IMPACT OF NIGHT SHIFT WORK ON

CENTRAL AND PERIPHERAL CLOCKS IN POLICE OFFICERS

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ABSTRACT (English)

A central clock and many peripheral clocks, located throughout our bodies, generate 24-hour rhythms in various physiological systems. Several studies have shown that the central clock becomes desynchronized with the environment during shift work. However, there is not much data on the peripheral clocks in shift workers. This study assessed 2 central clock markers and 2 separate peripheral clocks of police officers, before and after 7 consecutive night shifts.

Quantitative PCR was used to assess the circadian clock gene expression in peripheral blood mononuclear cells (PBMCs) and oral mucosa cells. Enzyme-linked immunosorbent assays (ELISA) were used to analyze saliva and urine samples for the assessment of central clock markers, salivary cortisol and urinary 6-sulfatoxymelatonin (aMT6s), respectively. Eleven police officers were studied for 24 hours at the laboratory, before and after a week of night shifts. At each visit, we collected saliva samples, before and after sleep periods, urine samples approximately every 3 hours, blood samples at 10h00 and 19h30, and oral mucosa samples approximately every 4 hours.

Before the week of night shifts, the rhythmic expression of clock genes, *PER1, PER2, PER3,* and *REV-ERBα,* in oral mucosa cells had a significant peak in the morning, while in PBMCs, a significant difference was observed for clock genes, *PER1, PER2,* and *PER3*, between 10h00 and 19h30. After 7 consecutive night shifts, the rhythmic expression of clock genes, *PER2, PER3,* and *REV-ERBα*, in oral mucosa cells showed either a loss of rhythmicity or a loss of its temporal relation with the sleep-wake cycle, while in PBMCs, the significant difference that was observed at baseline was lost in all clock genes after 7 consecutive night shifts. Salivary cortisol levels and rhythmic urinary aMT6s excretion were observed to show different levels of adaptation between individuals after 7 consecutive night shifts.

Overall, our results suggest that the 2 peripheral clocks that were studied were disrupted in police officers after 7 consecutive night shifts, while the 2 central clock markers tend to show a certain degree of adaptation.

RÉSUMÉ (Français):

L'horloge centrale et plusieurs horloges périphériques, situées dans notre corps, génèrent des cycles de 24 heures dans divers systèmes physiologiques. Plusieurs études ont montré que durant le travail posté, l'horloge centrale se désynchronise de l'environnement. Cependant, il y a peu de données sur les horloges périphériques chez les travailleurs postés. Ainsi, cette étude a évalué deux marqueurs de l'horloge centrale et deux marqueurs d'horloges périphériques distinctes chez des policiers en autopatrouille précédant et suivant une série de 7 quarts de nuit.

Un PCR quantitatif a été utilisé pour mesurer l'expression des gènes de l'horloge circadienne dans les cellules mononuclées sanguines (PBMC) et dans les cellules de la muqueuse orale. Des « Enzyme Linked Immunosorbant Assay » (ELISA) ont été utilisées pour analyser les échantillons salivaires et urinaires pour leur contenu en marqueurs de l'horloge centrale, soit le cortisol salivaire et 6-sulfatoxymélatonine urinaire. Onze policiers en autopatrouille ont été étudiés durant 24 heures dans notre laboratoire avant et après leur semaine de quarts de nuit. À chaque visite, des échantillons de salives ont été récoltés précédant et suivant leur période de sommeil, des échantillons d'urines ont été récoltés à chaque 3 heures environ, des échantillons sanguins ont été prélevés à 10h00 et 19h30, et des échantillons de cellules de la muqueuse orale ont été récoltés à chaque 4 heures.

Précédant leur semaine de quart de nuit, l'expression rythmique des gènes de l'horloge, *PER1*, *PER2*, *PER3* et *REV-ERBα*, dans les cellules de la muqueuse orale atteignaient un pic en matinée, tandis que dans les PBMC, une différence significative a été observée pour *PER1*, *PER2* et *PER3* entre 10h00 et 19h00. Suivant leur série de 7 quarts de nuit, l'expression rythmique des gènes de l'horloge *PER2*, *PER3* et *REV-ERBα*, dans les cellules de la muqueuse orale ont soit perdu leur rythmicité ou soit perdu leur relation temporelle avec le cycle veille-

sommeil, tandis que dans les PBMC, la différence significative observé au niveau de base a été perdue dans tous les gènes de l'horloge. Les niveaux de cortisol salivaire et la rythmicité de 6-sulfatoxymélatonine urinaire ont été observés pour montrer les différents niveaux d'adaptation entre les participants après leur semaine de quarts de nuit.

Pour conclure, ces résultats suggèrent que les deux marqueurs d'horloges périphériques étudiées ont été perturbés chez des policiers en autopatrouille après une série de 7 quarts de nuit, tandis les deux marqueurs de l'horloge centrale semble avoir un certain degré d'adaptation.

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GLOSSARY OF ABBREVIATIONS
(alphabetical order)

aMT6s – 6-sulfatoxymelatonin

ARNTL – Aryl hydrocarbon receptor nuclear translocator-like protein

B2M – Beta-2 microglobulin

BMAL1 – Brain and muscle ARNT-like; see ARNTL

CBT – Core body temperature

CLOCK – Circadian locomotor output cycles kaput

CMSP – Cellules mononucléaires du sang périphérique

CRY – Cryptochrome

ELISA - Enzyme-linked immunosorbent assays

ipRGC – Intrinsically photosensitive retinal ganglion cells

MEQ – Morningness-Eveningness Questionnaire

NR1D1 – Nuclear receptor subfamily 1, group D, member 1

PBMC – Peripheral blood mononuclear cells

PER – Period

RORα – RAR-related orphan receptor alpha

PPIA - Peptidylprolyl isomerase

qPCR – Quantitative Polymerase Chain Reaction

REV-ERBα – REV-ERB alpha; see NR1D1

RHT – Retinohypothalamic tract

RGC – Retinal ganglion cells

SCN – Suprachiasmatic nucleus

INTRODUCTION

1. BACKGROUND INFORMATION:

1.1. Description of the circadian system

The circadian system is one of the most important regulatory systems in human beings; through intricate signalling pathways, this system synchronizes the body with the 24-hour day, ensuring the proper functioning of various rhythmic physiological parameters [1, 2]. The circadian system is composed of oscillatory structures called "clocks" which are found in virtually all cells of the body [2]. Circadian clocks are characterized by their capability to generate endogenous rhythms of approximately 24 hours. Therefore, these rhythms persist under constant conditions, without the influence of any external factors [3, 4].

The clocks in the human circadian system include a central 'master' clock, situated in the suprachiasmatic nucleus (SCN) of the hypothalamus, and various peripheral clocks, located in non-SCN brain regions and non-neuronal cells throughout the body [2, 4, 5].

1.1.1. Central clock

The SCN is called the master clock since it is known to hierarchically control the rhythmicity of other clocks in the body [2, 6, 7]. It generates circadian rhythmicity through transcriptional and translational feedback loops involving clock genes [2, 8]. These clock gene oscillations subsequently regulate the expression of other genes, referred to as clock-controlled genes, which are involved in various biochemical, molecular, and physiological processes [9-11]. Some of the genes involved in the feedback loop are *BMAL1* (or *ARNTL1*) and *CLOCK,* whose products act as transcription factors and bind to *E-box* elements found on promoters of other genes such as the period

genes (*PER1, PER2,* and *PER3),* the cryptochrome genes (*CRY1* and *CRY2), RORα,* and *REV-ERBα* [2, 8]. BMAL1 and CLOCK, the positive elements of the loop, activate the transcription of several genes including the negative elements of the loop, *CRY* and *PER*, which then inhibit their own transcription by negatively regulating BMAL1 and CLOCK activity [2, 8, 12]. The genes *RORα* and *REV-ERBα* make up a secondary feedback loop in which their products act as transcription factors and activate or repress *BMAL1*, respectively, via competitive binding on ROR-specific elements located on the *BMAL1* promoter [2, 12, 13]. These transcription loops are synchronized to the 24-hour light-dark cycle and also have a period of approximately 24 hours [2]. In the primary feedback loop of most cells, the clock genes *PER1-3* are transcribed and expressed highly in the daytime while *BMAL1* is transcribed and expressed highly in the evening. In the secondary feedback loop of these cells, *REV-ERBα* is transcribed and expressed highly in the morning.

The SCN is comprised of approximately 100, 000 neurons and generates rhythmic oscillations via the transcriptional-translational feedback loops [14, 15]. Individually, each of these SCN rhythms have a variable acrophase (the time at which a peak occurs during a cycle; peak being the highest value of expression of the marker in question) and period (the total duration in time to complete a full cycle) [16]. However, the robustness of the SCN arises due to the synchronization of these rhythms, intercellularly, that leads to a highly-coupled network which generates very strong rhythmicity [6, 14, 16].

As it is impossible to directly assess the rhythms generated in the SCN of a living human subject, physiological parameters that are strongly regulated by the signals transmitted from the SCN serve as practical markers to study the central clock. These

markers include the endocrine hormone secretion of cortisol and melatonin, as well as core body temperature (CBT) [17, 18].

1.1.2. Peripheral clocks

This molecular clock mechanism is also found in peripheral tissues, throughout the human body [2, 8]. The core clock genes expressed in the periphery can generate circadian rhythmicity independent of the SCN, and thus, these peripheral cells are considered as autonomous clocks or peripheral clocks [19-22].

The peripheral clocks were studied in this project by assessing clock gene expression in peripheral tissues, such as in oral mucosa cells and peripheral blood mononuclear cells (PBMCs). Previous studies have assessed clock gene expression in hair follicle cells and in human epidermis as well as in *in vitro* studies using skin fibroblasts [23-27]. The *in* vitro studies in skin fibroblasts not only showed that the period length of the human circadian clock *in* vivo is proportional to the period length of cultured human fibroblast cells but it also demonstrated that peripheral clocks in humans can be cell-autonomous [25, 28].

1.2. Entrainment and realignment of circadian rhythms

1.2.1. Photic effects on the central circadian clock

Photic information from the environment directly enters the retina where it is received by rods, cones, and a subset of retinal ganglion cells (RGCs) which expresses melanopsin, called intrinsically photosensitive RGCs (ipRGCs) [29, 30]. The information then travels to the SCN, via the retinohypothalamic tract (RHT), synchronizing it with the light-dark cycle [31]. These particular photoreceptors, ipRGCs, are fundamental for the photic entrainment of the SCN and the control of circadian rhythms [29, 30]. Light at

night, in particular white light, is shown to have acute alerting effects, increasing cortisol, and improving performance [32-34]. Moreover, white light at night suppresses melatonin production, reduces sleepiness, and in mice, dim light decreases the circadian expression of clock genes - *Per1, Per2,* and *Cry2* [35-37]. The suppression of melatonin by light at night is due to the 'masking' effect of light which directly inhibits the synthesis of melatonin in the pineal gland [37]. Light during the daytime increases the nocturnal production of melatonin and improves moods but does not significantly affect clock gene expression [38-40].

1.2.2. Coordination of peripheral clocks by systemic synchronizing factors

Various systemic synchronizing factors coordinate peripheral clocks, via neuronal and hormonal signals that are integrated and transmitted from the SCN, as well as from feeding-fasting cycles and temperature variations [18, 22, 41, 42].

Neurons from the SCN project to different areas in the brain to control physiological parameters such as sleep and CBT [43]. SCN projections via hormonal signals, like melatonin and glucocorticoids, are also known to regulate peripheral structures, such as the liver, kidney, pancreas, heart, adrenal cortex, and brown adipose tissue, thus controlling the rhythmicity of various metabolic and physiological processes that these structures are involved in [44].

Studies in rodents have shown that food availability and timing of feeding have influences on the peripheral clocks [21, 22, 45, 46]. Feeding cues can reset peripheral clocks without involving the SCN. For example, when the time of feeding in rodents shifts and becomes desynchronized with the light-dark cycle, peripheral organs, such as

the liver and kidneys, can become synchronized to the new feeding schedule through food cues whereas the SCN remains synchronized to the light-dark cycle [22, 45, 47].

Hormonal signals, such as glucocorticoids and dexamethasone, a synthetic corticosteroid, are shown to entrain rhythms of gene expression. Recently, a study by Cuesta et al. demonstrated that molecular components of the human peripheral PBMC clocks can be entrained by exogenous glucocorticoid administration, while it has no entraining effects on central clock markers [48]. The clock genes, *PER2, PER3,* and *BMAL1,* were all responsive to the exogenous glucocorticoid administration [48]. In particular, *PER2, PER3,* and *BMAL1* were all phase shifted accordingly after 6 days of glucocorticoid administration [48]. Even studies in rodents have shown that injection of a glucocorticoid receptor agonist, dexamethasone, can reset rhythms of gene expression in peripheral tissues such as the liver, kidney, and heart [20, 21].

