and controls most processes, including those responsible for psychological issues, such as the dopamine and serotonin secretion pathways (Parekh, Ozburn and McClung, 2015; Moore and Speh, 2004). Considering that the same genes are responsible for chronotype, the results of a variation appearing in one of those genes can be multidirectional, simultaneously influencing diurnal preference and neural processes that lead to altered reward processing, cognitive biases, impulsivity, and impaired emotional regulation. On the other hand, it is important to notice that the same mechanisms can cause positive changes like improved cognitive ability/higher IQ so an exploration of these relationships can be highly beneficial not only from a clinical sense (treatment of mental illness) but also social sense (helping individuals to reach their full potential).

The molecular clock

Circadian rhythmicity and entrainment by environmental cues are orchestrated by a hierarchy of oscillators and governed by the molecular clock. In mammals, the circadian pacemaker that coordinates a number of independent central nervous system (CNS) and peripheral tissue oscillators, to regulate a coherent rhythm at the level of the whole organism is referred to as the central clock and lies in the suprachiasmatic nucleus (SCN), located in the anterior hypothalamus (Dibner, Schibler and Albrecht, 2010). Recently our view on this extraordinary organ has been revolutionised as we discovered its input and output mechanisms. These include the discovery of a new visual pathway from the retina to the SCN involving melanopsin and ipRGCs (intrinsically photosensitive retinal ganglion cells – photoreceptors which are particularly sensitive to the absorption of short-wavelength (blue) light), that entrains circadian rhythms to the solar day, and the clarification of ways in which the SCN clock generates output rhythms in physiology and behaviour (Reppert and Weaver, 2002).

The molecular machinery behind circadian rhythmicity is present in every cell of the body and works on the basis of two main transcriptional-translational loops. First discovered in the fruit fly *Drosophila Melanogaster*, through a series of experiments

assessing circadian rhythmicity in mutants with different variants of clock genes (*period* gene being the first one), it has now been studied in other species, including mammals, finally providing a relatively good understanding of how our own biological clock works (Panda, Hogenesch and Kay, 2002). The clock mechanism involves the interaction of positive and negative transcriptional feedback loops that drive recurrent rhythms in the RNA and protein levels of key clock components within the cytoplasm and nucleus of the cell (Figure 2) (Reppert and Weaver, 2002). The cycle starts when a heterodimer of two bHLH-PAS (basic Helix-Loop-Helix/Per-ARNT-SIM) domain-containing transcription factors, CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT-Like 1), binds to the E-box in *Per (Period)* and *Cry (Cryptochrome)* promoters, and promotes transcription of three *period* and two *cryptochrome* genes. The resulting PER and CRY proteins upon accumulation in the cytosol bind to each other and translocate into the nucleus, where they act as a negative regulator by directly interacting with CLOCK and BMAL1 to inhibit transcription, closing the first negative feedback loop (Shearman *et al.*, 2000). The second feedback loop involves opposing actions of REV-ERBα (nuclear receptor subfamily 1, group D) and RORα (RAR-related orphan receptor alpha) nuclear receptors, which control the rhythmic expression of BMAL1, with RORα promoting *BMAL1* transcription and REV-ERBα inhabiting it (Preitner *et al.*, 2002). Additionally, the CLOCK-BMAL1 heterodimer also promotes the transcription of several other noncore clock and metabolic genes, called CCGs (clock-controlled genes). This whole process takes approximately 24 h and repeats periodically to create anticipation and allow the organism to prepare for an extracellular stimulus before its onset (Edery, 2000; Bailey, Udoh and Young, 2014). However, it is important to mention that beyond the transcriptional-translational feedback loops, post-translational modifications also play an important role in ensuring the appropriate operation of the clock mechanism, as well as allowing for the entrainment of the mechanism with the environment. A good example of that is the process of phosphorylation of PER protein, which allows for regulation of PER stability and degradation of the excess of this protein to keep the protein turnover timing in check (Harms *et al.*, 2004).

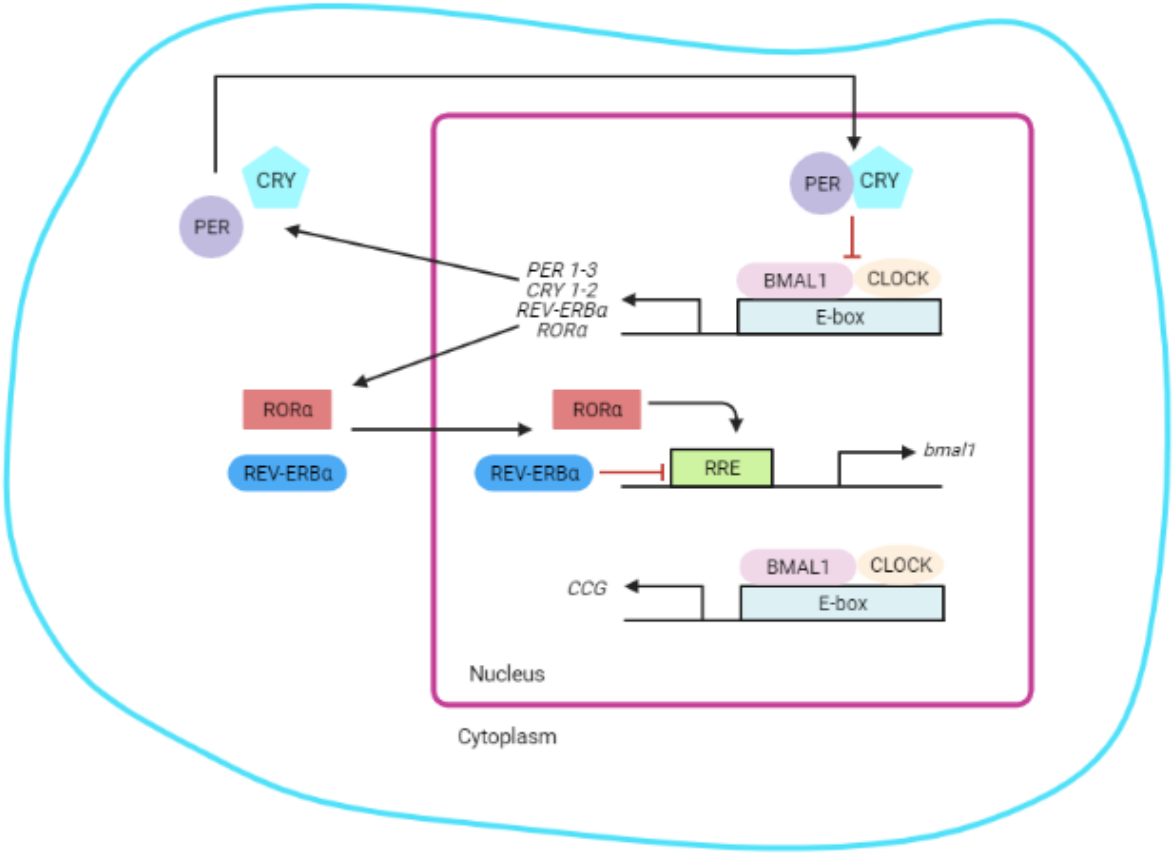

Figure 2. The mammalian circadian clock. The mechanism relies on transcriptional-translational feedback loops comprising the transcription factors BMAL1 and CLOCK. When BMAL1/CLOCK heterodimer binds to the E-box elements of specified genes, it induces transcription of *PER1-3, CRY 1-2, REV-ERBα, RORα,* and other clock-controlled genes (CCGs). When enough PER and CRY proteins accumulate in the cytoplasm, they form a heterodimer, which is then translocated back to the nucleus and interferes with BMAL1/CLOCK, inhibiting its action and repressing transcription until there is once again a lack of PER and CRY proteins. The second feedback loop involves RORα acting as an enhancer and REV-ERBα as an inhibitor for transcription of the *BMAL1* gene. This controls the rhythmic expression of *BMAL1*, which is essential for the generation of circadian rhythms.

BMAL1 - brain and muscle ARNT-like 1; CLOCK – circadian locomotor output cycles kaput; *CRY1-2* – cryptochrome 1-2; *PER1-3* – period 1-3; REV-ERBα – nuclear receptor family 1, group D; RORα – RAR-related orphan receptor alpha; RRE – retinoid response element. Adapted from Bailey et al. (2014).

Furthermore, the circadian system is among the most tractable models for providing a complete understanding of the cellular and molecular events connecting genes to behaviour. Through the dissection of the genetic bases of circadian behaviour, we can increase our knowledge of how gene mutations of the core clock genes contribute to not only chronophysiology (e.g. cell maintenance and tissue repair) but also psychopathology (e.g. depressive disorders) and cognitive ability (Schmidt *et al.*, 2007; Rogers, Hunt and Pekovic-Vaughan, 2018; Ujma *et al.*, 2020), which is the focus of this study.

Genetics of evening chronotype

It has been found that due to a difference in a fundamental aspect of circadian timing system - intrinsic period (shorter in morning-types and longer in evening-types), morning-types wake up significantly later within the circadian cycle than evening-types do, at a time when the drive from circadian timing system for sleep is decreasing and circadian rhythms of alertness and performance are rising. The exact opposite happens for evening-types, causing difficulties with awakening and decreasing morning performance (Duffy, Rimmer and Czeisler, 2001). Considering that interindividual differences in circadian period arise from differences at a genetic level and that circadian period and circadian phase (which is delayed in evening-types) are strongly correlated, the genetic code is one of the main contributing factors to the establishment of diurnal preference in an indiviudal.

Moreover, knowing how relatively small the effect of the environment is on the genome, genetic analysis could provide the information that would improve the causal understanding and knowledge of the relationships between chronotype, mental health, and intelligence. Indeed, four genome-wide association studies (GWAS) have already been carried out and identified a total of 351 chronotype-associated loci (Hu *et al.*, 2016; Lane *et al.*, 2016; Jones *et al.*, 2016; Jones *et al.*, 2019), which can now be used in studies using the candidate gene approach to perform an in-depth functional investigation into chronobiology. Moreover, the most recent study by Jones *et al*. (2019) not only found genetic correlations between well-being, schizophrenia, depressive syndromes, intelligence, and chronotype, but it also identified 10 coding variants with a high likelihood of being causal. Some of the most strongly associated loci were those containing well-known circadian rhythm genes including *RGS16, PER1, PER2, PIGK/AK5, FBXL3, INADL, HCRT2, and HTR6*, as well as *PER3* and *CRY1* genes. Polymorphisms in both of the latter genes have been previously found to be associated with eveningness and delayed sleep phase syndrome (Archer *et al.*, 2010; Patke *et al.*, 2017; Ebisawa *et al.*, 2001).

Out of the two, *PER3* has been investigated further, with numerous studies looking into the effects of genetic mutations in this gene on diurnal preference and mental health. Following an in-depth screening of *PER3* by Ebisawa *et al.*, (2001), which discovered that structural polymorphisms in the gene can be associated with DSPS, a number of other studies have focused on the variable number of tandem repeats (VNTR) polymorphism in *PER3*, validating its relevance for the prediction of chronotype and DSPS, as well as suggesting a relationship with mental health (Archer *et al.*, 2003; Viena *et al.*, 2016; Weiss *et al.*, 2020). It has been found that the frequency of the 5-repeat allele is significantly higher in morning-type individuals and the 4-repeat allele in evening-type individuals. The shorter allele was strongly associated with DSPS patients, 75% of whom were homozygous for the 4-repeat allele (Archer *et al.*, 2003). Moreover, a study by Lazar *et al*. (2012) revealed that individuals homozygous for the 5-repeat allele exhibited higher morning preference compared to both homozygous and heterozygous genotypes, suggesting a dominant effect of the 4-repeat allele. This association between dirunal preference and *PER3* VNTR has been shown to most likely stem from the differences in sleep homeostasis and structure between individuals with the different version of the polymorphism. Viola *et al*. (2007) found that sleep pressure builds faster for indivudals with the PER3 (5/5) genotype and sleep deprivation has a higher impact on their cognitive performance when comparaed to PER (5/5) indivudals. This would mean that the mornigness of those with the PER (4/4) genotype is not caused by a change in the circadian rhytm, but simply a stronger need for an earlier bed-time due to a different sleep structure.

Interestingly, on the contrary to what Viola *et al*.'s (2007) findings on cognitive performance during sleep deprivation might suggest, it has been shown that PER3 (4/4) genotypes are at greater risk for transient psychological effects when reduced sleep duration is reported (Viena *et al.*, 2016). However, a more recent study by Weiss *et al.*, (2020) found that VNTR genotypes had marginal effects in predicting depressive symptoms in regression of mid-sleep point on a free day (MSF) based chronotype and sleep disturbance. The authors suggested a possible gender effect in the association between the PER3 VNTR polymorphism and MSF chronotype, as males with PER3 (4/4) genotype were found to be nine times more likely to be evening-types than other

genotypes, with those evening types reporting a positive association with higher depression scores. Moreover, a follow-up to a study by Hida *et al*. (2014), which found a Single Nucleotide Polymorphism (SNP) rs228697 in *PER3* to be significantly associated with diurnal preference (where the presence of the major allele C was more common in morning types and the minor allele G more common in evening types), showed a sex-specific association of this SNP with the depressive syndrome (Shi *et al.*, 2016). Additionally, a study by Archer *et al*. (2010) has found a number of polymorphisms in the *PER3* promoter, which were associated with DSPS. Considering the role of the promoter in *PER3* expression, these polymorphisms might also have an effect on the phenotypic associations with previously described mutations in the gene itself.