Thus, the molecular clock mechanism and various systemic factors, both, allow robust circadian oscillations and alignment of peripheral clocks to the 24-h light-dark cycle.

1.3. Shift work and disruption of circadian rhythms

1.3.1. Shift work

As the existence of shift work becomes more prevalent in today's work environment, so are the issues associated with it. Approximately 28% of the Canadian workforce operates outside of the conventional daytime hours, working either regular evening or night shifts, rotating shifts, on-call jobs, or other irregular shifts [49].

The sleep-wake cycle is one possible pathway through which the SCN regulates the circadian system. In general, humans sleep at night, when it is dark outside. At this

time, the circadian rhythm of body temperature has its nadir and that of melatonin production has its peak. In contrast, when individuals work night shifts, they sleep during the day time, while their circadian systems often continue to function under a day-oriented schedule. Thus, the temporal relationship that exists between the individual's sleep-wake cycle and their circadian rhythms is disrupted, which could lead to the disruption of physiological and endocrine rhythms in their body. Additionally, an internal misalignment could also develop, between an individual's central circadian clock and the peripheral clocks, located in various cells and tissues of the body [45, 50].

Shift work can also lead to sleep deprivation from working odd hours [51]. This is important because circadian disturbances and/or sleep deprivation are major contributors of various health problems such as cancer, metabolic syndrome, autoimmune and cardiovascular diseases [51-56]. There are several negative impacts on an individual as a result of working shifts and in this study, we focussed on assessing the state of the circadian system and the circadian clocks in shift workers.

1.3.2. Consequences on the central clock

The central clock's main output includes hormonal systems, such as melatonin and cortisol, and core body temperature, which are all under strong circadian control [57, 58]. When the temporal relationship between the sleep-wake cycle and the circadian system is disrupted, it leads to the subsequent disruption of physiological and endocrine rhythms in the body [59].

In recent studies involving night-shift working nurses and police officers, it was shown that the circadian pacemaker does not rapidly adapt to shifted sleep schedules introduced by working 7-12 night shifts over 1-3 weeks. This was evidenced by a lack of entrainment of the salivary melatonin and cortisol rhythms as well as the core body temperature rhythms to a night-oriented schedule in both groups [17, 60-62]. Another study demonstrated that shifting the sleep episodes of healthy subjects by 12 hours led to the cortisol profile in these individuals to be misaligned with their shifted sleep-wake schedule [63]. Moreover, cortisol levels were also affected after restricting sleep by just 2 hours in healthy subjects; a significantly lower peak of cortisol secretion was observed in the morning after 1 week of sleep restriction compared to baseline, with particularly lower secretion immediately after the early awakening [64].

Also, during nocturnal sleep periods that follow sleep deprivation, plasma cortisol levels in the second half of the night are significantly reduced when compared to levels at control conditions [65]. This is because slow wave sleep is present at a higher than usual level during the second half of the sleep episode that follows a sleep deprivation period. Due to the inhibitory effect of slow wave sleep on cortisol release, there is subsequent reduction of plasma cortisol levels [65].

1.3.3. Consequences on the peripheral clock

When the sleep-wake cycle is desynchronized from the internal circadian system, clock gene rhythms are also disrupted, which can lead to disturbed physiological functioning [9]. Since the sleep-wake cycle is involved in coordinating the timing of molecular processes such as transcription and translation, sleeping at inappropriate times can affect these processes, in particular, the transcription and translation of core clock genes that are involved in the generation of circadian rhythms [59]. When working atypical schedules, like during the night, it also leads one to eat meals at atypical times during the day, which in turn lead to improper metabolism and nutrient uptake and various metabolic issues [66].

A recent study showed that mistimed sleep led to the disruption of gene expression in whole blood cells, with certain genes being up- or down-regulated when compared to baseline [59]. Moreover, sleeping at inappropriate times also led to a loss of rhythmicity in transcripts involved in key molecular processes, from 6.4% at baseline to 1% after participants underwent a laboratory protocol that enforced them to sleep at progressively delayed times over 3 days [59]. A study has also shown that shifting sleep periods of healthy subjects by 4 hours over 3 weeks led to clock gene expression of *PER2, PER3,* and *REV-ERBα* not being adapted to their shifted sleep-wake schedule [23].

In another study by Scheer et al., when subjects slept 12 hours out of phase from their usual sleep times, after progressively delaying their sleep schedules over 4 days, there was a subsequent decrease in leptin levels, an increase in glucose levels, and insulin resistance [53]. This was in accordance with a study that showed that circadian misalignment increases postprandial glucose, independent of circadian and behavioural

effects, decreases pancreatic B-cell function during the biological evening, and decreases insulin sensitivity [63].

It has also been demonstrated that when humans eat during the night, as do night shift workers, their total daily energy expenditure is reduced [55]. Thus, if food intake is not reduced as energy expenditure is reduced, it would contribute to weight gain and a higher risk of obesity [55].

In mice, daytime restricted feeding (feeding during the rest phase) leads to a misaligned expression of circadian clock-controlled homeostatic genes, which can eventually generate metabolic pathologies including diabetes and obesity [67, 68]. However, the SCN clock is left unaltered during restricted feeding which creates a misalignment between peripheral clocks and SCN clock-controlled phases of activity and rest [67]. Another study in mice demonstrated that stimuli such as light during the rest phase leads to a metabolic imbalance which can subsequently affect the circadian clock [69]. It was shown that a high-fat diet led to changes in the expression and rhythmicity of clock-controlled genes involved in energy metabolism in liver and adipose tissue [69].

Similar to mistimed sleep, lack of sufficient sleep or sleep deprivation can lead to adverse effects on the circadian system. A transcriptome analysis conducted in subjects that underwent a sleep restriction protocol resulted in a reduction of rhythmic genes in whole blood, from 1855 to 1481, and in an increase in certain genes that responded only to sleep deprivation from 122 to 856 [54]. The genes that were affected by insufficient sleep were clock genes such as *PER1-3* and *CLOCK*, and other genes that play a role in sleep homeostasis, oxidative stress, and metabolism [54]. Moreover, even one night of sleep deprivation under dim light conditions led to *BMAL1* mRNA expression in PBMCs

to be suppressed while that of other clock gene rhythms were not affected [70]. The changes observed for *BMAL1* are noteworthy because of its key role in circadian rhythm generation and therefore, the results from this study demonstrates that acute sleep deprivation negatively impacts the core clock mechanism in peripheral tissues. It was also observed that the amplitude of the melatonin rhythm was increased after this one night of sleep deprivation [70]. Amplitude is measured as the average value from peak to trough in a biological rhythm.

In a study by Davies et al., it was observed that the number of rhythmic metabolites in the plasma of subjects, after they stayed awake for 24 hours, was reduced when compared to the corresponding number during their normal sleep periods [71]. The researchers also noticed that certain metabolites, such as serotonin, tryptophan, and taurine, displayed increased levels after this acute sleep deprivation period. Interestingly, these molecules are linked with anti-depressive effects [71].

Studies involving peripheral clocks have also been conducted in actual shift workers. Reszka et al. recently showed that *PER1* and *PER3* transcript levels in rotating shift workers were significantly down-regulated during the later hours of the morning compared to the early hours, while they found no significant effect on the expression of genes *PER2, BMAL1, CLOCK,* and *CRY1-2* in peripheral blood leukocytes [72]. Another recent study has shown that mRNA expression of *PER2* in whole blood cells had higher levels, both in the evening preceding a night shift and in the morning, at the start of their day shift [73]. Thus, the authors concluded that *PER2* could serve as a biomarker of disrupted circadian rhythm in whole blood cells [73]. *PER2* was also shown to be affected in another peripheral clock, hair follicle cells. The maximum levels of *PER2*

gene expression in hair follicle cells was significantly different between shift work and daytime nurses [74]. However, this study had its limitations as there was no record of sleep logs or other methods to control between-subjects' variabilities [74].

Overall, the human body is clearly affected by disruption of sleep and/or insufficient sleep and can subsequently result in disruption of gene expression and proper metabolism.

1.3.4. Consequences on attention and vigilance

While sleeping, the brain is at work, processing complex information and connecting different pieces of this information together to form intricate neural networks – all of which prepares an individual to complete important tasks while awake [4].

Cognitive speed, working memory, and attention are all parameters that are sensitive to sleep deprivation and can lead to potential workplace accidents [17, 75]. Lack of sleep also affects flexible thinking, multitasking, risk assessment, insight, and temporal memory skills [75]. These skills are necessary to work efficiently, especially when this work involves the monitoring of health, safety and/or security of the public population.

Working night shifts also increases the risk of workplace injuries and accidents, independent of sleep [76]. In a constant routine protocol, tonic and phasic alertness as well as selective attention had circadian variation [77]. Thus, working during hours when alertness is at a circadian low, i.e. during the night, will lead to the risk of the occurrence of workplace errors [77]. High subjective ratings of alertness and motivation are also associated with high performance, independent of circadian phase and hours awake [78]. Thus, the circadian nature of attention itself can lead to various errors and workplace accidents while individuals work during the night.

1.3.5. Therapies and interventions to counteract disruption of circadian rhythms and effects of sleep deprivation

Although circadian disturbances and sleep restriction in shift workers leads to various health and safety issues, including risks of developing cancer and cardiovascular disorders, recent studies discuss about the various forms of therapies and interventions that are now available to be used to counteract the numerous negative outcomes of working shifts.

In a simulated shift work study, it was observed that administering bright light therapy during night shifts, helped to rapidly entrain centrally regulated circadian rhythms of cortisol and melatonin secretion to the shifted sleep-wake schedule (night-oriented schedule) following just 3 nights of therapy [50]. Bright light therapy has also led to phase shifts in actual shift working populations. After a series of night shifts, phases of salivary melatonin and CBT rhythms were reset to baseline phases only in the treatment group of nurses that received bright light and not in the control group [60]. In another study, nurses that received bright light treatment had subjectively better sleep quality but had no significant differences in their salivary melatonin levels [79].

Studies have also shown that the timing and careful control of light and darkness exposure can substantially affect the re-entrainment of central markers to a shifted sleep/wake schedule [61, 62]. In a study by James et al., night shift working nurses who worked 12 nights, underwent an intervention of 2000 lux bright light for 6 hours during work, after which they wore tinted neutral grey density lens on their return home, plus slept in total darkness 2 hours after the end of each shift, had significant phase shifts in times of peak cortisol expression and realignment with the night-shift schedule [62].

Thus, maintaining regular sleep schedules and sleeping in total darkness also helps to counteract circadian disruption [61, 62].

Human peripheral clocks can also be treated with pharmacological agents such as glucocorticoids as seen in the recent study by Cuesta et al. After 6 days of glucocorticoid administration, clock genes *PER2, PER3,* and *BMAL1* were all phase shifted accordingly [48].

Thus, interventions such as multimodal therapy that couples pharmacological treatment (peripheral clocks) with bright-light therapy (central clock) can be further developed and suggested as a possible way to counteract the deleterious effects of shift-work.

1.4. Circadian desynchrony and its complexity

Desynchrony of the circadian system is complex as it affects different levels of the circadian system and has major impact on us. More importantly, these circadian disturbances in circadian clocks could have significant clinical implications. Recently, rhythms of cytokine secretion have shown to be disrupted in subjects that underwent a simulated shift-working protocol, impacting their immune system [52]. Also, a recent study in mice has shown that a disruption in the biological clock can lead to rapid tumor growth, suggesting that this disruption can lead to the development of cancer [80]. There are also implications of disrupted sleep on our cardiovascular health, such that circadian desynchrony can be a factor that is implicated in the development of heart disease [56]. Moreover, circadian disturbances can also be associated with neurological issues such as Alzheimer's disease, as was seen with the altered expression of clock genes *PER1, PER2,* and *BMAL1* patients with this disease

[81]. Due to its immense number of health implications, it is important to carefully study the different types of desynchronization that can occur in our circadian systems

1.4.1. Desynchrony between circadian clocks and the environment

Desynchrony can occur between the circadian system and the environment (i.e. the shifted sleep-wake and feeding schedules). In a recent study conducted in police officers who worked 7 consecutive nights of shift work, 56% of the participants did not adjust their central clock (salivary melatonin rhythms) to the shifted sleep-wake schedule after 7 consecutive night shifts [17]. However, the remaining 44% of the police officers did indeed adapt their salivary melatonin rhythms to the shifted sleep-wake schedule after the 7 consecutive night shifts [17]. This adaptation was spontaneous and not influenced by any therapy. The 44% rate of adjustment was much higher than the previously reported ~3% adjustment rate of central markers (endogenous melatonin rhythm) that was estimated in permanent night shift workers, based on the results from prior studies [17, 82]. This discrepancy between studies could be explained by the specific design of the work roster in the latter study [17, 82]. Since police officers worked 7 consecutive night shifts in the Boudreau et al. study (44% adaptation), as opposed to only about 3 days in the studies analyzed by Folkard et al. (3% adaptation), there was a high possibility of spontaneous adaptation that might have occurred because they had about 4 additional days of shift work. This spontaneous adaptation could also be explained by a potential selection bias as the police officers who were recruited in the Boudreau et al. study were motivated individuals who had to maintain regular sleeping habits and be studied twice, in a time-isolation room. They also slept in total darkness and presumably

exposed themselves to higher light intensities in the late night/early morning compared to non-adapted officers in the control group [17].