Although the gene *CRY1,* has not been investigated so thoroughly, some good evidence has been found linking it to chronotype, DSPS, and mental health. A recent GWAS study carried out in the Finish general population has revealed 3 independent association signals with evening chronotype within *CRY1* (rs8192440, rs77706154, and rs1017168A), one of which (rs1017168A) has previously been discovered to be associated with evening type (Jones *et al.*, 2019; Maukonen *et al.*, 2020). Another example comes from a study by Patke *et al*. (2017), which has found an association between a hereditary form of DSPS with a dominant coding variation (*CRY1*Δ11c.1657+3A>C) in *CRY1*. This gain-of-function variant was discovered to cause reduced expression of key transcriptional targets and lengthen the period of circadian molecular rhythms. Moreover, the allele had a relatively high frequency of up to 0.6%, suggesting that it might affect sleep behaviour in a sizable portion of the human population (Patke *et al.*, 2017). Another study confirmed this claim by running a phenome-wide association study involving 9438 unrelated Europeans, which also showed a significant association with major depressive disorder, insomnia, and anxiety (Onat *et al.*, 2020). Furthermore, another study found a significant association between an SNP (rs2287161) in *CRY1* and mood disorders (in this case unipolar major mood depression (MDD) and bipolar disorder (BD)) (Soria *et al.*, 2010). Interestingly, this association within the MDD subsample remained statistically significant, even after permutation correction at the experiment level. An independent study by Hua *et al*. (2014) confirmed this finding for the Chinese population and found the rs2287161 *CRY1* polymorphism to be significantly

associated with major depressive disorder. However, the amount of research relating to the relationships between *CRY1,* chronotype, and mental health is still relatively low and these associations need further exploration.

The impact of COVID-19 lockdown on sleep habits and mental health

It is normally expected of most students and employees that they abide by the societal norms of waking up and going to bed early, working throughout the morning and early afternoon. This goes against the natural circadian rhythm of those individuals with evening chronotype, who usually achieve their peak productivity at the end of the day. It is suspected that such individuals might be characterized by generally high intelligence, which cannot be fully expressed due to social jet lag and associated social sleep restriction, resulting in overall lower academic performance and well-being. This misalignment with societal norms might also lead to higher rates of depression, especially if the individuals start experiencing societal pressure to achieve more than they are capable of when struggling with daytime sleepiness and low productivity.

The recent situation related to the global pandemic of COVID-19 created the possibility of the introduction of new work/study schedules. A few studies have already found that lockdown has significantly reduced social jet lag and allowed for a generally healthier sleep behaviour with participants sleeping longer (>7 hours per night) and more in line with their chronotype due to more flexible work hours (Leone, Sigman and Golombek, 2020; Blume, Schmidt and Cajochen, 2020; Wright *et al.*, 2020). However, decreased sleep quality was also reported, most likely due to the stress associated with a global pandemic. Moreover, when chronotype was not taken into account most studies found a significant decline in sleep quality, activity levels, and well-being, especially in university students (Gupta *et al.*, 2020; Romero-Blanco *et al.*, 2020; Martínez-de-Quel *et al.*, 2021; Marelli *et al.*, 2021). In this study, we want to follow up on these findings and investigate the impact of the COVID-19 pandemic on sleep schedules, mental health, and academic progression in relation to chronotype and polymorphisms

found in the clock genes. This should provide a deeper understanding of the importance of following one's internal clock and whether some of the changes introduced throughout the pandemic shouldn't be retained to create a healthier society.

Aims of the study

The aim of this study is to deepen the understanding of the role chronotype, specifically, eveningness plays in influencing brain function with a focus on mental health and intelligence. We hypothesize that evening-type people might be more capable of developing higher IQ due to genetic factors, while simultaneously becoming more exposed to mental health disorders. Therefore, we aim to look into the genotype of evening-type individuals with respect to a core clock gene, *CRY1*, known to be associated with eveningness and mental health disorders (Patke *et al.*, 2017). We want to investigate whether specific mutations in this gene are predictive for self-reported mental health and objectively measured intelligence and to what extent these genetic effects are modulated by chronotype, sleep, sex, and age. This study should help clarify the strength and nature of associations between these different factors as well as extend our understanding of the genetic bases that underline the evening chronotype. It could prove to be another step in the direction of more flexible working hours, that could accommodate individuals with later chronotypes and allow them to reach their full potential.

Moreover, the mutations that might be identified in the course of this study could provide other scientists in the fields of neurobiology and genetics with the material needed to carry out more sophisticated experiments using model organisms and gene-editing technologies such as the CRISPR-Cas9 system for the exploration of the knock-in/out effects of these specific mutations.

In this study, we are focusing on university students as they usually represent the young, healthy, and highly intelligent part of the population, which allows for the elimination of additional detrimental factors that may come with age, as well as opening

the door to investigation of the impact of chronotype on academic performance, class attendance, student satisfaction, and mental health. As previous studies have shown, students are a group that has been highly affected by COVID-19 lockdowns, and shows a high variety in mental health and sleep quality. We are aware that focusing on students means that the differences in IQ, as well as the age of our participants, might not be substantial, but we believe that we will still be able to observe interesting effects based on the differences in mental health and sleep quality relative to chronotype and genotype between sexes. This is a pilot study that we aim to keep explorative in nature, investigating associations between all of the studied traits and not only the genotype.

Materials and Methods

Ethics

The study was reviewed and accepted by the ethical committee of the University of East Anglia. It was conducted in accordance with the principles of the Declaration of Helsinki and the Human Tissue Act.

Recruitment of subjects

Subjects for the study were recruited through the following channels:

- *The sleep and brain research group website*- the study was advertised on this webpage providing visitors with the opportunity to contact the researcher if they were interested in taking part (https://www.uea.ac.uk/web/about/school-of-health-sciences/research/sleep-and-brain-health/sleep-and-brain-research-unit)
- *Advertisements (online, print, media, and public spaces)* – social media was used to post an advertisement of the study, as well as an invitation email being sent to student groups expressing interest in taking part in the research. Gatekeeper consent was obtained for the propagation of email invitations within specific courses or student organisations. Posters advertising the study were also used at the University of East Anglia campus.

Upon expression of interest all potential participants were notified of the preliminary inclusion criteria for participation in the study which included:

- Being aged 18-35 years old or/and being a student (students older than 35 were also accepted into the study)
- Proficiency in the English language
- Capacity to consent
- Availability to take part in the study

Additionally, participants who fulfilled these criteria and expressed interest to participate in the face-to-face portion of the study were included in that part of the study if they did not show symptoms of any acute infections or diseases.

Screening through online questionnaires

Participants identified through the recruitment sources mentioned above were invited to provide informed consent (in form of an electronic signature on an online consent form) and asked to complete a series of online questionnaires that would assess their mental health, general health, sleep quality, chronotype and the impact of COVID-19 on their mental health and sleeping habits. Morningness-Eveningness Questionnaire (MEQ) (Horne and Ostberg, 1976) and the Munich ChronoType Questionnaire (MCTQ) (Roenneberg, Wirz-Justice and Merrow, 2003) were implemented to assess the chronotype of the volunteers, combined with the 9-item Patient Health Questionnaire (PHQ-9) (Kroenke, Spitzer and Janet, 2003) and the Generalized Anxiety Disorder Questionnaire 7 (GAD-7) (Spitzer *et al*., 2006) to assess mental health. Sleep quality was measured by Pittsburg Sleep Quality Index (PSQI) (Buysse *et al.*, 1989). General health was additionally assessed by a shortened version of the General Medical Questionnaire (GMQ) designed by Dr. Alpar Lazar's research group. Moreover, a new questionnaire was created for this study to assess the impact of COVID-19 on the mental health, academic performance, sleeping habits and sleep quality of each individual (Appendix 1). Participants also received an option to fill in three additional questionnaires, including the Mannheim Dream Questionnaire (Schredl *et al*., 2014), the Positive and Negative Affect Schedule (Watson *et al*., 1988), and the Big-five questionnaire (BIG-5) (Goldberg, 1992) to assess their personality, emotional disposition, and dream patterns. A longer description of these questionnaires can be found in Appendix 2. All questionnaires were re-created using Microsoft Forms and made available in an online form. Each participant was also assigned an identification code to use while filling in the questionnaires. A total of 119 participants consented to take part in the study out of which 114 consented to take part in the genetic analysis involving a

buccal swab. However, due to the online nature of the screening that only allowed for partial supervision of the participants during the screening session, the priority questionnaires were fully filled in and returned by 94 subjects out of the 119 consenting volunteers.

Collection of genetic samples and intelligence testing

Additionally, individuals identified as either evening or morning types were invited to participate in the genetic analysis part of the study. Subject DNA was collected through a buccal swab, using the *Isohelix SK-1S* swabs. Participants were instructed not to consume any food or drinks other than water for half an hour before the swab was performed. The collection involved rubbing the swab against the inside of each cheek for at least 30 seconds (1 minute total). DNA was collected from a total of 39 participants during face-to-face meetings with the researcher. The same individuals were also asked to take a Raven's Advanced Progressive Matrices test (purchased from *Pearson*), which assessed their fluid intelligence. Subjects completed both Set I (10 questions) and Set II (36 questions) of the test, with Set I being used to familiarise participants with the type of questions used in the test. The result (in points) was converted into percentiles based on participants' age, according to the instructions provided. Percentile Ranks were then converted into IQ points using the table provided in the additional test materials (Appendix 3).

DNA isolation

The swabs were placed in 2ml *Eppendorf*™ tubes and DNA was isolated using the *QIAamp® DNA Mini* kit, following the manufacturer's instructions (spin protocol). This was done within 5 days of the collection of the sample to avoid unwanted DNA decay. Purified samples were stored in a freezer at -20°C until further use.

Measurement of DNA yield

The amount of pure DNA present in each sample was measured using *Thermo Scientific™ NanoDrop™ 8000* spectrophotometer. The machine was calibrated using Buffer AL (used for suspension of isolated DNA) and deionised water. Most samples showed a DNA yield in the range of 5-30 ng/µl, with some higher and lower yields being noted as well (Table 1)

Table 1. DNA yields.

Subject ID	DNA yield [ng/µl]
CHR000	9.81
CHR001	24.65
CHR002	32.93
CHR004	7.46
CHR005	7.19
CHR006	9.4
CHR007	11.6
CHR011	20.02
CHR012	12.5
CHR014	10.0
CHR017	24.1
CHR018	17.43
CHR022	5.16
CHR023	58.5
CHR025	5.37
CHR028	10.5
CHR032	5.5
CHR034	5.18
CHR038	36.4
CHR040	7.8
CHR042	10.64
CHR045	23.04
CHR049	12.41
CHR054	19.78
CHR065	7.23
CHR081	9.57
CHR082	7.87
CHR083	9.4
CHR086	9.65
CHR087	51.0
CHR091	7.55
CHR096	6.4
CHR098	27.73
CHR099	4.1
CHR102	6.1
CHR104	33.0
CHR106	5.0
CHR109	41.74
CHR114	32.85

Gene isolation for sequencing

Preparation of primers

To isolate the gene of interest (*CRY1*), six pairs of PCR primers covering 12 out of 13 exons of the gene, were designed for this study (Table 2). Exon 1 was excluded from the study as it consists mostly of a large untranslated region (UTR).

Table 2. Primers designed to amplify the coding regions (Ebisawa *et al.*) of *CRY1*. The sequences of the primers, start and stop positions on the gene, as well predicted size of each amplified fragment are given.

***CRY1* Exon**	**Direction**	**Sequence (5' – 3')**	**Start (in *CRY1* GenBank: EF015898.1)**	**Stop (in *CRY1* GenBank: EF015898.1)**	**Size of fragments (bp)**
2	Forward	GGAGGTAATAAGATGATAGGTTGG	76238	76724	487
2	Reverse	GGTAGTAGCTGTTGCTTCTGGG	76238	76724	487
3	Forward	GCATAATGCCTAGAATCTAATGGG	93095	93763	669
3	Reverse	GAAAGGCAAGACCACAGGAGGC	93095	93763	669
4-5	Forward	CAGAGGATGTCTAGAAGCTCAGG	96395	97392	998
4-5	Reverse	TTAACCATTTCTGTTAATCCTCCCCTTTCAA	96395	97392	998
6-7	Forward	TGAGTATGTGCTGCACAAGGACG	98256	99561	1306
6-7	Reverse	AGCTGAGATCACGTCATTGCACC	98256	99561	1306
8-10	Forward	CAGAACATAGGGAAGCTAACTTTGGAATGTTT	100331	101406	1076
8-10	Reverse	CTGGGTGCACATGTATGCATGC	100331	101406	1076
11-13	Forward	GAGCTGTCAACACTTCTGTGAGCCTAAA	105307	107307	2001
11-13	Reverse	GGTAGCTGTTCCTTCAAAATGATGATGTG	105307	107307	2001

PCR optimisation

To check if all the primers were working correctly, a test PCR was run using the standard protocol for use with Phusion® High-Fidelity DNA Polymerase (Table 3). No bands showed on a 1.5% agarose gel following electrophoresis, which indicated that the PCR reaction needed optimisation.

Table 3. Standard PCR protocol for use with Phusion® High-Fidelity DNA Polymerase.

Recommended Mix		
Component	**Amount in a 50 ul reaction**	**Final concentration**
Nuclease-free water	Make up to 50 ul	
5x Phusion HF Buffer	10 ul	1X
10 mM dNTPs	1 ul	200 uM
10 uM Forward Primer	2.5 ul	0.5 uM
10 uM Reverse Primer	2.5 ul	0.5 uM
Template DNA	variable	(Less than 250 ng)
DMSO (optional)	(1.5 ul)	3%
Phusion DNA Polymerase	0.5	1 unit
Recommended Programme		
Step	**Temperature**	**Time [sec]**
Initial denaturation	98°C	30
Cycle (x25-35)	98°C	5-10
	45-72°C	10-30
	72°C	15-60
		(30 sec per kb for genomic DNA)
Final extension	72°C	5-10

Table 4. PCR programme used for annealing temperature optimisation.

Programme		
Step	**Temp**	**Time [sec]**
Initial denaturation	98°C	30
Cycle (x35)	98°C	10
	Gradient	30
	72°C	30
Final extension	72°C	10

The first step was to check whether changing the annealing temperature would cause the PCR reaction to work correctly. To do this, two primer pairs were selected (Exon 2 and Exon 3) and a set of 8 reactions was prepared for each pair of primers using the recommended proportions of PCR reagents (Table 3). The reaction tubes were then

put into the MJ Research PTC-200 Gradient Thermal Cycler. A standard program was used, with a variation in the annealing temperature (Table 4). For this set of primers predicted melting temperatures (Liao *et al.*) were 63 and 64°, therefore a gradient of 60 to 69°C was set up, with the 8 samples starting on column 3 (60.8°C) of the thermocycler and finishing on column 10 (68.4°C). Following the PCR reaction, a 25ml 1.5% agarose gel was loaded with a 100 bp DNA ladder and the PCR samples and run at 120 V (10 V/cm) for 20 mins, then stained in a solution of ethidium bromide (6µl) and 1xTBE (100 ml). A scan of the gel was taken using the Typhoon™ FLA 9500 biomolecular imager. The image showed a DNA ladder, but no PCR bands, suggesting that further optimisation was needed.

Table 5. The changes in concentration of primers and dNTPs used in a PCR optimisation experiment.

Strip 1 – Variable Primer Concentration							
Tube	1	2	3	4	5	6	7
Forward primer volume (µl)	1	1.5	2	2.5	3	3.5	4
Reverse primer volume (µl)	1	1.5	2	2.5	3	3.5	4
Water volume (µl)	6	5	4	3	2	1	0
Total volume (µl)	8	8	8	8	8	8	8
Primer concentration in PCR (µM)	0.2	0.3	0.4	0.5	0.6	0.7	0.8

Strip 2 -Variable dNTPs concentration							
Tube	1	2	3	4	5	6	7
1 mM dNTPs volume (µl)	4	5	6	7	8	9	10
Water volume (µl)	6	5	4	3	2	1	0
Total volume (µl)	10	10	10	10	10	10	10
dNTP concentration in PCR (µM)	80	100	120	140	160	180	200

The next step was to vary the primer and dNTPs concentration in the PCR mix. Two sets of reactions (7 tubes per reaction) were set up with varying concentrations of the reagents mentioned above (Table 5). A PCR mix containing the rest of the necessary reagents in standard concentrations was then added to make up a 50 µl reaction. Primers for exon 2 were used for both sets of reactions. Additionally, a fresh 10 mM dNTPs mix was made to use in this experiment, as to eliminate the possibility that the

one which had been used previously, was either contaminated or out of date. A standard PCR programme was used (Table 3) with the annealing temperature set to 63°C. The same procedure as in the previous experiment was then repeated to run and scan the agarose gel loaded with a 100 bp DNA ladder and the products of the PCR reaction. In the case of both variables, the DNA ladders as well as the expected PCR products of the expected size could be seen on the image at every concentration (Figure 3). There was an exception at the 100 μM dNTPs concentration lane, where the band was quite faint, however, this was most likely caused by a loading error (Figure 3B). The result of these two optimisation experiments suggested that the main reason for the PCR not working previously was a bad set of dNTPs and a standard PCR protocol could be used for all of the following PCR reactions.

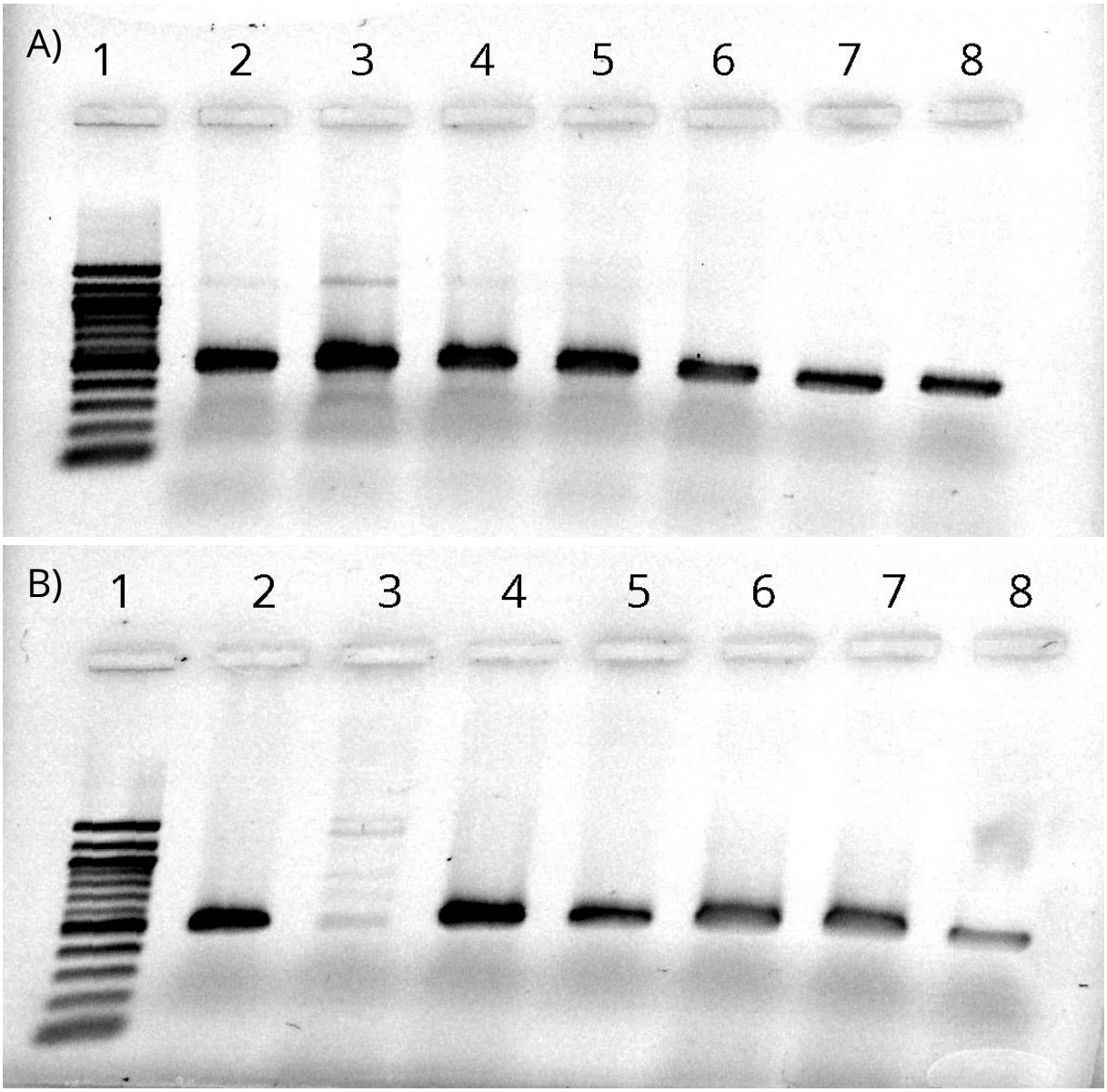

Figure 3. Gel electrophoresis analysis of PCR optimisation.

A) PCR with varying primer concentration.

Lane 1 – DNA ladder; Lane 2 – 0.2 μM primer concentration; Lane 3 - 0.3 μM primer concentration; Lane 4 - 0.4 μM primer concentration; Lane 5 - 0.5 μM primer concentration; Lane 6 - 0.6 μM primer concentration; Lane 7 - 0.7 μM primer concentration; Lane 8 – 0.8 μM primer concentration.

B) PCR with varying dNTPs concentration.

Lane 1 – DNA ladder; Lane 2 – 80 μM dNTPs concentration; Lane 3 - 100 μM dNTPs concentration; Lane 4 - 120 μM dNTPs concentration; Lane 5 - 140 μM dNTPs concentration; Lane 6 - 160 μM dNTPs concentration; Lane 7 - 180 μM dNTPs concentration; Lane 8 - 200 μM dNTPs concentration.

Validation of primers

A PCR reaction was run using an additional CHR001 sample to validate the use of the previously designed primers. The same PCR master mix (Table 6) was prepared and added to 8 tubes, each one containing a different primer. The samples were then put in the thermocycler and the standard program was run, with adequate annealing temperatures set for each primer (Table 7).

Table 6. PCR master mix components

Component	50 ul reaction	Final concentration
Nuclease-free water	27 ul	
5x Phusion HF Buffer	10 ul	1X
10 mM dNTPs	0.5 ul	100 µM
Template DNA	7 ul of DNA sample (@12 ng/ul)	84 ng
Phusion DNA Polymerase	0.5 ul	1 unit
10 uM Forward Primer	2.5 ul (in the tubes)	0.5 uM
10 uM Reverse Primer	2.5 ul (in the tubes)	0.5 uM

Table 7. Primer pairs for amplifications of different exons in *CRY1* gene and their corresponding annealing temperatures.

Primer pair	Tm (°C)
Exon 2	63
Exon 3	64
Exons 4-5	67
Exons 6-7	71
Exons 8-10	69
Exons 11-13	69

When the cycling was finished the samples were placed on a 1.5% agarose gel for electrophoresis. The gel was run at 100 V (5 V/cm) for c. 1.5 h and then stained in a 6% ethidium bromide solution (dissolved in 1xTBE) for 20 min. A scan of the gel was taken using the Typhoon™ FLA 9500 biomolecular imager. The image revealed that all primers were working, with primers for exon 3, exons 4-5, and exons 11-13 giving the most distinct bands with no other undesirable products showing in the same lane (Figure 4). Therefore, only these three primers were chosen for further use in the study.

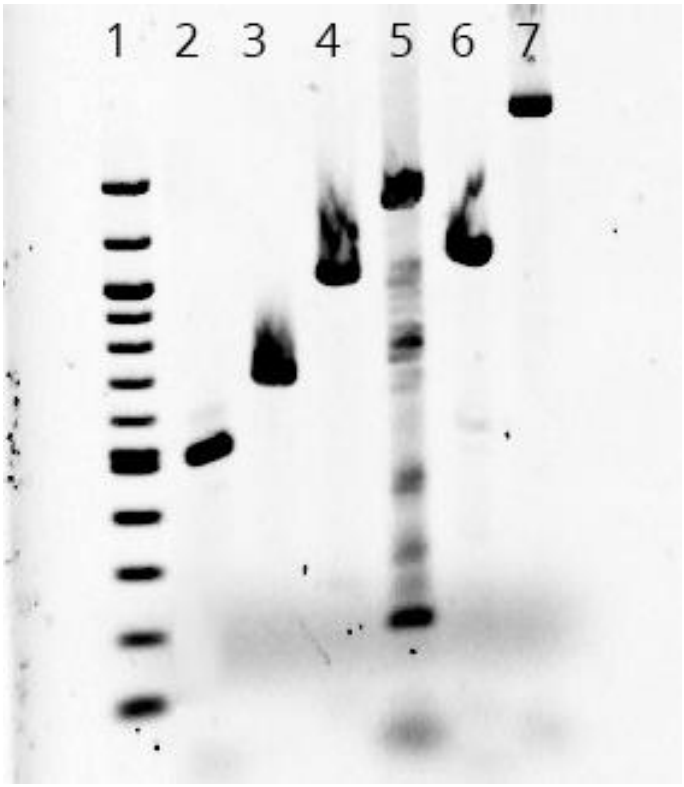

Figure 4. Gel electrophoresis analysis of primers designed for the study.
Lane 1 – DNA ladder; Lanes 2-7 – amplified fragments of CRY1 gene covering the following regions: Lane 2- exon 2, Lane 3 - Exon 3, Lane 4 – Exons 4-5, Lane 5 – Exons 6-7, Lane 6 – Exons 8-10, Lane 7 – Exons 11-13

Isolation and amplification of *CRY1*

Subsequently, several PCR reactions were carried out using the standard PCR protocol (Table 3), and participant DNA isolated from the buccal swabs. To minimise the chance of faulty results, the samples with very low and very high DNA yields (Table 1) were not used. Each DNA sample was amplified three separate times with three different sets of primers covering exon 3, exons 4-5, and exons 11-13 of the *CRY1* gene. Following the thermocycling, all samples were loaded onto 1.5 % agarose gels and run for c. 1.5 h at 100 V (5 V/cm) for validation. The gels were then stained in a 6% ethidium bromide solution (dissolved in 1xTBE) for 20 min and scanned using the Typhoon™ FLA 9500 biomolecular imager. The images revealed which PCR amplified samples were ready for sequencing and which needed further verification, as for some samples the expected band could not be seen (Figure 5). To avoid experimental error, electrophoresis was repeated for those samples that initially did not show on the gel. The one sample that consistently did not produce a band (CHR081) was deemed unusable for sequencing (Figure 5). Therefore, a total of 96 gene sequences (32 for each set of primers) were successfully isolated and deemed ready for sequencing.

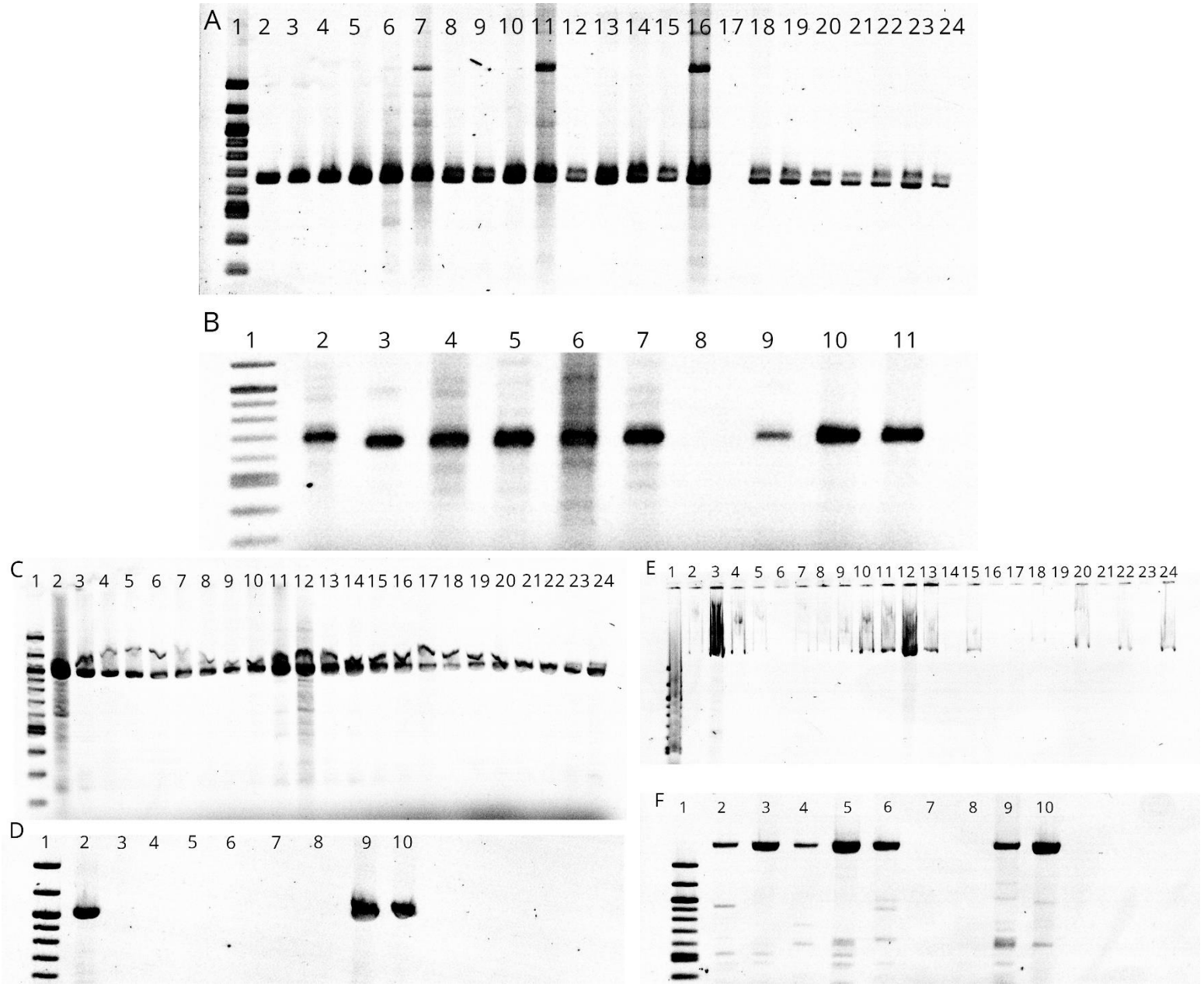

Figure 5. Gel electrophoresis analysis of PCR amplified DNA samples covering the following regions of *CRY1* gene:
A,B) Exon 3; C,D) Exons 4-5; E,F) Exon 11-13