1.4.2. Internal desynchrony between central and peripheral clocks

An internal desynchrony can occur within the circadian system itself, between the central clock and the peripheral clocks, leading to a misalignment between central and peripheral clocks [50]. A simulated shift work study by James et al. demonstrates that temporal misalignment can occur between an individual`s central clock and peripheral clocks with the use of bright light exposure at night [50]. Bright light was shown to entrain central clock markers within just 3 nights while rhythms of clock gene expression in PBMCs, were adjusted with the shifted sleep-wake schedule only after 10 nights of bright light therapy [50]. This study suggests that bright light cannot rapidly entrain peripheral clocks, possibly due to the indirect pathway that the photic cue needs to take in order to act on the peripheral cells, although further studies are needed to provide more evidence for this [18].

This internal desynchrony shows that peripheral clocks can still be disrupted even if the central clock is realigned with a night-oriented schedule using therapeutic measures. Peripheral clocks were also shown to be entrained by glucocorticoids without any entrainment effects on central clock markers [48]. Finally, restricted feeding in mice has also shown that it leads to the entrainment of the liver clock independent of the SCN [45].

Altogether, these studies indicate that central and peripheral clocks could be desynchronized from each other when our sleep is disrupted or insufficient.

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1.4.3. Desynchrony between different peripheral clocks themselves

Different peripheral clocks can also be desynchronized between each other. As mentioned previously, feeding time can reset the circadian phase of tissues such as the liver, heart, pancreas, and kidneys but each of these tissues get reset at different times; i.e. the liver clock gets reset by daytime feeding within 2 days while the other tissues only get reset in about a week; however, after this one week, gene expression rhythms in all of these peripheral tissues were similar [22, 47]. This initial difference in resetting time demonstrates circadian desynchrony that can occur between the different peripheral clocks themselves.

Another study conducted in *Per1-luciferase* rats showed that when they were adrenalectomized, tissues such as in kidney, cornea, pituitary gland, and the liver, while it had no effect on the lung, pineal or salivary gland. On the other hand, when they treated these adrenalectomized animals with glucocorticoids, it advanced the phase of *Per1-luciferase expression* in the pituitary while delaying the phase of gene expression in the cornea and lung [83]. Thus, the variability in shifting and entraining rhythms of gene expression in different tissues clearly demonstrates the extent of desynchronization between different peripheral clocks.

2 *RATIONALE:*

There have been several studies done in the past, studying the desynchrony of the circadian system in both central and peripheral clocks, conducted in simulated shift work conditions [50, 53, 54, 59, 70]. There have also been studies focussing on central clock markers and their disruption in actual shift workers [17, 60, 84, 85]. However, there are very few studies, to date, that have addressed peripheral clock disruption in actual shift workers who work during the night, and these studies either looked at only 1 peripheral clock, observed significant gene expression of a very limited number of genes, or did not include very well-controlled protocols [73, 74]. It is important to study the impact of shift work on peripheral clocks since a disruption of clock gene expression can lead to the disrupted expression of clock-controlled genes – genes that underlie the appropriately-timed regulation of rhythmic physiological parameters in the body. The project presented in this thesis will include the assessment of 2 central clock markers and 2 separate peripheral clocks, and will particularly focus on their possible desynchrony, both internally within the circadian system, and externally with the environment.

Police officers that were recruited for this study work on atypical schedules, including nights that are organized as a series of 7 consecutive shifts followed by a week off (see Figure 1 for these police officers' work roster). Their work roster includes 3 weeks on a day-oriented schedule before their 1 week of night shifts. However, it is possible that some individuals were asked to work overtime with night shifts during that 3-week period. The only record of this happening with the subjects in our study was with one individual who had to work an extra night shift, 2 days prior to his 1st laboratory visit.

Due to working a week of night shifts, these workers are prone to experience desynchronization in their circadian rhythms relative to their sleep-wake cycle and this study was conducted to see the extent of this desynchronization.

3 AIM:

The aim of this study was to document the extent of desynchronization of circadian rhythms in actual shift workers, in both the central clock and 2 distinct peripheral clocks, before and after working 7 consecutive night shifts.

4 HYPOTHESES:

At baseline, before the series of 7 night shifts, we expect that:

- The central and peripheral clocks of individuals will remain adjusted to the day-oriented schedule that they are living under.

After working 7 consecutive night shifts, we expect that:

- Circadian misalignment will occur between an individual's central clock and their shifted sleep-wake cycle.
- Circadian misalignment will occur between an individual's peripheral clocks and their shifted sleep.
    - The misalignment in peripheral clocks will be greater than in the central clock.
    - The two peripheral clocks will respond differently to night shifts.
- Temporal desynchrony will occur between an individual's central and peripheral clocks.

METHODS

1 SUBJECTS:

We studied a total of 11 police officers (7 males and 4 females), aged 28.27±3.04 years, in this project thus far. As specific details that correspond to the studied subjects, such as the genders, ages, and seasons that each subject was studied at, could lead to the identification of individuals by coworkers at the police department, we have not provided individual details in this document. However, the chronotypes of individuals as per the Morningness-Eveningness questionnaire (MEQ) that they filled out are presented in the appendix - Table 4. The MEQ was used to assess subjects' chronotypes as either moderately morning type, intermediate, or moderately evening type [86]. Individuals who are moderately morning type have been reported to have earlier phases of circadian parameters, compared to moderately evening types, who tend to have later phases of circadian markers [86]. Individuals who are intermediate type do not tend to have earlier of later phases of circadian markers, compared to the norm [86]. Their individual sleep schedules, collected from sleep logs and verified by actigraphs, during the weeks preceding their laboratory visits and during their laboratory visits are also detailed in Table 2 (see Appendix). All 11 individuals were studied between the winter of the year 2016 to the winter of the year 2017. As for the phase of menstrual cycle at which the females were in during the time of their study, we were only able to obtain these details from 2 out of the 4 females that were studied. The 2 females were studied during their follicular phase and both females were taking oral contraceptive pills at the time of study. The intake of contraceptive pills was not part of the exclusion criteria since we wanted to study these police officers while they underwent their natural lifestyles.

2 RECRUITMENT AND SCREENING:

All subjects were required to be mentally and physically fit. Moreover, each subject filled out a general recruitment/screening questionnaire, as well as certain self-administered, validated screening questionnaires such as the Seasonal Pattern Assessment Questionnaire (SPAQ), used to screen for the existence of Seasonal Affective Disorder (SAD) [87], and the Berlin Questionnaire, used to screen for individuals who may be at risk for the sleep apnea syndrome [88]. Subjects who were deemed doubtful of being at risk for sleep-related pathologies also underwent a telephone interview with a sleep physician. Physical examinations were also conducted for each subject in order to exclude any participants who had chronic medical conditions that could affect the study results.

3 RESEARCH ETHICS BOARD:

As human experiments require very extensive and detail-oriented ethics approval applications, we have submitted the appropriate documents and have obtained the approval from the Douglas Mental Health University Institute's research ethics board for all aspects of this study.

4 AMBULATORY PERIODS:

Each police officer underwent one ambulatory monitoring session during the week preceding the first laboratory visit and one ambulatory monitoring session during the week of night shifts, between their first and second laboratory visits. During these periods, officers were required to complete sleep/wake logs, food logs, as well as wear an actigraph device that monitors light and activity levels at 15 second intervals (Actiwatch, Spectrum Plus, Philips/Respironics, OR, USA). The Actiwatch recorded light intensities in the range of 400-

700nm, specifically from the red, blue, and green light spectrums. All light intensity analyses were conducted using results from the combined white light spectrum. During the first ambulatory period, police officers worked on a day-oriented schedule with a mix of morning and evening shifts, as well as days off (appendix: Figure 2). The second ambulatory period consisted of 7 consecutive night shifts – 5 consecutive nights of 9-h shifts followed by 2 nights of 12-h shifts (appendix: Figure 2).

5 LABORATORY VISITS:

The first visit (segment B on experimental protocol; Figure 1), which will also be referred to as the baseline visit, occurred before the series of 7 night shifts (segment C on experimental protocol; Figure 1), while the second visit (segment D on experimental protocol; Figure 1), occurred on the day after working the 7th night shift. During the 2 laboratory visits, the officers were required to fill out pre-sleep and post-sleep questionnaires, shiftwork-related and wellness questionnaires that assessed the officers' overall sense of wellbeing and ability to concentrate, as well as continue wearing their Actiwatches. During their first laboratory visit, subjects were given breakfast, lunch, dinner, snack, and another breakfast around +0:45 h, +4:45 h, +10:30 h, +12:10 h, +23:10 h, respectively, after their lab entry time. During their 2nd visit, they were given a snack, breakfast, lunch, dinner, and another snack at approximately +0:02 h, +12:10 h, +14:45 h, +20:45 h, +22:45 h, respectively, after their lab entry time. During both visits, subjects entered the laboratory at 9h00 and exited the laboratory at 10h05. Also during both visits, subjects had 8-hour sleep episodes; from 23h00 to 7h00 during visit 1 and from 11h00 to 19h00 during visit 2.

6 *EXPERIMENTAL PROTOCOL:*

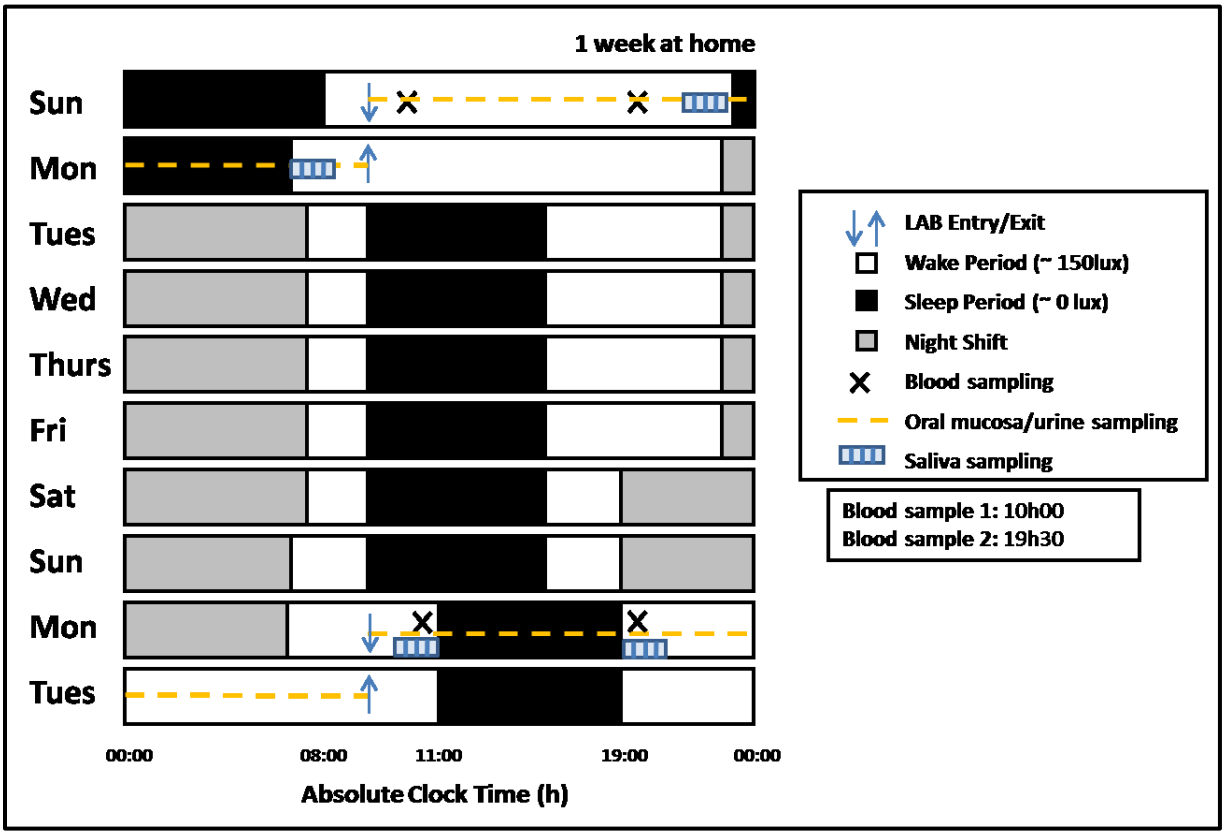


Figure 1: Experimental Protocol

Prior (A) to the first visit, police officers maintained a regular day-oriented schedule for ≥1 week at home. Before (B) and after (D) a series of 7 consecutive night shifts (C; grey bars), police officers visited the laboratory for 24 hours (entry at 9h00 and exit at 10h05 the next day, during both visits). During each laboratory visit, blood samples were collected in the morning (X) and in the evening (X). In addition, oral mucosa samples were collected every 2-4 hours (yellow dashed line). Sleep is represented by black bars and wakefulness by white bars. During visit 1 (B), police officers slept during the night (from 23h00 to 7h00) and during visit 2 (D), they slept during the day (from 11h00 to 19h00). The procedure illustrated above is for a participant with a night work schedule from 00:00 to 09:00 (first 5 nights) and from 0:00 to 12:00 (last 2 nights) and a daytime sleep schedule from 11:00 to 19:00 and from 14:00 to 22:00, respectively.

7 *MEASURES – BIOLOGICAL SAMPLES:*

7.1 Two markers of the central clock

The following biological samples served as 2 markers of the central clock and were analyzed as detailed further:

7.1.1 Saliva samples

Saliva samples were collected using Salivette cotton swabs without prep (Sarstedt Montréal, QC, Canada), every 15 minutes x 4 before bedtime (planned around -0:05 min, -0:20min, -0:35min, -0:50min before bedtime) and after awakening (planned around +0:06min, +0:21min, +0:36min, +0:51h after waketime). The samples were centrifuged immediately upon collection at 2900 revolutions per minute and stored at -20°C until further processing. Of important note is that precise times of sampling were documented for each individual and the analyses have considered these real times and not the planned ones.

Saliva samples were assayed for their content in salivary cortisol, which was also quantified in duplicate through commercially available enzyme-linked immunosorbent assays (ELISA) kits (Salimetrics, State College, PA, USA). The lower detection limit of the kit was 0.007 µg/dL, and its intra- and inter-assay coefficients of variations were experimentally determined to be approximately 3 - 7% and 3 - 11%, respectively.

7.1.2 Urine samples

During each of the laboratory visits, urine samples were collected approximately every 1-3 hours during a 24-h sampling period (planned around +0:05 h, +2:30 h, +5:00 h, +7:30 h, +9:30 h, +11:30 h, +13:47 h, +22:15 h, +24:15 h after

lab entry on visit 1 and +0:10 h, +1:47 h, +10:12 h, +12:40 h, +15:20 h, +17:20 h, +19:20 h, +21:20 h, +23:20 h after lab entry on visit 2). Of important note is that the subjects were requested to empty their bladder completely prior to each sleep period. Also, the precise times of sampling were documented for each individual and the analyses have considered these real times and not the planned ones.

Urine samples were assayed for their content of urinary 6-sulfatoxymelatonin (aMT6s), a metabolite of melatonin in urine, which was quantified in duplicate through commercially available enzyme-linked immunosorbent assay (ELISA) kits (ALPCO, Salem, NH, USA). The lower detection limit of the kit was 1.0 ng/mL, and its intra- and inter-assay coefficients of variations were experimentally determined to be between 5.2 – 12.2% and 5.1 – 14.9%, respectively.

7.2 Markers of two peripheral clocks

The following biological samples served as markers of 2 separate peripheral clocks and were analyzed as detailed further:

7.2.1 Blood samples

During each of the laboratory visits, blood samples were collected at 10h00 and 19h30. Since collecting blood samples at regular intervals over 24 hours to see the rhythmic pattern of expression of clock genes in PBMCs is a fairly invasive technique that involves an indwelling catheter, we decided to assess clock gene expression in PBMCs at only 2 critical time points during the day. These 2 time points were carefully chosen based on prior knowledge of peak expression times of the clock genes studied in PBMCs: *PER1, PER2, PER3, BMAL1, REV-ERBα. PER1, PER2, PER3*, and *REV-ERBα* are all clock genes that peak in the morning and have

their nadir in the evening [48, 89]. Of note, *REV-ERBα* is a gene that peaks very early in the morning [13]. *BMAL1* on the other hand is a gene that peaks in the evening but is known to have large inter-individual variability in its phase [48, 89]. Considering the circadian expression of these clock genes, we selected time points so that we can observe the gene expression of *PER1, PER2, PER3,* and *REV-ERBα*, when it is high in the 10h00 sample, and of BMAL1, when it is high in the 19h30 sample.

The PBMCs were isolated from whole blood samples which were collected in heparin-coated tubes, centrifuged for 30 minutes at 1600 rpm using Histopaque-1077 (Sigma-Aldrich, Oakville, ON, Canada) gradient centrifugation, washed with 1X phosphate-buffered saline, lysed in Trizol (Life Technologies, Burlington, ON, Canada), and stored in -80°C until further processing.

7.2.2 Oral mucosa samples

Oral mucosa samples were collected approximately every 4 hours during wake periods and once in the middle of the officer's sleep period (planned around +2:00 h, +6:00 h, +10:00 h, +13:53 h, +18:00 h, +22:03 h, +24:55 h after lab entry on visit 1 and around +1:53 h, +6:00 h, +10:03 h, +14:00 h, +18:00 h, +22:00 h, +24:55 h after lab entry on visit 2). Of important note is that precise times of sampling were documented for each individual and the analyses have considered these real times and not the planned ones.

The oral mucosa samples were collected from scraping inside of the officers' right and left cheeks with a Cytobrush (Medical Packaging CytoSoft, Panorama City, CA, USA), immediately placing the brush in Trizol (Life Technologies) to lyse and homogenize the cells, and stored in -80°C until further processing. The samples that

were analyzed further were obtained from the right cheek for all subjects except for subjects 5, 6, and 11, whose analyzed samples were obtained from the left cheek due to technical issues that arose while scraping their cheeks.

For oral mucosa collection, all samples were collected by the same individual, namely the Master's student, in order to reduce any potential variability that may arise between different technicians.

For both PBMCs and oral mucosa cells, total RNA was extracted and purified from the lyzed cells in Trizol. The concentration and purity of the RNA extracted from PBMCs were also checked on a NanoDrop 1000 spectrophotometer (ThermoFisher, Ottawa, ON, Canada) since there was a sufficient amount of PBMCs collected in order to do so. The RNA from both tissues was then reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative Polymerase Chain Reaction (qPCR) was then performed on the cDNAs using the TaqMan Gene Expression Kit (Life Technologies), according to the manufacturer's specifications, to determine the levels of expression of the clock genes *PER1* (assay ID: Hs00242988_m1), *PER2* (assay ID: Hs00256144_m1) *PER3* (assay ID: Hs00213466_m1), *REV-ERBα* (assay ID: Hs00253876_m1), and *BMAL1* (assay ID: Hs00154147_m1). The fold changes in clock gene expression of each of the 5 clock genes were determined for every sample collected (measured in triplicate) using the $2^{-\Delta\Delta CT}$ method. All clock gene expression results were described relative to the averaged expression of 2 control genes, *B2M* (β-2 microglobulin; assay ID: Hs99999907_m1) and *PPIA* (peptidylprolyl isomerase A; assay ID:

Hs99999904_m1), in which an absence of observable 24-h rhythmicity had been previously verified in another study [90].

8 MEASURES – OTHER PHYSIOLOGICAL PARAMETERS:

The following parameters were collected as additional markers of the circadian clocks, mood, and performance, during both 24-hour laboratory visits. These will be analyzed in the upcoming months at our lab during which we will test additional hypotheses related to this project.

8.1. Distal body temperature using iButtons → skin temperature of left and right feet and hands

8.2. Proximal body temperature using iButtons → skin temperature of left and right infraclavicles

8.3. Psychomotor Vigilance Task → completed on smart-phone; tests reaction time and speed

8.4. Short Mood Scales → completed on smart-phone; assessment of mood, alertness, and levels of calmness, over a 10-mm visual linear scale

8.5. Pre- and post-sleep questionnaires

8.6. Light levels → measured using a photometer (IL1400A, International Light Inc., Newburyport, MA, USA) every 2 hours during the waking periods, throughout each of the laboratory visits

9 *ANALYSES:*

The following parameters were analyzed and compared between visit 1 and visit 2 in order to conclude whether the officers' circadian clocks were adapted or not to a shifted sleep-wake schedule, after working 7 consecutive night shifts. All modifications in the different markers' expression levels before and after the week of night shifts in visit 1 and visit 2, respectively, were analyzed by 2-way ANOVA for repeated measures. The 2 factors used for the ANOVA were: condition (i.e. visit 1 or visit 2 [for PBMCs, salivary cortisol, and urinary MT6s levels] and ambulatory period 1 or ambulatory period 2 [for light intensity]) and time-of-sample (i.e. 10h00 vs. 19h30 [for PBMCs], waketime vs. bedtime [for salivary cortisol], wake vs. sleep [for urinary MT6s levels], and each 1 hour time bin over 24 hours [for light intensity]). When the 2-way ANOVA resulted in a significant interaction, we performed pairwise multiple comparisons using the Sidak's post-hoc test.

In order to test our 1st hypothesis, that subjects' circadian clocks will remain adjusted to their day-oriented schedules before working 7 consecutive night shifts, we analyzed salivary cortisol levels, rhythms of MT6s, and clock gene expression of PBMCs and oral mucosa, during each of the two laboratory visits. This allowed us to statistically compare the results we observed for visit 1, before the week of night shifts, with those at visit 2, after the week of night shifts.

To test our 2nd hypothesis, which stated that subjects' central clock markers will be misaligned with their shifted sleep schedules, we analyzed results of 2 markers of the central clock, salivary cortisol levels and aMT6s rhythms, before and after the week of night shifts.

To test our 3rd hypothesis, which stated that subjects' peripheral clock markers will be misaligned with their shifted sleep schedules, we analyzed clock gene expression in 2 peripheral clock markers, oral mucosa and PBMCs, before and after the week of night shifts.

Our final hypothesis stated that there would be a misalignment between the central and peripheral clocks of the police officers after they work 1 week of night shifts. This was addressed by comparing the overall results of the 2 central clock markers with those of the 2 peripheral clocks.

9.1. Analyses of the two central clock markers

9.1.1. Salivary Cortisol

The concentration of salivary cortisol for the 4 samples that were collected during the hour prior to bedtime and during the hour upon awakening were first averaged per subject, during each of the 2 laboratory visits (please note that due to technical circumstances during the experimental procedure, two individuals only provided 3 saliva samples instead of the required 4 samples at a certain timepoint). This produced one single, average concentration value for cortisol at waketime and one single average concentration value for cortisol at bedtime.

These mean values were then averaged across subjects to determine the group concentration values at waketime and bedtime during both visits. A 2-way ANOVA for repeated measures was conducted with factors: condition and time-of-sample. The ratios of the average concentration levels of salivary cortisol at waketime over those at bedtime were also determined during each visit.

To calculate the ratio of cortisol, the following equation was used:

$$\text{Ratio of Cortisol} = \frac{\text{Mean Concentration of Cortisol Secretion at Waking } \left(\frac{\mu g}{dl}\right)}{\text{Mean Concentration of Cortisol Secretion at Bedtime } \left(\frac{\mu g}{dl}\right)}$$

Since in day-active subjects, cortisol levels are greater at waketime than at bedtime, the ratio would be greater than 1. We thus decided to illustrate the ratio and interpret its meaning by analogy to a ratio of 1.

At baseline, this ratio should be greater than 1 since cortisol has its peak expression time at waketime and its lower expression time at bedtime. If circadian adjustment of salivary cortisol did occur after 7 consecutive night shifts, the resulting ratio would be greater than 1 during visit 2 as well.

Since concentration of salivary cortisol was only assessed at 2 separate time points, an additional central clock marker, urinary aMT6s excretion, was also assessed, at more frequent intervals, to observe the rhythmic behavior of the central clock.