A) Lane 1 – DNA Ladder; Lanes 2-24 – amplified fragments coming from different participants (Lane 2 – CHR032; Lane 3 – CHR096; Lane 4 - CHR040; Lane 5 – CHR054; Lane 6 – CHR025; Lane 7- CHR082; Lane 8 – CHR011, Lane 9 – CHR028, Lane 10 – CHR018; Lane 11 – CHR098; Lane 12 – CHR106; Lane 13 – CHR083; Lane 14 – CHR017; Lane 15 – CHR054; Lane 16 – CHR002; Lane 17 – CHR012; Lane 18 – CHR006; Lane 19 – CHR045; Lane 20 – CHR114; Lane 21 – CHR042; Lane 22 – CHR038; Lane 23 – CHR014; Lane 24 – CHR087)

B) Lane 1 – DNA Ladder; Lanes 2-11 - amplified fragments coming from different participants (Lane 2 – CHR004; Lane 3 – CHR091; Lane 4 – CHR065; Lane 5 – CHR086; Lane 6 – CHR000; Lane 7 – CHR049; Lane 8 – CHR081; Lane 9 – CHR012; Lane 10 – CHR109; Lane 11 – CHR104)

C) Lane 1 – DNA Ladder; Lanes 2-24 – amplified fragments coming from different participants (Lane 2 – CHR011; Lane 3 – CHR002; Lane 4 - CHR054; Lane 5 – CHR017; Lane 6 – CHR098; Lane 7- CHR018; Lane 8 – CHR028, Lane 9 – CHR014, Lane 10 – CHR006; Lane 11 – CHR082; Lane 12 – CHR025; Lane 13 – CHR034; Lane 14 – CHR040; Lane 15 – CHR096; Lane 16 – CHR032; Lane 17 – CHR114; Lane 18 – CHR087; Lane 19 – CHR012; Lane 20 – CHR042; Lane 21 – CHR038; Lane 22 – CHR045; Lane 23 – CHR106; Lane 24 – CHR083)

D) Lane 1 – DNA Ladder; Lanes 2-10 - amplified fragments coming from different participants (Lane 2 – CHR004; Lane 3 – CHR091; Lane 4 – CHR065; Lane 5 – CHR086; Lane 6 – CHR000; Lane 7 – CHR049; Lane 8 – CHR081; Lane 9 – CHR109; Lane 10 – CHR104)

E) Lane 1 – DNA Ladder; Lanes 2-24 – amplified fragments coming from different participants (Lane 2 – CHR011; Lane 3 – CHR002; Lane 4 - CHR054; Lane 5 – CHR017; Lane 6 – CHR098; Lane 7- CHR018; Lane 8 – CHR028, Lane 9 – CHR014, Lane 10 – CHR082; Lane 11 – CHR025; Lane 12 – CHR034; Lane 13 – CHR040; Lane 14 – CHR096; Lane 15 – CHR032; Lane 16 – CHR083; Lane 17 – CHR114; Lane 18 – CHR042; Lane 19 – CHR038; Lane 20 – CHR006; Lane 21 – CHR045; Lane 22 – CHR012; Lane 23 – CHR106; Lane 24 – CHR087)

F) Lane 1 – DNA Ladder; Lanes 2-10 - amplified fragments coming from different participants (Lane 2 – CHR004; Lane 3 – CHR091; Lane 4 – CHR065; Lane 5 – CHR086; Lane 6 – CHR000; Lane 7 – CHR049; Lane 8 – CHR081; Lane 9 – CHR104; Lane 10 – CHR109)

PCR purification

All PCR products were purified using the *QIAquick PCR Purification Kit*, following the protocol provided by the manufacturer (microcentrifuge method). For appropriate elution efficiency, DNA was eluted in 30 µl DNase/RNase-free water.

DNA sequencing

The amplified DNA samples were sent to *Eurofins Genomics* for sequencing by the Sanger Sequencing method. The original reads were corrected using the CodonCode Aligner 10.0.2 software and compared to the reference *CRY1* gene sequence obtained from the National Center for Biotechnology Information (NCBI). The EMBOSS Water internet software was used for pairwise alignment of samples with the reference gene sequence and identification of SNPs. Additionally, the comparison of chromatograms allowed for the identification of specific genotypes (differentiation between homozygotes and heterozygotes) (Appendix 4).

Out of the 96 PCR products that were identified as suitable for sequencing, 5 samples (one covering exon 3, two covering exons 4-5, and two covering exons 11-13) were not sequenced successfully. These samples were freshly prepared and sequenced again, which did not change the low quality of the sequences, therefore deeming them unusable for further study.

Statistical Analyses

All statistical analyses were conducted using IBM SPSS Statistics 28.0.0.0 software. Before a further assessment, all numerical data was tested for normality by the Kolmogorov-Smirnoff test and further analysed accordingly to the result (using parametric or nonparametric tests respectively).

For the correlation analyses, Pearson and Spearman's correlation tests were used. This was followed by a comparison of means between the two sex groups using a *t-s* test for parametric data and a Mann-Whitney *U* test for nonparametric data. A one-way ANOVA test (or Kruskal-Wallis for nonparametric data) was used for all comparisons between three or more groups. Due to the pilot nature of the study and given that a clear priori hypothesis related to chronotype and IQ as well as mental health was established, no correction for multiplicity was performed and alpha was set at 0.05.

For the assessment of differences between categorical variables and comparison of observed genotype frequencies with expected Hardy-Weinberg equilibrium values, a chi-square test was used.

Results

Sample characteristics

The participants taking part in this study were all university students aged 18 to 41 years old. Participation was completely voluntary and no exclusion criteria related to ethnicity or gender were applied. Nonetheless, the majority of the individuals reported to be of European origin (90%) and female (76%).

The relationships between chronotype and the non-genetic variables

Out of all the participants who completed the MEQ (N=100), 42% were found to be evening-type, 49% intermediate-type, and 9% morning-type. When treated as a continuous variable (presented as total scores from the MEQ, where lower scores correspond to eveningness and higher scores to morningness), chronotype was found to be significantly associated with four out of the studied variables, namely - age, depression scores, nonverbal intelligence and positive affect scores (Table 8). In the case of age and positive affect scores, the correlation was positive, indicating an association

with morningness, whereas for depression scores and nonverbal intelligence the correlation was negative, indicating an association with eveningness (Figure 6). Moreover, other variables that have been previously found to be associated with eveningness, such as worse sleep quality and higher anxiety scores, were confirmed to be negatively correlated with the MEQ scores. However, most likely due to a small sample size these correlations were not found to be significant (Table 8).

Overall, no major effect of sex on the main studied variables was noted (Table 9). On average, males scored higher on the MEQ questionnaire, but lower on the PSQI, PHQ-9, and GAD-7 questionnaires, showing a slight lean towards morningness and better sleep quality and mental health than females. Male participants also achieved higher mean IQ test scores (Figure 7). However, most of these differences were not found to be statistically significant ($p>0.05$) with an exception of the difference in GAD-7 scores which was statistically significant ($U=526,5$; $N=94$; $P=0,017$) and showed that anxiety levels among men were much lower than in females. However, it is possible that this difference was caused by the fact that men represented only 24% of the sample and were not a completely accurate representation of the population.

Table 8. The relationships between chronotype and the non-genetic variables.

						Relationship with Chronotype*		
Areas	Questionnaires	Studied variables	*N*	Mean	SD	Type of relationship	r/r_s	p
Demographics	Demographics Questionnaire	Age (yrs)	100	22.60	3.84	Positive correlation	0.262	**0.009**
		BMI (kg/m^2)	98	23.76	4.91	Positive correlation	0.057	ns
Mental Health	PHQ-9	Depression (total score)**	94	6.76	6.87	Negative correlation	-0.242	**0.019**
	GAD-7	Anxiety (total score)**	94	7.62	5.47	Negative correlation	-0.175	ns
Sleep Quality	PSQI	General sleep quality (total score)***	97	6.24	3.22	Negative correlation	-0.138	ns
	MADRE	Occurrence of Nightmares (score on the nightmare-related question) ****	88	3.82	1.99	Negative correlation	-0.078	ns
Psychology	Raven's Advanced Progressive Matrices (APM)	Nonverbal intelligence (IQ points)	39	110.51	12.28	Negative correlation	-0.481	**0.002**
	Positive Affect-Negative Affect Scales (PANAS)	Positive affect (total score)	76	28.47	7.51	Positive correlation	0.337	**0.003**
		Negative affect (total score)	76	23.68	8.12	Negative correlation	-0.004	ns
	Big Five Inventory (BIF)	Extroversion (total score)	76	24.46	6.21	Positive correlation	0.2	ns
		Agreeableness (total score)	76	33.63	5.33	Negative correlation	-0.071	ns
		Conscientiousness (total score)	76	31.16	5.82	Positive correlation	0.222	ns
		Neuroticism (total score)	76	26.26	6.16	Negative correlation	-0.06	ns
		Openness (total score)	76	35.3	5.34	Negative correlation	-0.069	ns

*Here chronotype is represented by a total score from the Morningness-Eveningness Questionnaire where lower scores correspond to eveningness and higher scores to morningness
**Higher scores indicate a more severe condition
***Higher scores indicate worse sleep quality
****Higher scores indicate a higher frequency of nightmares, where 0=Never and 7=Several times a week

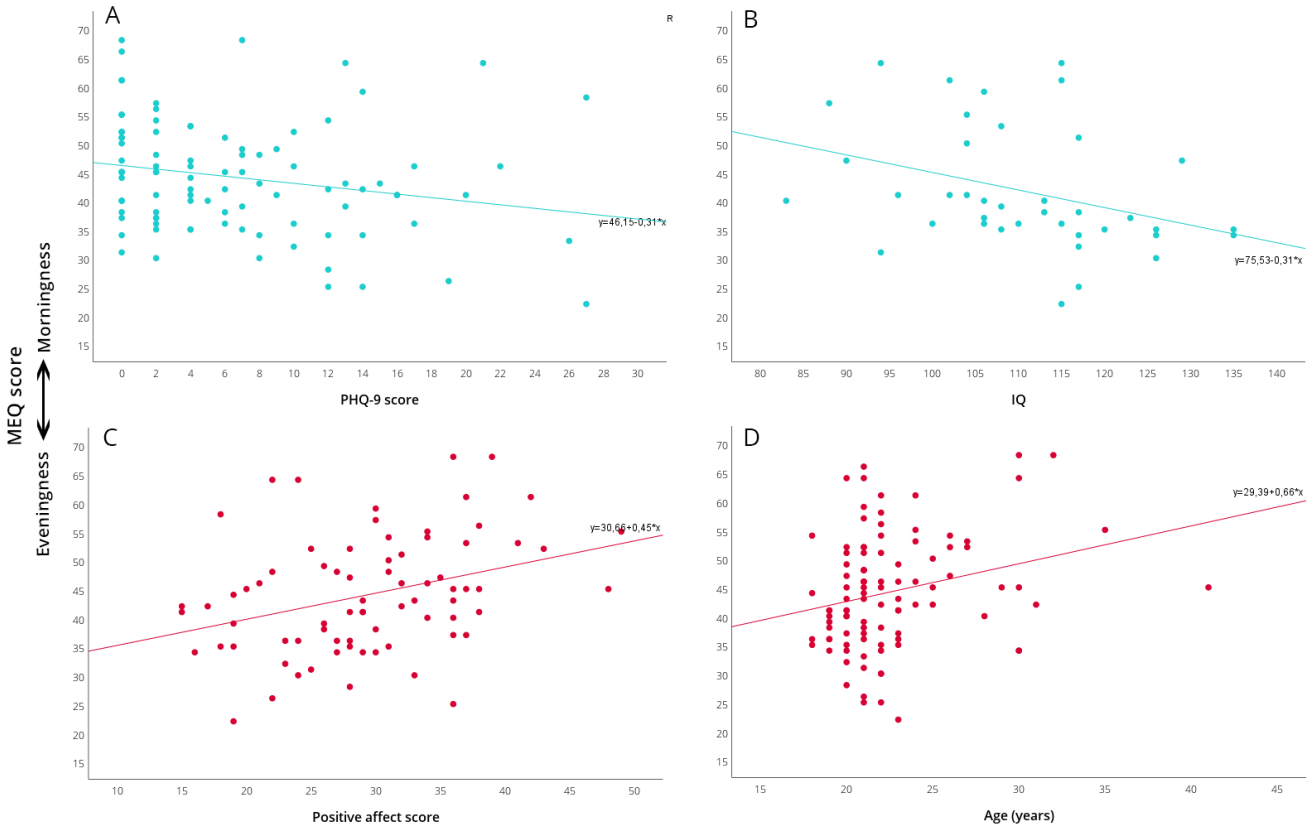

Figure 6. Correlations between total MEQ scores (chronotype) and the different variables that were found to be statistically significant. **A)** Negative correlation with PHQ-9 score (depression severity) (r_s=-0.242, N=94, *p*=0.019); **B)** Negative correlation with IQ points (r_s=-0.481, N=39, *p*=0.002); **C)** Positive correlation with positive affect score (r_s=0.337, N=76, *p*=0.003); **D)** Positive correlation with age (r_s=0.262, N=100, *p*=0.009). Lower total MEQ scores correspond to eveningness and higher to morningness with cut-off points for classification of chronotype as follows: evening: <42; intermediate >= 42 and <= 58; morning >58.