9.1.2. Urinary aMT6s

Concentrations of aMT6s varies greatly between individuals; in day-active, healthy subjects, it has been shown that both nighttime and 24-hour melatonin excretion could vary by approximately 20-fold, inter-individually [17, 91]. This variability could be explained by factors such as exposure to different light intensities or drug ingestion [92]. However, we controlled for these factors by asking subjects to stop the use of any drug intake for 2 weeks prior to the start of study, as well as by measuring the different intensities of light that the subjects were exposed to during their ambulatory periods.

We measured urine volume and concentration of aMT6s for every sample that was collected. These values of volume and concentration were used to calculate the

amount of metabolite excreted between every 2 consecutive sample time points. We then converted these amounts to amount excreted per hour in each subject, at a time point which corresponded to the centre of the bin.

All aMT6s rhythms were analyzed first, at an individual level which assessed each subject's results independently of one another, and second, at a group level which combined the results of all individuals to produce the mean urinary aMT6s rhythm.

Since urine samples were collected at various time points for different subjects, the urinary aMT6s data was first averaged per 3-hour time bins for each individual, during both visits, at a time point which corresponded to the centre of the bin. This data was then averaged across subjects to produce the mean urinary aMT6s rhythm, for each of the laboratory visits.

Upon visual inspection of the individuals' data, we noticed that the 24-hour rhythm was bimodal with 1 large peak and another peak that was smaller for 10 of the 11 subjects. We thus applied dual-harmonic regressions for the group data and the individual data (user-defined equation using Prism 6; GraphPad, La Jolla, CA, USA) to conduct further analyses. The phase and amplitude of the regressions were then calculated using a manual process to determine the fitted amplitudes and phase values from the 2 harmonics with the 12 and 24 hour periods [48]. For each of the 2 components of the regression, if the 95% confidence interval of the estimated amplitude did not include the zero value, it was considered significant [48].

If a certain regression was significant for both visits, we were then able to determine the phase shift (i.e. the shift in time of peak expression) of the aMT6s rhythms. This was done by calculating the difference between the acrophase at each visit

($Acrophase_{Visit\ 1}$ – $Acrophase_{Visit\ 2}$) [52]. A positive value for the phase shift, by convention, indicates a phase advance, while a negative phase shift indicates a phase delay [93].

Another circadian measure that can be analyzed includes the phase angle (i.e. difference in time between the acrophase of 2 different measured markers, i.e. aMT6s rhythm and the average bedtime of the group. However, due to the large inter-individual variability between subjects' bedtimes and due to the lack of significance in urinary aMT6s rhythms at an individual level, the phase angles were not determined.

Moreover, for each laboratory visit, we determined the average aMT6s excretion rate in urine during subjects' sleep and wake periods, independently. To determine these average quantities, we used the absolute amounts of aMT6s excreted in each sample collected during the sleep and wake periods, respectively, and divided it by the total number of hours the subjects spent sleeping or awake. A 2-way ANOVA for repeated measures was conducted with factors: condition and time-of-sample.

The average total quantity of aMT6s excreted per 24 hours was also calculated and analyzed to compare the total amounts of the urinary metabolite excreted before and after 7 consecutive night shifts. Since subjects' visits were not always comprised of exactly 24 hours, the calculations used to determine the amount of urinary aMT6s excreted were corrected such that values were representative of a full 24-hour cycle.

9.2. Analyses for the markers of 2 peripheral clocks

9.2.1. Clock gene expression in PBMCs

Clock gene expression levels in PBMCs in the morning (10h00) was compared with those in the evening (19h30), for each subject, during each of the 2 laboratory visits.

These values were then averaged across subjects to determine the group clock gene expression levels in the morning and evening, during both visits. The comparisons were made using a 2-way ANOVA for repeated measures, with factors: condition and time-of-sample.

Since clock gene expression was only assessed at 2 separate time points in PBMCs, oral mucosa samples were also collected, at more frequent intervals, to observe the rhythmic pattern of expression of these genes over a 24-h period.

9.2.2. Clock gene expression in Oral Mucosa Cells

The relative quantity of clock gene expression was first calculated based on the amplification results of control genes and the calibrator samples (that was used to normalize results between different qPCR plates). An important note is that the n value (i.e. number of available subjects from which samples were used to assess gene expression) was different for different genes. Due to technical issues while collecting oral mucosa cells, some subjects' oral mucosa samples yielded approximately 60 times less RNA. As a result, we were not able to detect *PER1, PER2, PER3, BMAL1*, and *REV-ERBα* gene amplification in these few subjects. Namely, *BMAL1* had a n of 6 subjects, *PER2* and *PER3* had a n of 7 subjects, *PER1* had a n of 9 subjects, and *REV-ERBα* had a n of 10 subjects. Of important note is that the samples used to assess gene expression, for a certain gene, between the 2 laboratory visits were obtained from the same subjects.

The raw data (gene expression values expressed as relative quantities) were first converted to z-scores ($z = (x-\mu)/\sigma$, where x is the raw data value, μ is the mean (calculated from all results during both visits, from specific subject) and σ is the standard

deviation (calculated from all results during both visits, from specific subject), in order to normalize between different officers and reduce the inter-individual variability.

All clock gene expression rhythms were analyzed first, at an individual level which assessed each subject's results independently of one another, and second, at a group level which combined the results of all individuals to produce the mean clock gene expression rhythm.

Oral mucosa samples were collected at the same time for every individual and thus, it was not necessary to bin any of the data.

In order to analyze the 24-hour rhythms of clock gene expression in the oral mucosa cells during visits 1 and 2, we applied single-harmonic regressions for the group data and the individual data (user-defined equation using Prism 6; GraphPad, La Jolla, CA, USA). The fitted phases of the regressions were determined and these corresponded to the peak expression time. Since we had controlled for the variability between subjects during both the qPCR procedure as well as by converting raw values to z-scores, we did not carry out an addition non-linear mixed model approach to determine the group average phases of the regressions. For each rhythm, if the 95% confidence interval of the estimated amplitude did not include the zero value, it was considered significant [48].

If a certain regression was significant for both visits, we were then able to determine the phase shift (i.e. the shift in time of peak expression) of the clock gene expression rhythms. This was done by calculating the difference between the acrophase at each visit ($\text{Acrophase}_{\text{Visit 1}} - \text{Acrophase}_{\text{Visit 2}}$) [52].

If a certain regression was significant for both visits, we were then able to determine the phase shift of the clock gene expression rhythms.

The phase angles between the acrophases of average clock gene rhythms (of genes which were significant during both visits) and the group aMT6s rhythms were also determined. Due to the lack of significance at an individual level, in both urinary aMT6s and clock gene rhythms, phase angles were not determined for each subject.

9.3. Actigraph analyses

The light exposure and rest-activity information obtained from the Actiwatches during both ambulatory periods, before each of the laboratory visits, were also analysed. The light intensity data that was collected from each subject was binned every hour, over 24 hours, for each of the days during ambulatory period 1 and 2. The results between both ambulatory periods were then compared with each other using a 2-way ANOVA for repeated measures, with factors: condition and time-of-sample. The average light intensities that the subjects were exposed to during their laboratory visits were also calculated (mean ± SD).

Moreover, the bedtimes and waketimes of each subject, for all sleep episodes, were averaged across each of their ambulatory periods, using mean ± SD. This averaged data was then averaged across the group, for both ambulatory periods (mean ± SD). The shifts in bedtimes and waketimes in visit 2 vs. visit 1 were also determined.

Finally, total sleep duration of each subject, for every sleep episode, was also averaged across each of their ambulatory periods, using mean ± SD. This averaged data was then averaged across the group, for both ambulatory periods (mean ± SD).

9.4. Explanation of terms/numbers used

Of particular note is that all harmonic regressions were considered significant if the 95% confidence interval of the calculated amplitude did not include the zero value. Results are expressed as mean ± SEM on all visual graphs while all numerical results, such as age, bedtimes, waketimes, total sleep times, and light intensity levels at the laboratory, which are all expressed as mean ± SD. All time values are expressed over a 24-hour clock (e.g. 6:00 PM would be expressed as 18h00) and standard deviations of time are expressed as hh:min.

9.5. Future analyses to be conducted

This project is currently ongoing since a few more subjects have expressed interest in the study and thus, we will pursue further analyses over the next few months. We will continue with the clock gene expression analyses and assays for cortisol and melatonin for these subjects as well. However, we do not expect to observe any dramatic changes to the results we have already found from the eleven completed subjects, except for an increase in statistical power due to an increase in the n value.

RESULTS

1 INTRODUCTION TO RESULTS:

In this study, we assessed 2 central clock markers and markers of 2 separate peripheral clocks in police officers who work 7 consecutive night shifts as part of their 35-day work roster. Based on the data collected from 11 police officers for these 4 separate circadian markers, we observed significant results both in individuals and as a group. We also observed large inter- and intra-individual variability in subjects during each of their visits to the laboratory. The results demonstrate that a disturbance of circadian parameters does occur in police officers, both before and after working a week of night shifts.

2 CENTRAL MARKERS:

2.1. Salivary Cortisol

The mean salivary cortisol concentrations for the group, at waketime and bedtime for each visit, were determined by averaging across all the subjects and analyzed using a 2-way ANOVA for repeated measures, with factors: condition and time-of-sample. The interaction between the 2 factors were significant with a $p=0.01$, a main effect of condition with $p=0.01$, a main effect of time-of-sample with $p\leq0.0001$. Multiple comparisons using Sidak's post-hoc test yielded that at visit 1, waketime vs. bedtime had a $p\leq0.001$; at visit 2, waketime vs. bedtime had a $p\leq0.05$, at waketime, visit 1 vs. visit 2 had a $p\leq0.05$; and at bedtime, visit 1 vs. visit 2 was non-significant. The results from the multiple comparisons are shown in Figures 2.1 and 2.2. A closer observation at an individual level showed that all 11 subjects had higher average levels of cortisol at waketime over bedtime during visit 1 (Figure 2.2 A) while 9 out

of 11 subjects (all except S01 and S02) had higher average levels of cortisol at waketime over bedtime during visit 2 (Figure 2.2 B).

The concentrations of cortisol for each subject along with their ratios of cortisol between visits 1 and 2 are shown in Table 1. The individuals' ratios are also plotted in Figure 2.3. All cortisol ratios are smaller in visit 2 vs. visit 1 except for subjects 3, 5, 6, and 10. Subjects 3, 5, and 10 have similar ratios during both visits while subject 6 has a very large increase in ration during visit 2. Ratios smaller than 1 are only seen during visit 2 (i.e. in subjects 1 and 2). However, ratios close to 1 are seen during both visits.

More critical observation of the results show that subjects 1, 2, 4, 7, and 11, not only have reduced ratios at visit 2 but these ratios are also very close to a ratio of 1. Subjects 3 and 5 had similar ratios during both visits which were close to a ratio of 1. Subject 10 had similar ratios during both visits as well, but these ratios were higher than 1. Finally, subject 6 particularly had a ratio that was close to 1 during visit 1 which largely increased during visit 2. Finally, subjects 8 and 9 had reduced ratios at visit 2 vs. visit 1, but these were ratios much greater than 1.

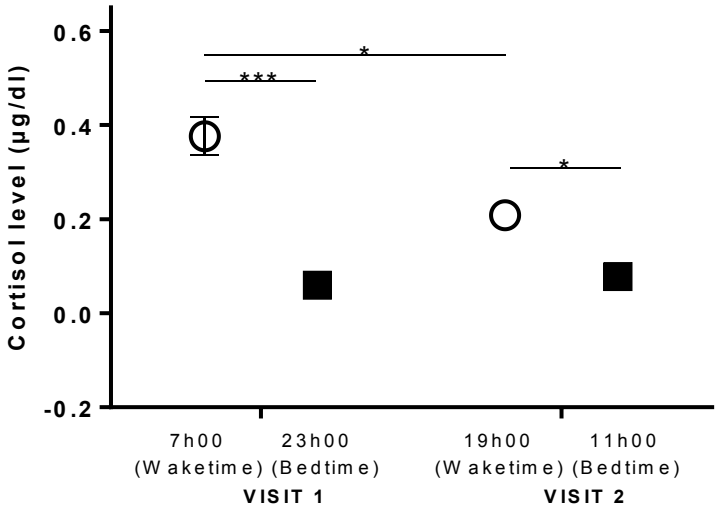


Figure 2.1: 2-way ANOVA on group data of salivary cortisol levels

The group averaged data for salivary cortisol concentration at waketime (7h00 during visit 1 and 19h00 during visit 2) and at bedtime (23h00 during visit 1 and 11h00 during visit 2). The open, white circles represent the results at waketime while the black squares represent the results at bedtime.