Table 9. Sex differences across the main studied variables (chronotype, mental health, sleep quality, IQ).

Studied Variables	Women			Men			Main Effect				Effect Size*
	N	Mean	SD	N	Mean	SD	t	Mann-Whitney U	df	p	d_{Cohen}
Age (yrs)	76	22.43	3.81	23	23.13	3.97	0.766	-	98	ns	0.182
Chronotype (MEQ score)	75	43.51	10.27	24	46.54	8.67	1.425	-	45.44	ns	0.306
Depression (PHQ-9 score)	71	7.54	7.26	23	4.35	4.9	-	612.5	-	ns	0.377
Anxiety (GAD-7 score)	72	8.46	5.67	22	4.86	3.62	-	526.5	-	**0.0167**	0.504
Sleep Quality (PSQI scorer)	73	6.59	3.31	24	5.17	2.7	-	645.5	-	ns	0.399
IQ	31	109.42	12.57	8	114.75	10.74	1.206	-	12.47	ns	0.441

*Guidelines for assessing effect size are as follows: d≥0.2 -small, d≥0.5- medium, d≥0.8- large

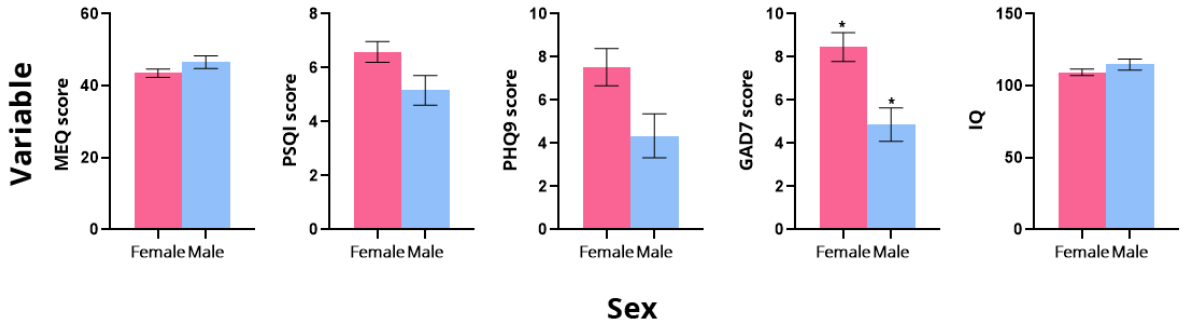

Figure 7. The effect of sex on studied variables. The bars represent a mean score (±SEM) achieved by males and females on the different questionnaires and an intelligence test. Statistically significant differences are highlighted by an asterisk (*).

The impact of the COVID-19 pandemic on diurnal preference, sleep, and mental health

This study was conducted during the global pandemic caused by the COVID-19 virus. Therefore, the impact of the newly enforced laws, including country-wide lockdowns and working-at-home policies had to be taken into account. Most people experienced a complete change in work and social schedules, often being presented with more freedom in planning their day. This allowed individuals to follow their diurnal preference and establish healthier sleeping habits. Indeed, over 50% of participants in this study, reported that their sleep duration got longer (55%) and both wake-up and bed-times on weekdays became delayed by one or more hours (66% and 56% respectively). On the weekends this change was smaller, with 45% of individuals reporting delayed bedtimes and 48% delayed wakeup times. A relatively high percentage of participants also reported no change when it came to wake-up and bed-times on the weekends (29% and 40% respectively), highlighting the shift in diurnal preference (Figure 8).

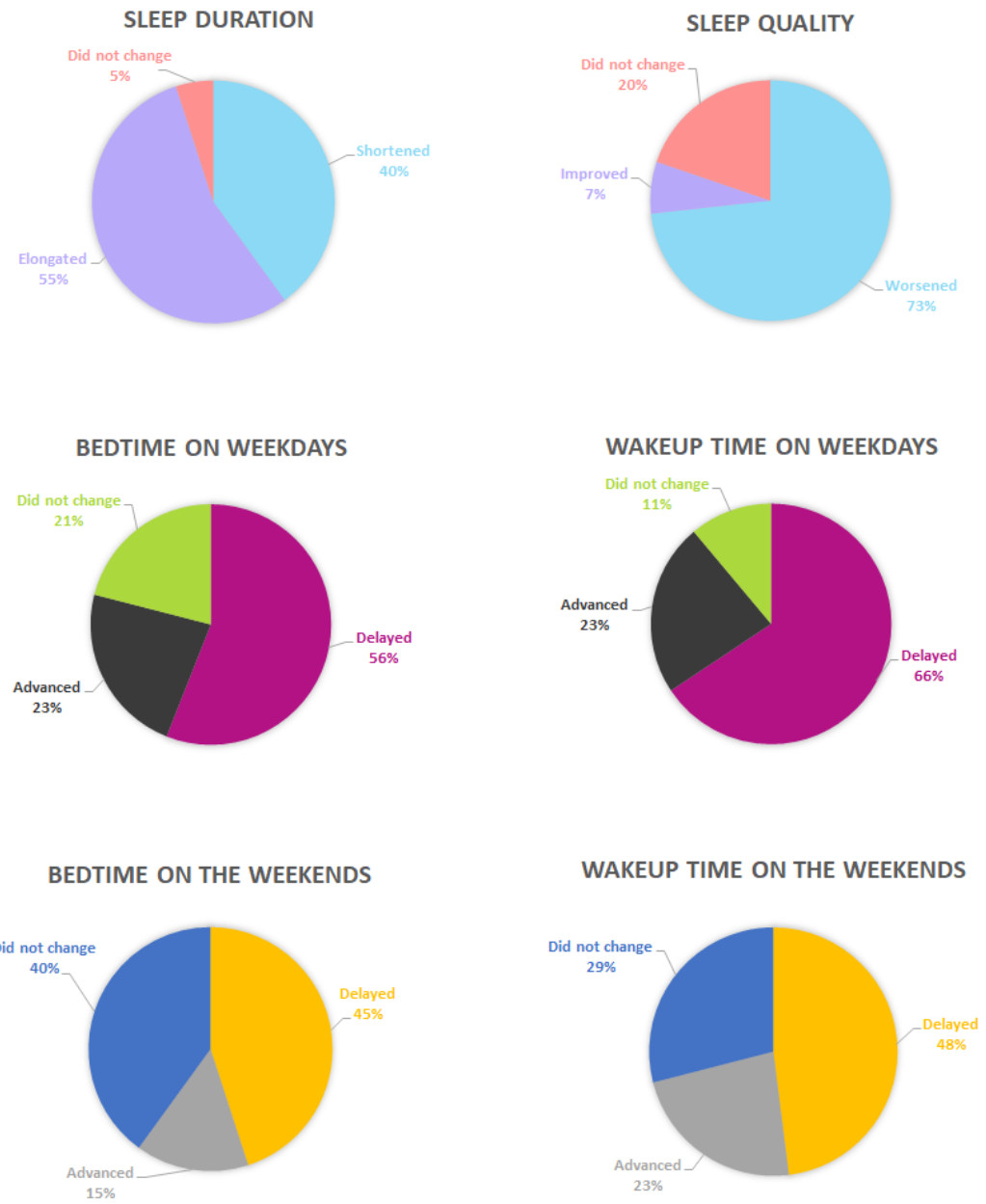

Figure 8. Self-reported changes in main sleep characteristics during lockdown. The diagrams show percentage of answers given by subjects on a COVID-19 impact questionnaire designed for this study (Appendix 1). A clear shift towards more delayed chronotype can be seen on both working and free days as well as an elongated sleep duration and overall worsened sleep quality.

However, in line with previous research on this topic, a significant decrease in sleep quality was also reported by the participants of this study, with 73% of responders indicating a worsened sleep quality during the pandemic (Figure 8). The most prevalent sleep issues reported by the subjects were daytime tiredness, difficulty falling asleep and difficulty waking up, with more than 50% of responders experiencing these

problems to a high extent (4 or 5 scores on a 5-point scale) (Figure 9). Moreover, no significant difference was found between the average MEQ scores of the subjects who reported a negative or positive change in sleep quality (U=101, N=44, *p*=0.656) (Figure 10), suggesting that there was no direct relationship between chronotype and the worsening of sleep quality.

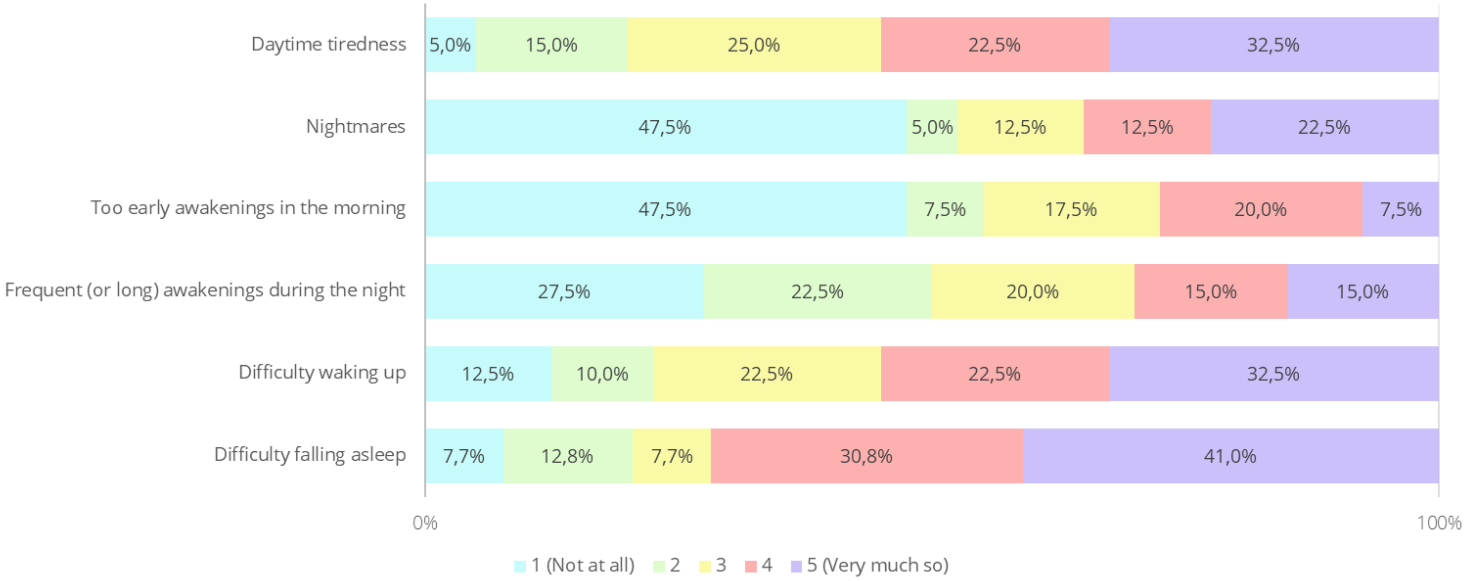

Figure 9. Self-reported experiences of sleep-related issues during lockdown. If participants reported worsened sleep quality they were asked to what extent did they experience different sleeping problems on a 5-point scale where 1 is “Not at all” and 5 “Very much so”. The size of each box corresponds to the percentage of given responses. Difficulty falling asleep was found to be the most extensively experienced issue among responders.

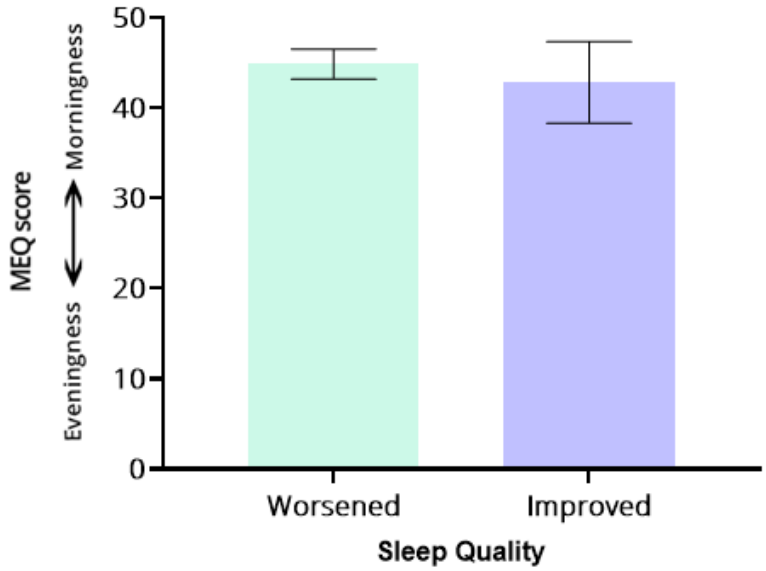

Figure 10. Difference between mean MEQ scores (±SEM) of participants who reported change in sleep quality during COVID-19 lockdown. Lower total MEQ scores correspond to eveningness and higher to morningness with cut-off points for classification of chronotype as follows: evening:<42; intermediate >=42 and <= 58; morning >58. This difference was found not to be significant (U=101, N=44, *p*=0.656).

Furthermore, the subjects reported a significant change in their emotional well-being during the pandemic (89% versus. 11%), with a majority feeling more pessimistic and anxious, as well as less focused and motivated (Figure 11). The same number of responders (89%) also felt that their academic progress at the university was affected,

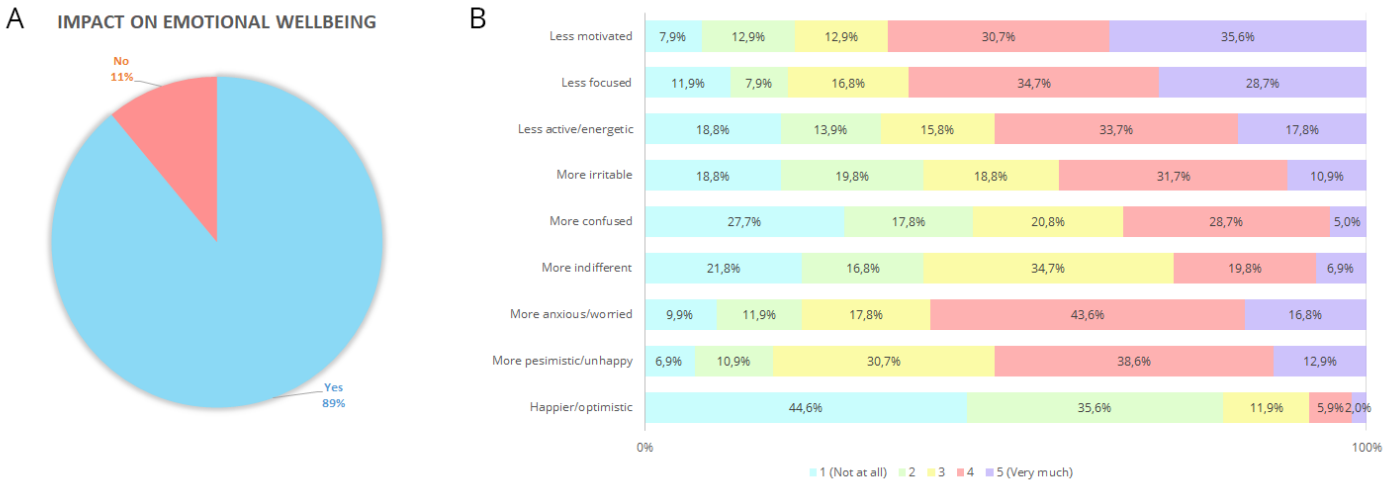

Figure 11. The impact of COVID-19 pandemic on the emotional well-being of participants. **A)** Percentage of subjects agreeing (Yes) or disagreeing (No) with the statement "The COVID-10 pandemic has affected your emotional well-being"; **B)** Diagram showing to what extent the subjects experienced certain feelings during the pandemic on a 5-point scale, where 1 is "Not at all" and 5 "Very much so". The size of each box corresponds to the percentage of given responses. It was found that lack of motivation and focus, as well as feeling more pessimistic and anxious were the most prevalent issues among the studied group.

with 84.4% of subjects indicating that the change was for the worse and 81.2% reporting that they found it harder or much harder to engage with their studies (Figure 12).

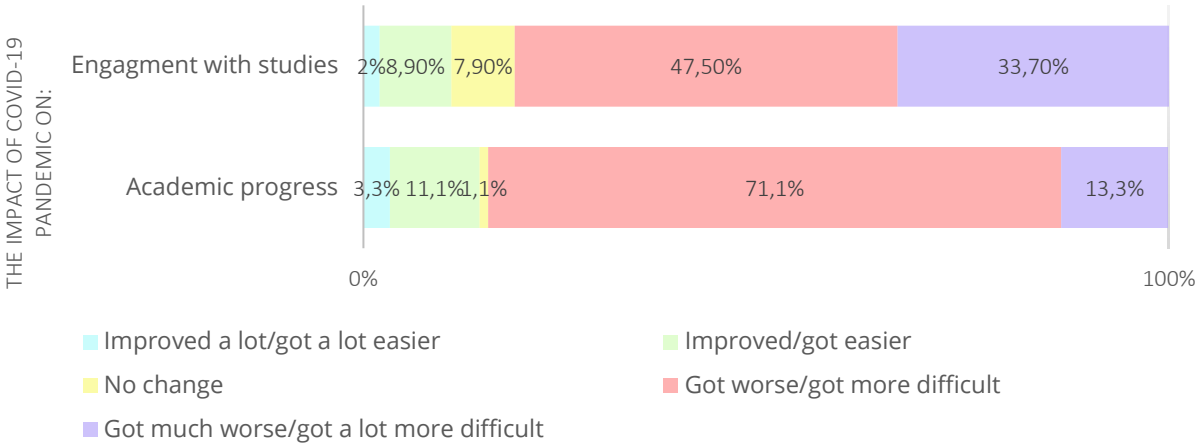

Figure 12. The self-reported impact of the COVID-19 pandemic on engagement with studies and academic progress shown in percentage of given responses.

Finally, the participants were asked to indicate whether they felt as if the change in their sleeping habits had any influence on their emotional well-being and academic progress. In the case of mental well-being, most responders expressed that this change had either a negative influence on their well-being (53%) or it did not make any difference (40%), with only 8% stating that the influence was positive. Similar results were found concerning the effect on academic progress, with 43% indicating a negative impact, 45% no difference, and 13% a positive impact (Figure 13).

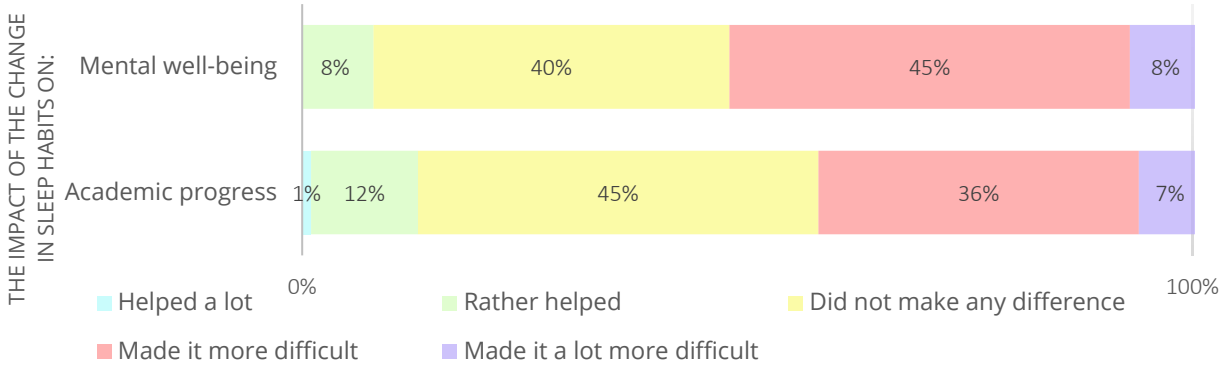

Figure 13. The self-reported effect of the change in sleep habits during COVID-19 pandemic on mental well-being and academic progress shown in percentage of given responses.

All of the above data was gathered during the COVID-19 pandemic while normal student activities (e.g. in-person lectures, meetings of student societies and sports clubs, club nights) were very limited or completely forbidden due to nation-wide lockdowns, therefore the measurements are representative of the home environment and were not influenced by typical university social schedules that would usually include evening or night-time activities.

Genetic analyses

Sequencing of the participant DNA revealed the presence of three different SNPs within the tested population (n=32) (Table 10). Two of those SNPs were found in exon 13 (rs1056560, rs8192441) and one in exon 5 (rs8192440) of the *CRY1* gene. Considering that the rs8192441 variation was found in only one participant, it was excluded from the statistical analysis.

Table 10. Characteristics of the SNPs found in the studied population.

SNP	Variation	Location on CRY1	Position	Residue change in protein	Genotypes	Genotype frequency (%)
rs8192440	T>C	Exon 5	chr12:107001328	Synonymous G [Gly] > G [Gly]	TT TC CC	7 (23,3%) 13 (43.3%) 10 (33,3%)
rs1056560	G>T	Exon 13 (non-coding UTR-3 region)	chr12:106991832	N/A	GG GT TT	9 (30%) 13 (43.3%) 8 (26,7%)
rs8192441	A>C	Exon 13 (non-coding UTR-3 region)	chr12:106991676	N/A	AA AC CC	29 (97%) 1 (3%) 0 (0%)

N/A – not applicable

Genotype frequencies of both rs8192440 and rs1056560 variations did not significantly deviate from the Hardy-Weinberg equilibrium (χ^2=0.544, p=0.762 for rs8192440; χ^2=0.535, p=0.765 for rs1056560).

Table 11. Genotype differences across the main studied variables (chronotype, mental health, sleep quality, IQ)

Studied Variables	rs8192440												rs1056560										
	CC			CT			TT			Main Effect			GG			GT			TT			Main Effect		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	Kruskal-Wallis H	df	p	N	Mean	SD	N	Mean	SD	N	Mean	SD	Kruskal-Wallis H	df	p
Chronotype (MEQ score)	11	39.91	11.4	13	40.54	9.395	7	36.71	3.988	0.336	2	ns	9	40.44	7.876	13	41.85	10.81	8	37.63	12.76	1.441	2	ns
Depression (PHQ-9 score)	9	8.222	9.909	13	5.769	5.388	7	4.429	4.541	0.324	2	ns	9	6.333	6.782	13	6.308	6.511	7	7.143	10.17	0.251	2	ns
Anxiety (GAD-7 score)	9	8.333	6.671	13	8.692	6.499	7	5.714	2.812	0.313	2	ns	9	8.0	4.637	13	8.769	7.108	7	8.571	6.803	0.088	2	ns
Sleep Quality (PSQI score)	10	7.1	3.665	13	5.462	3.526	7	5.714	2.215	1.583	2	ns	9	5.444	2.297	13	6.385	3.906	8	7.25	3.284	1.011	2	ns
IQ	10	107.1	15.34	13	114.8	8.745	7	112.7	9.499	1.892	2	ns	9	107.4	8.323	13	114.4	9.803	8	109.8	14.77	3.503	2	ns

No statistically significant differences or associations between the different genotypes formed by these two polymorphisms and the studied variables (chronotype, mental health, intelligence, or sleep quality) have been found (Table 11). Nevertheless, two interesting trends were noticed when it comes to the rs8192440 variation. It was found that while the CC and CT genotypes were associated with more than one chronotype, the TT genotype was only found in participants with an evening chronotype (Table 12). This was not the case for rs1056560 variation where a mix of at least two different genotypes could be seen for each chronotype (Table 13). Furthermore, TT homozygotes of the rs8192440 variant on average scored lower than the CC homozygotes or the heterozygotes on both of the mental health questionnaires (PHQ-9, GAD-7) (Figure 14A). This could suggest a possible protective effect of the T allele. The almost opposite pattern was noted for the rs1056560 variation, although the differences in scores were smaller than in the case of rs8192440 (Figure 14B). Considering the small sample size and the lack of statistical significance, to confirm whether these findings were not accidental, a study on a much larger scale would need to be conducted. However, looking at the effect sizes of these differences (Table 14), most of which were found to be moderate or almost moderate, there is much promise in the further exploration of these SNPs, especially the rs8192440 variation.

Table 12. Number of cases with the particular combination of genotype and chronotype for the rs8192440 SNP. Cut-off points used for classification of chronotype through MEQ questionnaire were as follows: evening:<42; intermediate >=42 and <= 58; morning >58

		Genotype		
		CC	CT	TT
Chronotype	Morning	0	2	0
	Intermediate	3	1	0
	Evening	7	10	7

Table 13. Number of cases with the particular combination of genotype and chronotype for the rs1056560 SNP. Cut-off points used for classification of chronotype through MEQ questionnaire were as follows: evening:<42; intermediate >=42 and <= 58; morning >58

		Genotype		
		GG	GT	TT
Chronotype	Morning	1	2	0
	Intermediate	0	2	2
	Evening	8	9	6

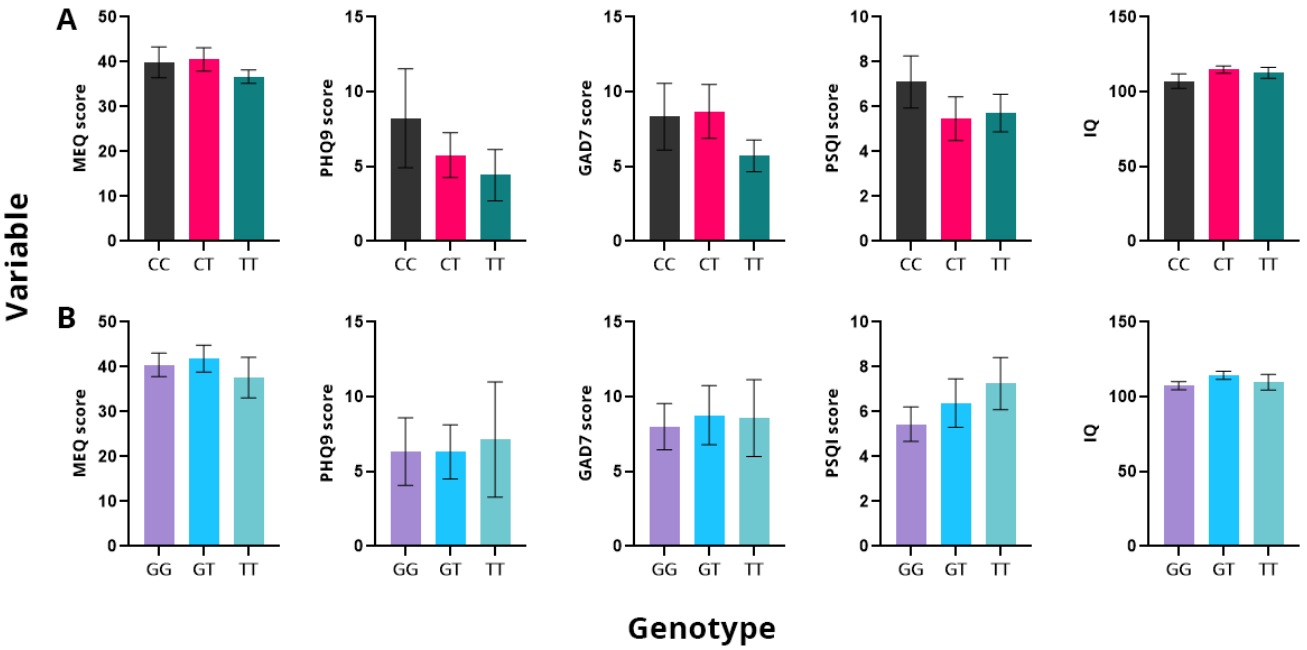

Figure 14. The differences between mean questionnaire and IQ scores (±SEM) of participants with different genotypes of the studied variants: **A)** rs8192440 **B)** rs1056560. In all cases the differences between the scores were found to be statistically non-significant.