Analysis: Sidak's post-hoc test results. 2-way ANOVA, repeated measures, factors: condition vs. time-of-sample. The group averages are mean ± SEM. *$p \leq 0.05$; ***$p \leq 0.001$.

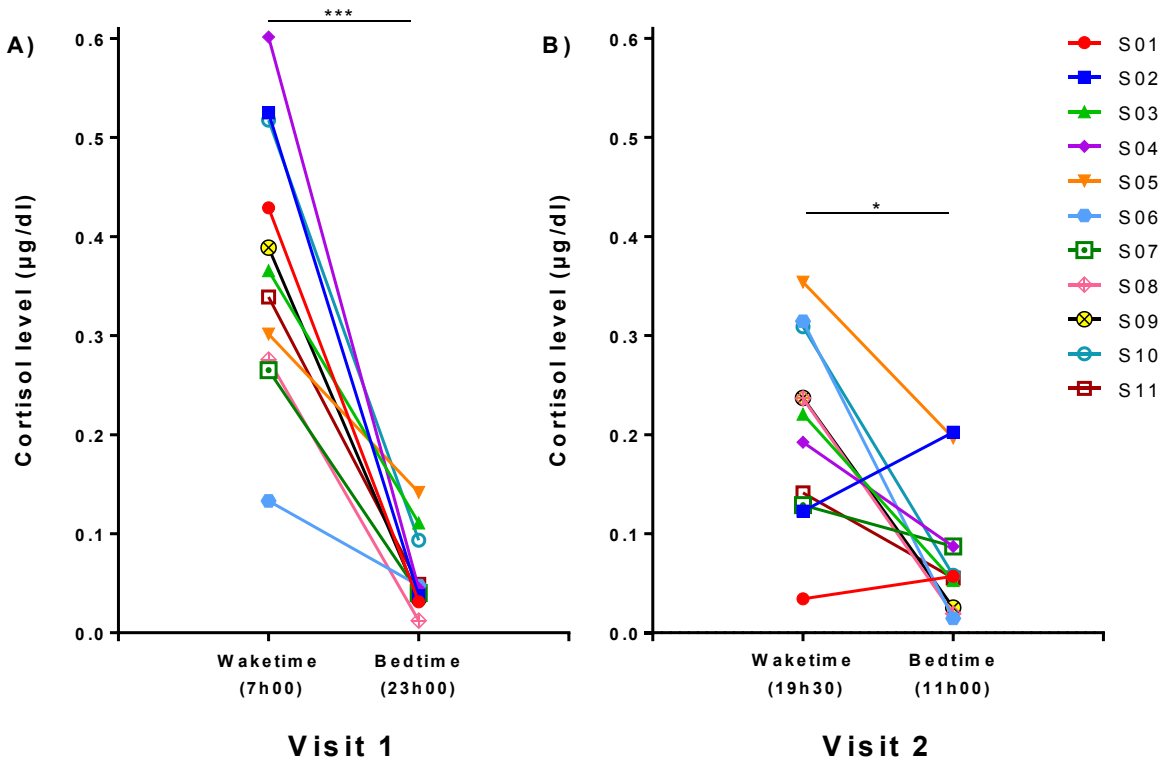


Figure 2.2: 2-way ANOVA on individuals' data of salivary cortisol levels

(A) shows the individual data points (see side legend with subjects' colour codes), comparing levels of salivary cortisol (µg/dl) at waketime and bedtime during visit 1 (7h00 and 23h00) while (B) shows the individual data points, comparing salivary cortisol levels at waketime and bedtime during visit 2 (19h00 and 11h00).

Analysis: Sidak's post-hoc test results. 2-way ANOVA, repeated measures, factors: condition vs. time-of-sample. The group averages are mean ± SEM. *$p \leq 0.05$; ***$p \leq 0.001$.

Table 1: Individuals' data of salivary cortisol levels and ratios

Subject ID	Visit 1 - Cortisol Concentration (µg/dL)		Visit 2 - Cortisol Concentration (µg/dL)		Cortisol ratio (waketime/bedtime)	
	At Waketime	At Bedtime	At Waketime	At Bedtime	Visit 1	Visit 2
Sx1	0.43	0.03	0.03	0.06	13.68	0.60
Sx2	0.52	0.04	0.12	0.20	14.11	0.61
Sx3	0.37	0.11	0.22	0.05	3.30	4.18
Sx4	0.60	0.04	0.19	0.09	13.39	2.21
Sx5	0.30	0.14	0.35	0.20	2.13	1.80
Sx6	0.13	0.05	0.31	0.01	2.80	21.58
Sx7	0.27	0.04	0.13	0.09	6.63	1.48
Sx8	0.28	0.01	0.24	0.02	22.51	12.21
Sx9	0.39	0.03	0.24	0.03	11.24	9.33
Sx10	0.52	0.09	0.31	0.06	5.53	5.33
Sx11	0.34	0.05	0.14	0.06	6.93	2.56

The concentrations of salivary cortisol levels (µg/dL) for each individual at waketime and at bedtime, corresponding to each laboratory visit. The individual ratios of salivary cortisol at waketime/bedtime during each visit are also displayed on the last 2 columns.

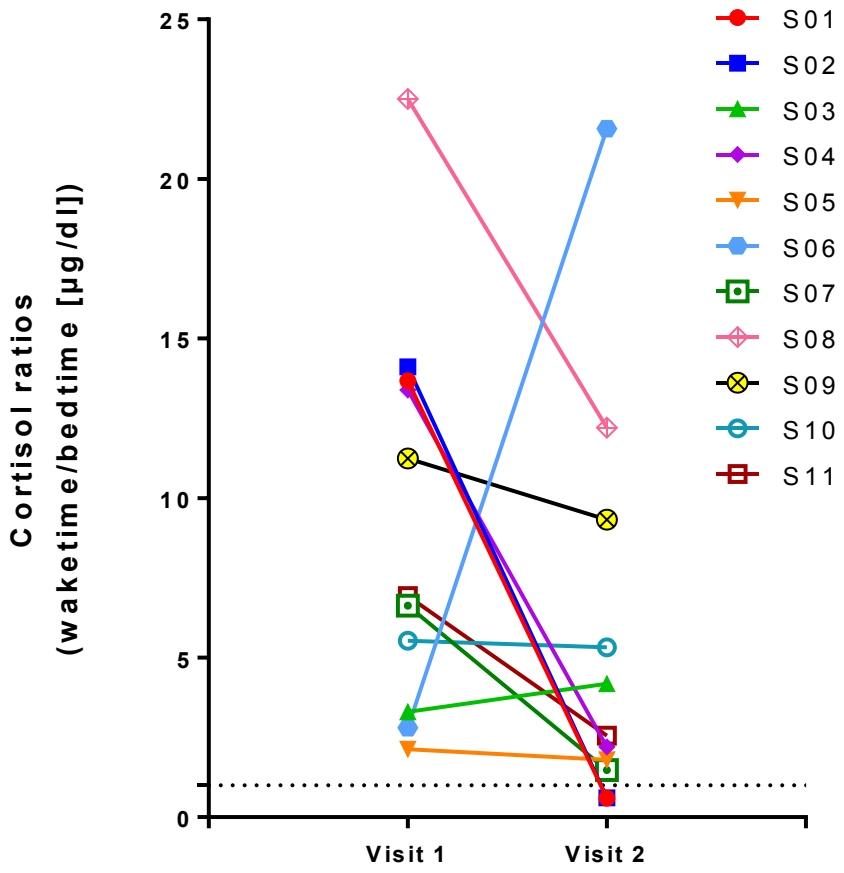


Figure 2.3: Individuals' data of salivary cortisol ratios

The calculated ratios of salivary cortisol levels at waketime over bedtime, compared between the 2 laboratory visits. The individual data points which represent the ratios are shown (see side legend with subjects' colour codes) for each subject.

2.2. Urinary aMT6s

The dual-harmonic regression carried out on the group data during both visits resulted in phases and amplitudes for both the 12-h and 24-h components of the regression. During visit 1, the phase and amplitude of the 24-h component was 4h20 and 692.92 ng/hr, respectively, and the phase and amplitude of the 12-h component was 16h52 and 73.74 ng/hr.

During visit 2, the phase and amplitude of the 24-h component was 11h58 and 395.19 ng/hr, respectively, while no phase and amplitude of the 12-h component could be calculated.

During visit 1, both the 24-h and 12-h components of the dual-harmonic regression were significant (95% confidence interval not crossing zero and thus indicating that $p \leq 0.05$), while during visit 2, only the 24-h component of the regression was significant ($p \leq 0.05$). These results are shown in Table 2.1 and Figures 3.1 A and B.

The phase shift between the significant 24-h components of the 2 regressions, resulted in the phase being delayed by 7h37 in visit 2 vs. visit 1 (Table 2.1).

When observing the individual dual-harmonic regressions, certain subjects had significant circadian rhythms while others did not and this varied between visits as well (Table 2.2). Subjects 1, 4, 5, 6, and 11 had regressions with a significant 24-h component during visit 1 with phases occurring at 5h40, 6h00, 5h00, 5h55, and 5h19 respectively, during their sleep periods (Table 2.2 and Figure 3.2). Moreover, subjects 4, 5, 6, and 11 had regressions with a significant 12-h component during visit 1, with phases occurring at 18h00, 17h00, 17h48, and 18h00, respectively.

During visit 2, subjects 3, 5, 6, 7, 8, and 9 had regressions with a significant 24-h component with phases at 15h35, 15h34, 15h45, 9h33, 14h39, and 14h00, respectively, during their shifted sleep periods (see Table 2.2 and Figure 3.2). Moreover, subjects 3, 5, 6, and 8 had regressions with a significant 12-h component during visit 2, with phases occurring at 5h03, 2h04, 2h50, and 2h47, respectively.

Since subjects 5 and 6 had significant 12-h and 24-h components in their dual-harmonic regressions during both visits, further analyses were conducted on their results. Subject 5 had delayed his acrophase, produced by the 24-h component of his regression, by

10h34 in visit 2 vs. visit 1 while the acrophase produced by the 12-h component of his regression was delayed by 9h04 in visit 2 vs. visit 1 (Table 2.2). Subject 6 had delayed his acrophase, produced by the 24-h component of his regression, by 9h49 in visit 2 vs. visit 1 while the acrophase produced by the 12-h component of his regression was delayed by 9h02 in visit 2 vs. visit 1 (Table 2.2).

Furthermore, the averaged amount of urinary aMT6s excreted per hour during subjects' sleep and wake periods were also analyzed using a 2-way ANOVA for repeated measures, with factors: condition and time-of-sample. Although the interaction between the 2 factors were non-significant, the main effect of condition had a $p=0.0244$, and the main effect of time-of-sample has a $p=0.0102$. No further analyses were conducted as the interaction was non-significant. These results are shown in 3.3 A and B.

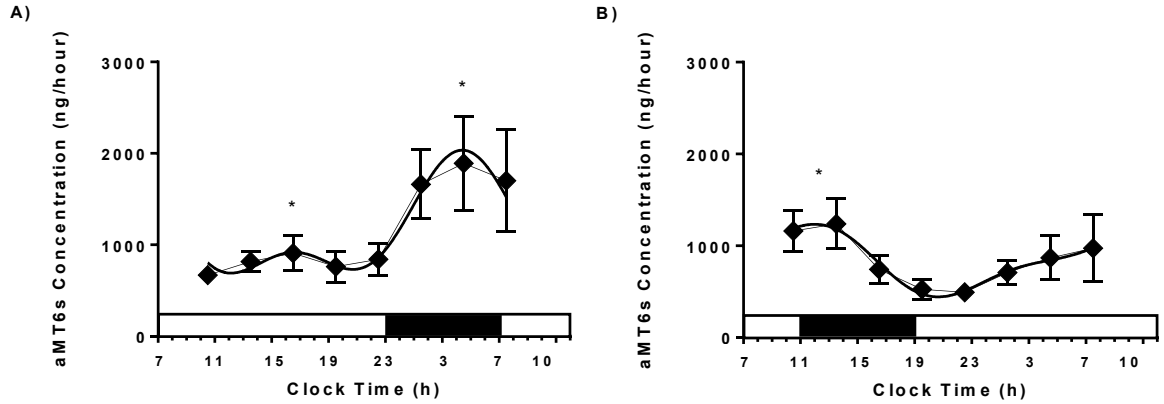


Figure 3.1: Dual-harmonic regressions on group data of urinary aMT6s excretion

The dual-harmonic regression for the 24-h rhythms of aMT6s urinary excretion rate of the group during visit 1 is shown in (A) whereas that of visit 2 is shown in (B). The regressions are significant and indicated by a solid, black line. The black bars indicate the subjects' 8-hour sleep episodes at the laboratory and the white bars indicate their waking periods at the laboratory.