Table 14. The effect size of the studied SNPs on the main studied variables. Medium or higher effect sizes are shaded grey.

Variable	Effect size*	
	rs8192440	rs1056560
Chronotype (MEQ score)	0.503	0.291
Anxiety (GAD-7 score)	0.527	0.563
Depression (Kroenke, Spitzer and Williams)	0.525	0.537
Sleep Quality (Romero-Blanco *et al.*)	0.25	0.39
IQ	0.127	0.486

*Guidelines for assessing effect size are as follows: d≥0.2 -small, d≥0.5- medium, d≥0.8- large

Discussion

This study, although based on a priori hypothesis regarding the direction of the investigated relationships, remained explorative in nature, looking for associations between different variables, all connected by the idea of chronotype and its influence on the day-to-day lives of students. A combination of online questionnaires, in-person meetings, and molecular biology techniques was used to reach a higher level of understanding of these relationships and their origins – from genes to environment.

Eveningness and mental health

The findings of this study replicate an association between eveningness and depression (Table 8, Figure 6), confirming previous findings regarding this relationship (Merikanto *et al.*, 2013; Abe *et al.*, 2011; Drennan *et al.*, 1991; Chelminski *et al.*, 1999; Hidalgo *et al.*, 2009) and providing further evidence of depression prevalence in subjects with evening chronotype. This association seemed to be independent of sleep quality, which in this study was found to be at a relatively similar level among all chronotypes.

Moreover, while a significant correlation between eveningness and negative affect (NA) could not be reported, greater morningness was found to be significantly associated with positive affect (PA) (Table 8, Figure 6). This converges with previous research conducted by Hasler *et al.*, (2010) and suggests that eveningness itself does not cause negativity and depressive thoughts, but rather limits positive thinking/attitude, which fuels mood dysregulation. Psychologically, the most likely cause for this trend is the unstable social and lifestyle rhythms observed among evening types (Monk *et al.*, 2004), as well as the associated pressure to perform at the time of the day when evening types are usually experiencing the effects of social jet-lag (Levandovski *et al.*, 2011).

Biologically, previous findings suggest that the association between eveningness and depression is derived from the presence of clock genes in the mesolimbic dopamine system, which is the neural circuit mediating response to reward (Nestler and Carlezon,

2006; Parekh, Ozburn and McClung, 2015; McClung, 2007). It's been proven that virtually all aspects of dopaminergic activity including neuronal firing patterns, neurotransmitter synthesis, release, degradation, and postsynaptic actions display diurnal variation and are subject to circadian transcriptional influence. This means that even small changes in the expression of the core clock genes (e.g. a protein residue change caused by an inherited or random mutation) could cause not only the circadian alterations already associated with eveningness (e.g. delayed circadian phase), but also changes in dopamine secretion and, therefore, mood and addictive disorders. Indeed, several SNPs in the clock genes have been found to be associated with mood disorders including rs2287161 *(CRY1*), rs11123857 (*NPAS2*), rs885861 (*VIPR2*), rs10462028 (*CLOCK*), rs17083008 (*VIP*), rs738499 (*TEF*), rs4132063 *(CRY2),* and rs10838524 *(CRY2)* (Byrne *et al.*, 2014; Soria *et al.*, 2010; Hua *et al.*, 2014). Although, none of the SNPs found in this study were found to be associated with either chronotype or mental health, the moderate effect sizes of the relationships between the rs8192440 (*CRY1*) investigated in this study and both chronotype and mental health suggest that it is yet another variation that can possibly be responsible for the association between eveningness and mental health.

Additionally, some evidence was found by DeYoung *et al.*, (2007) of the metatrait Stability, representing emotional (reversed Neuroticism), social (Agreeableness), and motivational (Conscientiousness) domains being positively related to morningness and serotonergic function. This could be another possible explanation for the elevated PA scores of morning-type individuals since the serotonergic function has been linked to the extent of PA before (Williams *et al.*, 2006). However, in this study, no significant correlations with any of the Big Five Inventory domains were found (Table 8), which suggests that this mechanism is not the most likely explanation of the relationships discovered within the tested sample. Although, it is important to notice that DeYoung and his colleagues used a different method of analysis which could be the reason for the difference in results.

Eveningness with regard to sex and age

This study supports the reported trend for increasing morningness tendencies with age. The positive correlation we found between age and morningness was significant, even though the study population mostly consisted of subjects in their 20s. Interestingly, a study by Kim *et al.*, (2010) showed that the relationship of depressive symptoms with eveningness is most prominent in younger (≤20) and older (≥50) age groups, which reinforces our belief that focusing on younger individuals in this study was the right choice for deepening the understanding of mental health issues in evening-types.

Furthermore, It has previously been shown that sex is associated with chronotype and that young men (before the age of 40) usually present with a later chronotype than women (Fischer *et al.*, 2017). Moreover, Hidalgo *et al.*, (2009) discovered an association between higher levels of depressive symptoms and the female gender, which could be another factor affecting the relationship between chronotype and mental health. Although in the current study we haven't found an effect of sex on chronotype or sleep quality, the anxiety scores were significantly higher for female participants, which would support Hidalgo *et al.*'s findings. This trend could also be seen for depression and sleep quality scores as well (Figure 7). The fact that these differences were not found to be significant is most likely due to the overrepresentation of females in the sample (75%), as well as an overall small sample size. However, it can also mean that the effects of sex and chronotype on such variables as mental health and intelligence are independent of each other and/or mediated by different mechanisms. Indeed, a recent study by Muzni *et al.*, (2021) showed that when sleep quality, sleep duration, and chronotype are included in the same multivariate model, sleep quality has the strongest independent effect on mental health. Moreover, they have found that these sleep quality associations were stronger in women and there was a significantly stronger correlation between chronotype and mental health in women compared to men. This goes along with the reports that poor sleep quality and prolonged sleep latency cause greater psychological distress in women relative to men (Suarez, 2008).

However, considering that in this study sex was not found to affect sleep quality, it is still possible that other factors that we have yet to discover might be contributing to the differences in chronotype and mental health between the sexes.

Eveningness and intelligence

The significant association between eveningness and intelligence reported in this study is consistent with findings of previous studies, which used different measures of intelligence and different subject populations (Roberts and Kyllonen, 1999; Kanazawa and Perina, 2009; Preckel *et al.*, 2011; Piffer *et al.*, 2014). Here we show that this relationship is present among both undergraduate and postgraduate university students independent of sex, age, or sleep quality. Considering that most students are on a rather strict working schedule, our findings go against the hypothesis of Ujma *et al*. (2020) that higher intelligence in evening types depends on work timing. We believe it is far more likely to be the result of genetic and physiological differences between morning and evening types such as differences in brain structure (Rosenberg *et al.*, 2014) or the presence of certain gene mutations and gene expression-regulating mechanisms that have yet to be identified.

Furthermore, it has been proposed that besides random genetic mutations, the most likely cause for the association between eveningness and intelligence is an evolutionary adaptation. Surveys of ethnographies of traditional societies suggest that nocturnal activities were probably rare in the ancestral environment since night-time conditions would have been tough to work or hunt in (low levels of light and higher levels of predatory activity). In line with this theory more nocturnal individuals would have developed higher intelligence for the sake of survival (Kanazawa and Perina, 2009). Another explanation is based on the theory of sexual selection, where being active late in the evening provided more opportunities for reproduction, and therefore, greater intelligence of evening-type individuals could be related to their mating intelligence (Piffer *et al.*, 2014).

More recently, a training effects hypothesis has been proposed, which suggests that evening types have a frequent need to overcome social jet lag and this need would, in turn, lead them to develop higher problem-solving abilities (Preckel *et al.*, 2011). However, considering the previously established relationship between chronotype and depression, this possible gain in intelligence might not be enough to compensate for the constant struggle against societal expectations that evening-types have to endure. We suggest that based on this and previous research, chronotype should be taken into account in both educational and professional settings whenever high cognitive performance is essential to complete a task. For example, this could mean giving students a choice of the time of day at which tests are administered or assigning shifts at certain workplaces based on chronotype.

Chronotype in lockdown

Furthermore, this research further solidifies that whenever the work/study schedule becomes more flexible the majority of the population naturally shifts to later wake-up and bed-times with prolonged duration of sleep (Romero-Blanco *et al.*, 2020; Wright *et al.*, 2020; Blume, Schmidt and Cajochen, 2020; Leone, Sigman and Golombek, 2020; Gupta *et al.*, 2020; Saalwirth and Leipold, 2021). This might suggest that current norms are too strict and do not allow for the establishment of healthy sleeping habits that are in line with both the molecular clock and the mind and body's need for recovery. Therefore, we believe that delaying the starting time of classes at schools and universities by at least one hour and introducing flexible working hours at workplaces could lead to a generally healthier and more productive society.

On the other hand, we have also found that during lockdown most people reported a decrease in sleep quality. Although this might seem to go against the suggestions made above, considering that this change was irrespective of chronotype and was mostly characterized by issues with falling asleep, we think the cause for this change lies in the overall stress and uncertainty that comes with a global pandemic.

Moreover, the finding that most of the subjects in this study reported a negative change in their mental health (feeling less motivated/focused and more pessimistic/anxious) and the fact that multiple previous studies have obtained very similar data, seems to confirm this theory (Romero-Blanco *et al.*, 2020; Wright *et al.*, 2020; Martínez-de-Quel *et al.*, 2021; Marelli *et al.*, 2021; Blume, Schmidt and Cajochen, 2020; Leone, Sigman and Golombek, 2020; Gupta *et al.*, 2020). However, it is important to note that even though the association between sleep quality and mental health is clear, we cannot be completely certain whether sleep deterioration is a function of psychological distress or vice versa. The results of this study do not rule out the fact that delayed sleep could result in poorer sleep quality. This is due to the possibility of a reduction in slow-wave sleep that is known to be associated with phase delay (Borbély *et al.*, 2016). Indeed, 53% of our subjects indicated that the change in sleep habits had a negative impact on their mental health, which suggests that the (seemingly healthier) longer sleep duration did not enhance well-being in lockdown conditions.

Thus to determine whether additional freedom in choosing bed and wake-up times is beneficial or derogative for mental and physical health, a further study would need to be conducted once COVID-19 is no longer a big threat, allowing for a natural reduction in overall stress and anxiousness that most people experienced during the COVID-19 pandemic.

The genetic variable

No significant associations between genetic polymorphisms and chronotype, mental health, or intelligence were found in the present study (Table 11, Figure 14). This is most likely due to the small sample size and does not eliminate the possibility of such relationships existing. Indeed, the findings of this study indicate that a closer look at the *CRY1* rs8192440 SNP could reveal new insights into the genetic bases of chronotype, especially with regard to the role of the rare TT genotype and the possible protective role of the T allele. Moreover, this mutation had already been linked to circadian rhythmicity before, specifically to the regulation of glucose homeostasis in a season-

dependent manner (Renström *et al.*, 2015), which further implies that the presence of this SNP can influence physiological processes dependent on the molecular clock. However, to confirm this theory and discover the exact way in which the rs8192440 polymorphism affects gene expression and/or protein assembly, a study on a much larger scale would be needed.

Furthermore, we would like to draw attention to the largely understudied *CRY1* rs8192441 polymorphism, that we have discovered in one of our study participants. Although this might be purely coincidental, the individual with this mutation is an evening type that suffers from severe depression and anxiety. To the best of our knowledge, only one study has looked into the possible effects of this mutation on mental health (or any other variables) (Kovanen *et al.*, 2013), and no studies exploring its relationship with chronotype have been conducted. Considering that it's a rather rare SNP (only 1.1% globally carry the G allele), and thus could have a major effect on those who carry it, we believe that further study is in order.

Limitations

This study had a number of limitations. First, due to the ongoing pandemic, the sample size was smaller than expected and only a restricted cohort of participants was assessed (university students). For this reason, our results cannot be extended to the general population. Second, part of this study was carried out through a web-based survey, introducing the possibility of response bias, as well as limiting the control we had over the completion of all questionnaires. This meant that some data was missing in certain variables, further limiting the sample size and statistical significance of our results. Moreover, such factors as comfort with self-reporting emotional and behavioural symptoms, stress related to the lockdown, or even lack of internet access, could all have influenced not only participation rates, but also the responses elicited. Third, the online survey was composed of self-reported questionnaires that were not backed with clinical or instrumental examinations (e.g. measurement of melatonin levels). Fourth, the explorative nature of this study meant that no long-term effects of

lockdown were measured and the focus of the genetic analysis shifted from investigating already known mutations to the exploration of the entire *CRY1* gene. This combined with the small sample size, meant that we were not able to identify any fully meaningful relationships and further studies would be needed to confirm our genetic findings as well as evaluate long-term outcomes of social isolation. Fifth, we used Raven's Advanced Progressive Matrices to measure intelligence, which is not a fully comprehensive examination of one's IQ as it omits verbal, mathematical, or linguistic portions of human intelligence. Sixth, although there were no exclusion criteria regarding sex or perceived chronotype, most of the participants in this study were female and presented as either intermediate or evening chronotype. This meant that in both cases our data was skewed and unintentional selection bias was introduced.

As mentioned previously, further longitudinal studies will be needed to expand on the findings of the current study. We would be especially interested to see the genetic aspects explored in a large population, using equipment that would allow for a more throughout examination of the SNPs found in the *CRY1* gene and their specific molecular effects (e.g. change in gene expression or protein structure). We also suggest that a study focusing on the genetic bases of the difference in intelligence between evening and morning-type individuals would be highly beneficial for our understanding of human evolution from a molecular point of view.