Analysis: Dual-harmonic regression with 12-h and 24-h components. The group averages are mean ± SEM. *$p \leq 0.05$.

Table 2.1: Group acrophase data of urinary aMT6s rhythms

Visit	Phase (1st peak) (hh:min)	Phase (2nd peak) (hh:min)	Phase Shift†
Visit 1	4:21*	16:52*	7h37 (delayed)
Visit 2	11h58*	--	

The grouped average phase of the 24-h (1st peak) and 12-h (2nd peak) components of the aMT6s regressions, before and after 7 consecutive night shifts. All shown regression components were significant are indicated by an asterisk.

† The phase shift was determined for regression components that were significant during both visits.

Analysis: Dual-harmonic regression with 12-h and 24-h components. *$p \leq 0.05$.

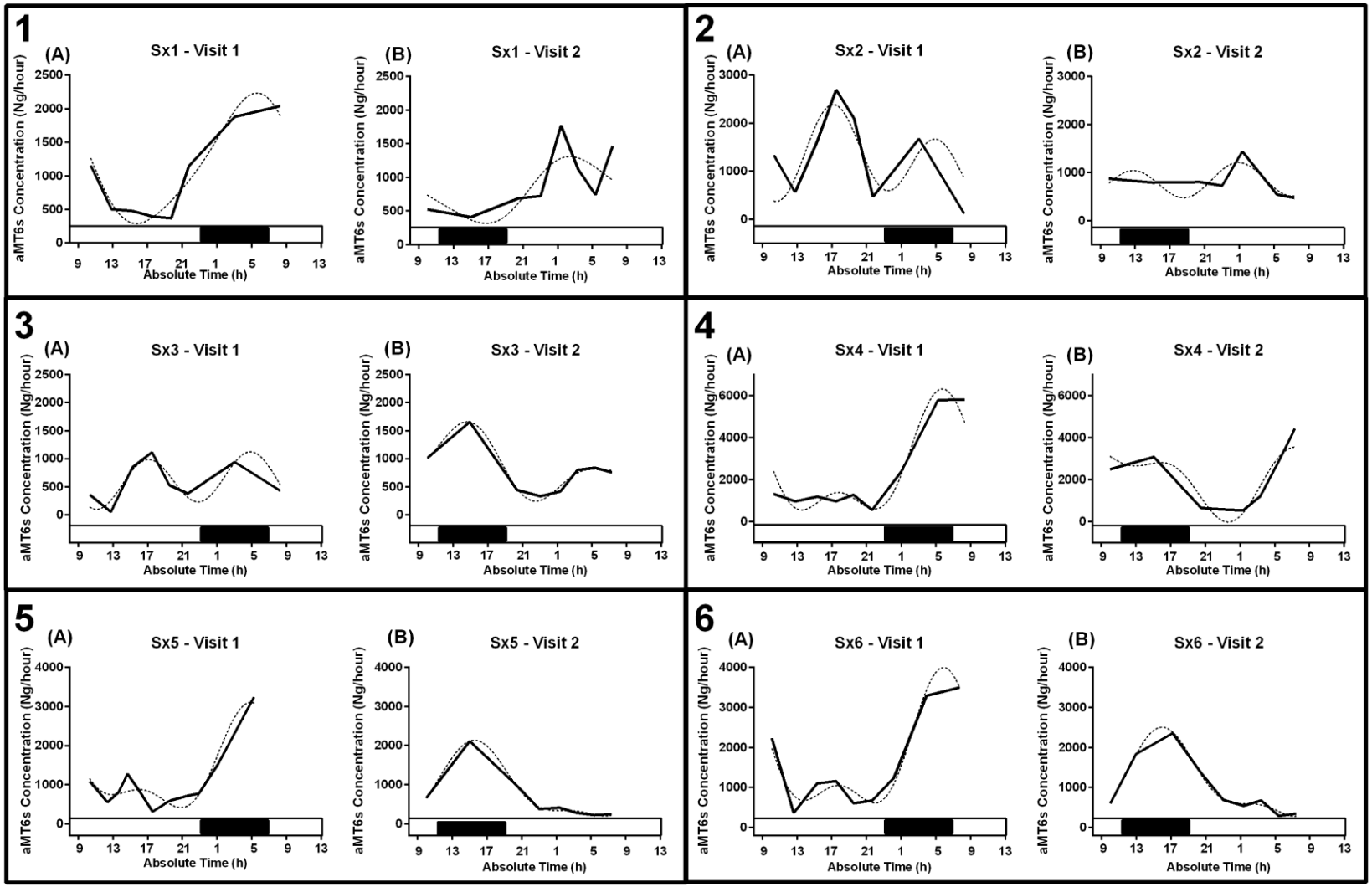
Table 2.2: Individuals' acrophase data of urinary aMT6s rhythms

Subject ID	Visit 1 aMT6s Acrophase (1st peak)	Visit 2 aMT6s Acrophase (1st peak)	Visit 1 aMT6s Acrophase (2nd peak)	Visit 2 aMT6s Acrophase (2nd peak)	PHASE SHIFTS† (1st peak)	PHASE SHIFTS† (2nd peak)
Sx1	5:40*	2:07	---	---	---	---
Sx2	17:06	0:54	4:51	12:47	---	---
Sx3	4:51	14:35*	17:08	5:03*	---	---
Sx4	6:00*	7:33	18:00*	15:54	---	---
Sx5	**5:00***	**15:34***	**17:00***	**2:04***	10:34 (delay)	9:04 (delay)
Sx6	**5:55***	**15:45***	**17:48***	**2:50***	9:49 (delay)	9:02 (delay)
Sx7	5:44	9:33*	15:43	---	---	---
Sx8	19:55	14:39*	7:39	2:47*	---	---
Sx9	17:13	14:00*	7:27	3:20	---	---
Sx10	15:57	7:14	4:37	19:25	---	---
Sx11	5:19*	15:03	18:00*	6:04	---	---
Individual regressions (n significant/n total)						
Visit 1	5/11 (45.45%) – 1st peak		4/11 (36.36%) – 2nd peak			
Visit 2	6/11 (54.55%) – 1st peak		4/11 (36.36%) – 2nd peak			

The phase of the 24-h (1st peak) and 12-h (2nd peak) components of the aMT6s regressions for each subject, before and after 7 consecutive night shifts. The regression components that were significant are indicated by an asterisk. The total number of individual regression components that were significant in comparison with total number of regression components.

† The phase shift was determined for regression components that were significant during both visits. The bolded/asterisk values indicate such regression components.

Analysis: Dual-harmonic regression with 12-h and 24-h components. The group averages are mean ± SEM. *$p \leq 0.05$.


1
(A)
Sx1 - Visit 1
(B)
Sx1 - Visit 2
2
Sx2 - Visit 1
Sx2 - Visit 2
3
Sx3 - Visit 1
Sx3 - Visit 2
4
Sx4 - Visit 1
Sx4 - Visit 2
5
Sx5 - Visit 1
Sx5 - Visit 2
6
Sx6 - Visit 1
Sx6 - Visit 2
aMT6s Concentration (Ng/hour)
Absolute Time (h)

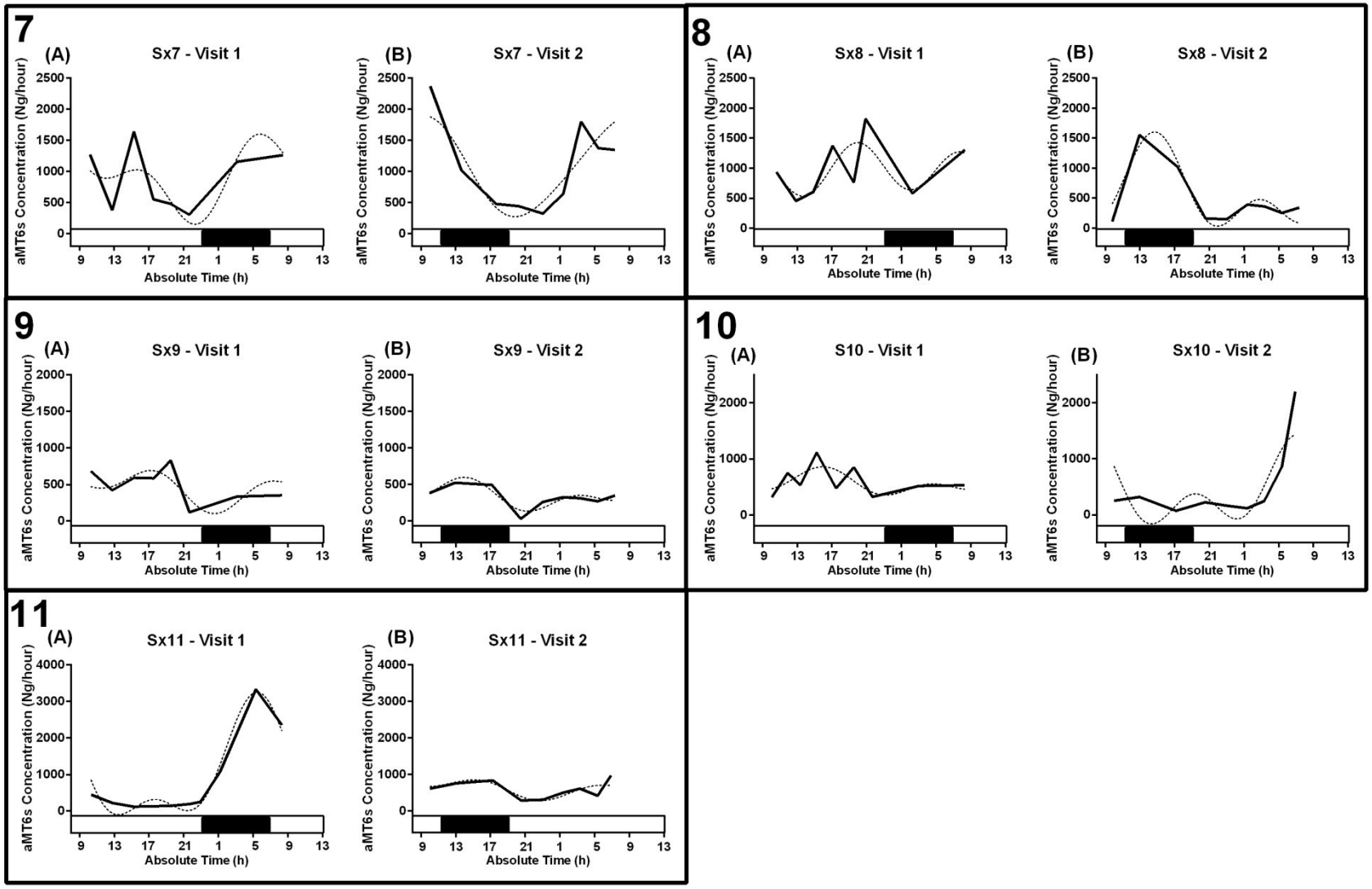

7
(A)
Sx7 - Visit 1
(B)
Sx7 - Visit 2
8
(A)
Sx8 - Visit 1
(B)
Sx8 - Visit 2
9
(A)
Sx9 - Visit 1
(B)
Sx9 - Visit 2
10
(A)
S10 - Visit 1
(B)
Sx10 - Visit 2
11
(A)
Sx11 - Visit 1
(B)
Sx11 - Visit 2
aMT6s Concentration (Ng/hour)
Absolute Time (h)

Figure 3.2: Dual-harmonic regression on individuals' data of urinary aMT6s excretion

Each of the panels (1-11) on the graph corresponds to the individual dual-harmonic regressions of subjects 1-11, during visit 1 (A) and visit 2 (B). The regressions that are significant are indicated by a solid, black line while the regressions that are non significant are indicated by a dotted, black line. The black bars indicate the subjects' sleep episodes at the laboratory and the white bars indicate their waking periods at the laboratory.

Analysis: Dual-harmonic regression with 12-h and 24-h components.