Conclusion

In conclusion, despite a relatively small sample size and limited age range, our study confirms the existence of previously established associations between eveningness, mental health, and intelligence. Moreover, it provides further proof of negative changes in sleep quality and mental well-being during lockdown that should be taken into account in the case of the return of COVID-19 or any other situation that would once again cause national lockdowns. We show that an introduction of a flexible schedule causes a phase delay and increases sleep duration, which under normal circumstances could lead to lower levels of social jetlag and therefore improved quality

of life for evening-type individuals. However, since such delay might also have disruptive effects on sleep, we suggest that a longitudinal study should be conducted on this topic. Last but not least, we uncover the presence of three SNPs within the *CRY1* gene, out of which the rs8192440 and rs8192441 polymorphism show promise for further study regarding possible associations with chronotype and mental health. Overall the results of this study may provide support for the implementation of chronotype-based interventions at schools and workplaces and form a great starting point for further studies on the genetic bases of chronotype and its relationship with intelligence and mental health.

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Appendix 1

COVID-19 impact Questionnaire

Do you feel the COVID-19 pandemic affected your sleep in either a positive or a negative way?	**Yes**	**NO**			
My sleep duration got	**shorter**	**longer**			
By:	< 10 min	10 - 30 min	30 min – 1 h	1h -2 h	>2 h
My sleep quality	**improved**	**worsened**			
Not at all	1	2	3	4	Very much so
If worsened, did you experience any of the following and to what extent did this impact your sleep quality?	**Difficulty falling asleep**	**Difficulty waking up**	**Nightmares**	**Inability to stay asleep throughout the night**	**Daytime tiredness**
No impact	1	2	3	4	Extreme change
My bedtime on the weekdays	**Delayed**	**Advanced**	**Didn't change**		
By:	< 10 min	10 - 30 min	30 min – 1 h	1h -2 h	>3 h
My wakeup time on the weekdays	**Delayed**	**Advanced**	**Didn't change**		
By:	< 10 min	10 - 30 min	30 min – 1 h	1h -2 h	>3 h
My bedtime on the weekends	**Delayed**	**Advanced**	**Didn't change**		
By:	< 10 min	10 - 30 min	30 min – 1 h	1h -2 h	>3 h
My wakeup time on the weekends	**Delayed**	**Advanced**	**Didn't change**		
By:	< 10 min	10 - 30 min	30 min – 1 h	1h -2 h	>3 h
Do you feel the COVID-19 pandemic affected your emotional wellbeing in either a positive or a negative way? If yes to what extent?	**Yes**	**NO**			
I feel	Happier	More anxious	More worried	More indifferent	More confused
No impact	1	2	3	4	Extreme change
Do you feel that COVID-19 pandemic changed your activity level? If yes by how	**Yes Positively**	**Yes Negatively**	**No**		

much?					
Not at all	1	2	3	4	Very much so
Do you feel the COVID-19 pandemic affected your academic progress at the university in either a positive or negative way? If yes to what extent?	**Yes**	**No**			
My academic progress	Became easier	More difficult			
Not at al	1	2	3	4	Very much so
How did you find the transition of face-to-face teaching to online teaching? And to what extent?	**Easier**	**Harder**			
No impact	1	2	3	4	Extreme change
During the COVID-19 pandemic, did you find it easier or harder to engage with your studies? And to what extent?	**Easier**	**Harder**	**No change**		
No impact	1	2	3	4	Extreme change
Do you feel that your productivity changed during the COVID-19 pandemic? If yes how and to what extent?	**Yes**	**No**			
I became:	More productive	Less productive			
No impact	1	2	3	4	Extreme change
If COVID-19 impacted you in any other ways, what were they?					

Appendix 2

Description of questionnaires used in the study

Assessments of health, mental health and general well-being

Demographics a few simple questions will regarding age, sex, ethnicity, marital status and BMI of the participant.

COVID-19 impact is a set of questions relating to the impact the COVID-19 pandemic has had on the participant.

General Medical Questionnaire *(GMQ)* a questionnaire designed by the study team that asks participants to provide details of their health, lifestyle and medication.

The Patient Health Questionnaire 9 (PHQ-9) (Kroenke *et al*., 2001) is a multipurpose instrument for screening, diagnosing, monitoring and measuring the severity of depression.

The Generalized Anxiety Disorder Questionnaire 7 (GAD-7) (Spitzer *et al*., 2006) is a brief self-rated questionnaire measuring for symptoms of generalized anxiety disorder.

Assessment of emotional state and personality

Positive and Negative Affect Schedule (PANAS) (*Development and validation of brief measures of positive and negative affect: The PANAS scales*, 1988) is a scale that consists of different words that describe feelings and emotions. One of these scales measures positive affect, and the other measures negative affect. Positive affect refers to the propensity to experience positive emotions and interact with others positively, even through the challenges of life. Negative affect, on the other hand, involves experiencing the world in a more negative way.

Big-five questionnaire (BIG-5) (Goldberg, 1992) measures personality differences over five major dimensions of personality: openness, conscientiousness, agreeableness, extraversion and neuroticism.

Assessment of subjective sleep

*Pittsburgh Sleep Quality Index (PSQI) (*Buysse *et al.,* 1989) is 24-item questionnaire that generates seven component scores (Romero-Blanco *et al.*, 2020 habitual sleep efficiency, sleep disturbances, use of sleep medications, and daytime dysfunction).

The Mannheim Dream Questionnaire (Schredl *et al.,* 2014) is a 21 item questionnaire which examines different aspects of dreaming.

*Karolinska Sleepiness Scale (KSS) (*Akerstedt & Gillberg, 1990) assesses sleepiness during the previous 5 minutes on a 1 (extremely alert) to 9 (extremely sleepy) scale.

Assessment of chronotype and diurnal preference

Morningness-Eveningness Questionnaire (MEQ) (Horne and Ostberg, 1976) is a simple questionnaire that assesses one's preferences towards morningness or eveningness where the respondent is asked to indicate when, for example, he/she would prefer to wake up or start sleep, rather than when he/she actually does.

Munich ChronoType Questionnaire (MCTQ) (Roenneberg, Wirz-Justice and Merrow, 2003) estimates chronotype based on the midpoint between sleep onset and offset on work-free days.

Assessment of fluid intelligence

Raven's Advanced Progressive Matrices (APM) (Raven and John Hugh, 1962) measures high-level observation skills, clear thinking ability, and intellectual capacity as a non-verbal estimate of abstract reasoning or fluid intelligence.

Appendix 3

Official Advanced Progressive Matrices table used for conversion of percentile points into IQ points

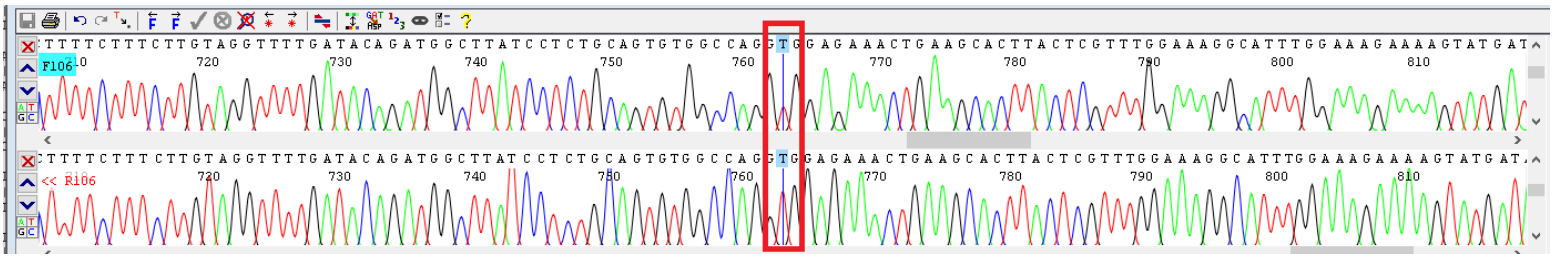
Table APM36
Table for Conveniently Comparing Percentile Points and PM Grades with IQs, Standard Deviations, Standard Scores, and Stanines

IQ	Percentile rank	PM grade	Standard score	Stanine	Standard deviation	IQ	Percentile rank	PM grade	Standard score	Stanine	Standard deviation
135	99.0	I	9.4	9	+2.33	99	47.2	III–	4.8	5	–0.07
134	98.8	I	9.3	9	+2.27	98	44.8	III–	4.7	5	–0.13
133	98.6	I	9.2	9	+2.20	97	42.1	III–	4.6	5	–0.20
132	98.3	I	9.1	9	+2.13	96	39.4	III–	4.4	4	–0.27
131	98.1	I	9.0	9	+2.07	95	37.1	III–	4.3	4	–0.33
130	97.7	I	8.8	9	+2.00	94	34.5	III–	4.2	4	–0.40
129	97.3	I	8.7	9	+1.93	93	31.9	III–	4.1	4	–0.47
128	96.9	I	8.6	9	+1.87	92	29.8	III–	3.9	4	–0.53
127	96.4	I	8.5	9	+1.80	91	27.4	III–	3.8	4	–0.60
126	95.8	I	8.4	8	+1.73	90	25.1	III–	3.6	4	–0.67
125	95.2	I	8.3	8	+1.67	89	23.3	IV	3.5	4	–0.73
124	94.5	II+	8.2	8	+1.60	88	21.2	IV	3.4	3	–0.80
123	93.7	II+	8.1	8	+1.53	87	19.2	IV	3.3	3	–0.87
122	92.9	II+	7.9	8	+1.47	86	17.6	IV	3.2	3	–0.93
121	91.9	II+	7.8	8	+1.40	85	15.9	IV	3.0	3	–1.00
120	90.8	II+	7.7	8	+1.33	84	14.2	IV	2.8	3	–1.07
119	89.8	II	7.6	8	+1.27	83	12.9	IV	2.7	3	–1.13
118	88.5	II	7.5	7	+1.20	82	11.5	IV	2.5	3	–1.20
117	87.1	II	7.3	7	+1.13	81	10.2	IV–	2.4	2	–1.27
116	85.8	II	7.2	7	+1.07	80	9.2	IV–	2.3	2	–1.33
115	84.1	II	7.0	7	+1.00	79	8.1	IV–	2.2	2	–1.40
114	82.4	II	6.9	7	+0.93	78	7.1	IV–	2.1	2	–1.47
113	80.8	II	6.7	7	+0.87	77	6.3	IV–	1.9	2	–1.53
112	78.8	II	6.6	7	+0.80	76	5.5	V	1.7	2	–1.60
111	76.7	II	6.4	6	+0.73	75	4.8	V	1.6	2	–1.67
110	74.9	III+	6.3	6	+0.67	74	4.2	V	1.4	2	–1.73
109	72.6	III+	6.2	6	+0.60	73	3.6	V	1.2	1	–1.80
108	70.2	III+	6.0	6	+0.53	72	3.1	V	1.1	1	–1.87
107	68.1	III+	5.9	6	+0.47	71	2.7	V	1.0	1	–1.93
106	65.5	III+	5.7	6	+0.40	70	2.3	V	0.9	1	–2.00
105	62.9	III+	5.6	6	+0.33	69	1.9	V	0.8	1	–2.07
104	60.6	III+	5.5	6	+0.27	68	1.7	V	0.7	1	–2.13
103	57.9	III+	5.4	5	+0.20	67	1.4	V	0.7	1	–2.20
102	55.2	III+	5.2	5	+0.13	66	1.2	V	0.6	1	–2.27
101	52.8	III+	5.1	5	+0.07	65	1.0	V	0.6	1	–2.33
100	50.0	III+	5.0	5	0						

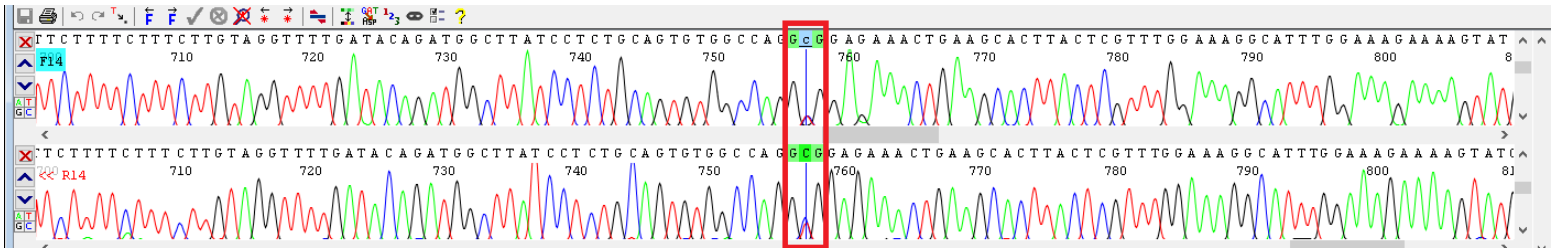
Note: This table is provided to facilitate general comparisons only. RPM scores should not be converted to deviation IQs because the distribution of the scores is not Gaussian for most of the populations which have been studied.

Appendix 4

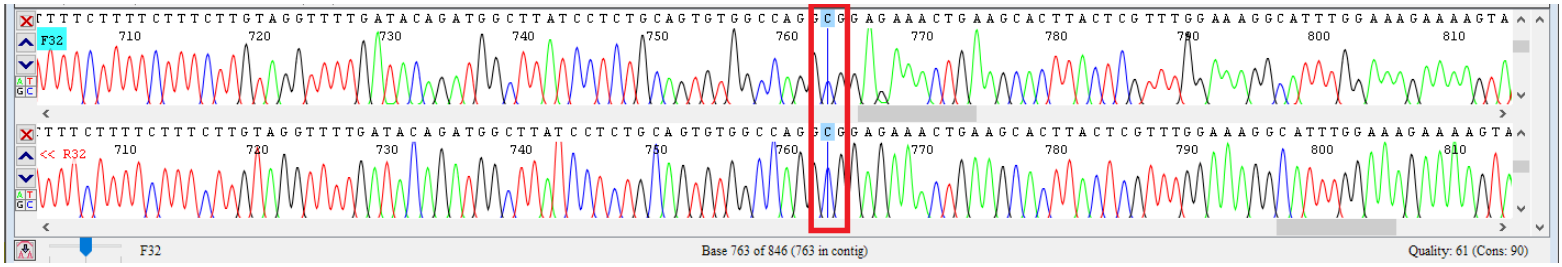
Exemplary chromatograms showing different genotypes of the SNP found in this study



Appendix 4.1 rs8192440 TT homozygote



Appendix 4.2 rs8192440 TC heterozygote



Appendix 4.3 rs8192440 CC homozygote