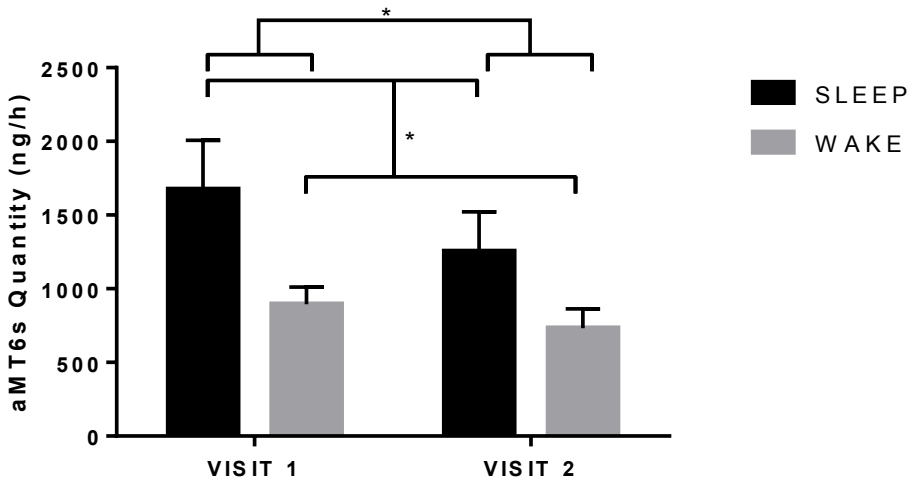


Figure 3.3 A: Group data of excreted amounts of urinary aMT6s during sleep and wake

The total quantity of urinary aMT6s excreted per hour for the 8-hour sleep periods (black bars) and 16-hour wake periods (grey bars), during each visit to the laboratory.

Analysis: 2-way ANOVA, repeated measures, factors: condition vs. time-of-sample. The group averages are mean ± SEM. *$*p \leq 0.05$*.

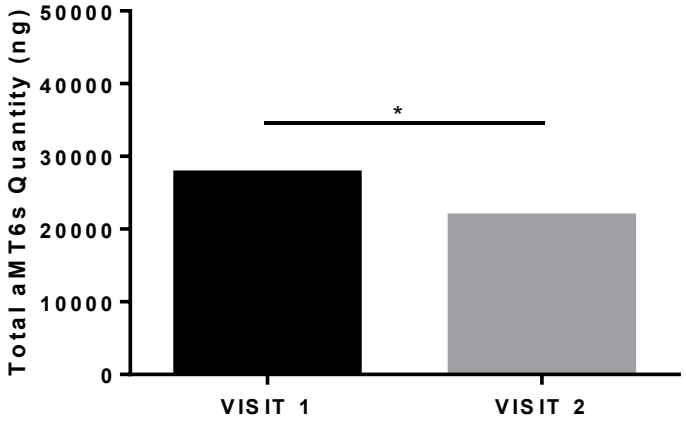


Figure 3.3 B: Group data of total excretion of urinary aMT6s levels

The total amount of urinary aMT6s excreted per 24 hours, during the first (black bar) and second (grey bar) laboratory visits.

Analysis: Sidak's post-hoc test results. 2-way ANOVA, repeated measures, factors: condition vs. time-of-sample. The group averages are mean ± SEM. *$p \leq 0.05$.

3 PERIPHERAL MARKERS:

3.1. Clock gene expression in PBMCs

First, the clock gene expression values of each individual were plotted in Figure 4.1 A. The mean clock gene expression for the group, at 10h00 and 19h30, for each visit, were determined by averaging across all the subjects and analyzed using a 2-way ANOVA for repeated measures with factors: condition and time-of-sample.

For *PER1,* the interaction between the 2 factors were significant with a p=0.0006, a main effect of condition that was non-significant, and a main effect of time-of-sample with p=0.04. Multiple comparisons using Sidak's post-hoc test yielded that at visit 1, 10h00 vs.

19h30 had a $p \leq 0.001$; at visit 2, 10h00 vs. 19h30 was non-significant; at 10h00, visit 1 vs. visit 2 had a $p \leq 0.01$; and at 19h30, visit 1 vs. visit 2 had a $p \leq 0.05$.

For *PER2,* the interaction between the 2 factors were significant with a $p=0.011$, a main effect of condition that was non-significant, and a main effect of time-of-sample that was also non-significant. Multiple comparisons using Sidak's post-hoc test yielded that at visit 1, 10h00 vs. 19h30 had a $p \leq 0.01$; at visit 2, 10h00 vs. 19h30 was non-significant; at 10h00, visit 1 vs. visit 2 was non-significant; and at 19h30, visit 1 vs. visit 2 had a $p \leq 0.05$.

For *PER3,* the interaction between the 2 factors were significant with a $p=0.014$, a main effect of condition that was non-significant, and a main effect of time-of-sample with a $p=0.017$. Multiple comparisons using Sidak's post-hoc test yielded that at visit 1, 10h00 vs. 19h30 had a $p \leq 0.001$; at visit 2, 10h00 vs. 19h30 was non-significant; at 10h00, visit 1 vs. visit 2 was non-significant; and at 19h30, visit 1 vs. visit 2 had a $p \leq 0.01$.

For *BMAL1,* the interaction between the 2 factors were non-significant, a main effect of condition that was non-significant, and a main effect of time-of-sample that was also non-significant. Multiple comparisons using Sidak's post-hoc test yielded that at visit 1, 10h00 vs. 19h30 was non-significant; at visit 2, 10h00 vs. 19h30 was non-significant; at 10h00, visit 1 vs. visit 2 was non-significant; and at 19h30, visit 1 vs. visit 2 was non-significant.

For *REV-ERBα,* the interaction between the 2 factors were significant with a $p=0.008$, a main effect of condition with a $p=0.028$, and a main effect of time-of-sample that was non-significant. Multiple comparisons using Sidak's post-hoc test yielded that at visit 1, 10h00 vs. 19h30 was non-significant; at visit 2, 10h00 vs. 19h30 had a $p \leq 0.01$; at 10h00, visit 1 vs. visit 2 had a $p \leq 0.01$; and at 19h30, visit 1 vs. visit 2 was non-significant.

All of the results from the multiple comparisons are shown in Figures 4.1 A and B.

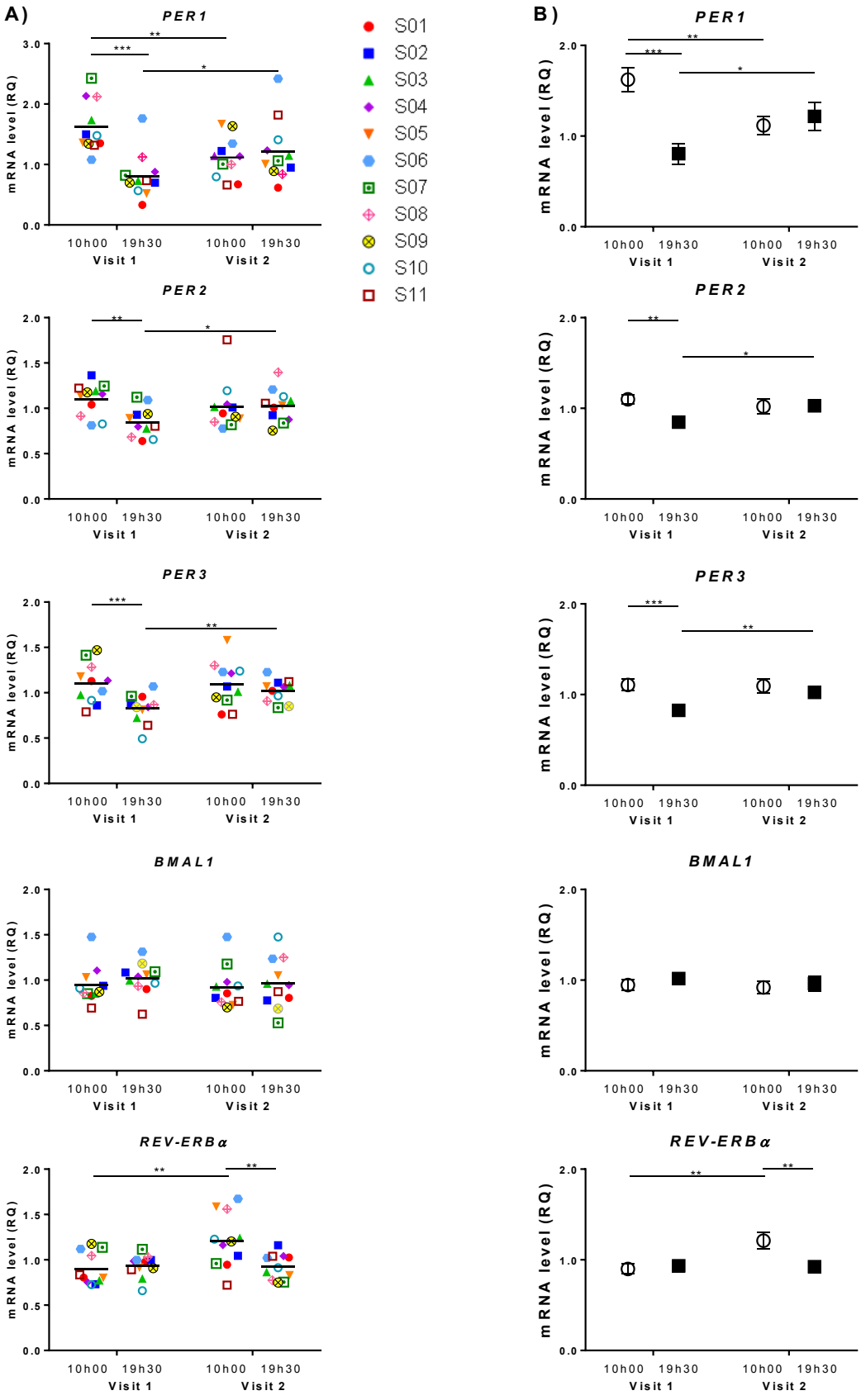

A)
B)
PER1
PER2
PER3
BMAL1
REV-ERBα
mRNA level (RQ)
10h00 19h30 10h00 19h30
Visit 1 Visit 2
S01
S02
S03
S04
S05
S06
S07
S08
S09
S10
S11

Figure 4.1: Individuals' and group data of gene expression levels in PBMCs

(A) shows the individual data points (see side legend with subjects' colour codes) for the relative quantity (RQ) of clock gene expression in PBMCs at 10h00 and 19h30 during visits 1 and 2. (B) shows the group average (mean ±SEM) for clock gene expression in PBMCs at 10h00 and 19h30 during visits 1 and 2. The open, white circles represent the 10h00 group average results while the black squares represent the 19h30 group average results.

Analysis: Sidak's post-hoc test results. 2-way ANOVA, repeated measures, factors: condition vs. time-of-sample. The group averages are mean ± SEM. *$p \leq 0.05$; **$p \leq 0.01$ ***$p \leq 0.001$.

3.2. Clock gene expression in oral mucosa

The single-harmonic regression carried out on the group data during visits 1 and 2 produced the fitted phases for the clock gene expression rhythms of *PER1-3, BMAL1,* and *REV-ERBα.*

For *PER1,* the phase was at 14h50±1h13 and the rhythm was significant (95% confidence interval not crossing zero and thus indicating that $p \leq 0.05$) during visit 1. During visit 2, the phase of *PER1* was at 2h08±1h21 and the rhythm was also significant ($p \leq 0.05$).

For *PER2,* the phase was at 16h34±1h12 and the rhythm was significant ($p \leq 0.05$) during visit 1. During visit 2, the phase of *PER2* was at 17h38±3h10 and the rhythm was non-significant.

For *PER3,* the phase was at 15h55±0h51 and the rhythm was significant ($p \leq 0.05$) during visit 1. During visit 2, the phase of *PER3* was at 21h45±2h36 and the rhythm was non-significant.

For *BMAL1,* the phase was at 21h05±2h13 and the rhythm was non-significant during visit 1. During visit 2, the phase of *BMAL1* was at 8h36±1h57 and the rhythm was non-significant.

For *REV-ERBα,* the phase was at 16h00±1h04 and the rhythm was significant ($p \leq 0.05$) during visit 1. During visit 2, the phase of *REV-ERBα* was at 22h51±3h43 and the rhythm was non-significant. These results are shown in Table 3.1 A and Figures 5.1.

Since *PER1* had a significant rhythm of gene expression in both visits, the phase shift was assessed. *PER1* expression had a phase that was delayed by 11h18 in visit 2 vs. visit 1 (Table 3.1 A).

When observing the expression of subjects' clock gene expressions, at an individual level, we noticed that they had varying expression patterns, compared to each other, and between the 2 visits (Figure 5.2). In Table 3.1 B, we indicate the percent of significant rhythms during each of the visits for all genes.

Although some of the subjects had several missing clock gene expression data, explained by technical difficulties, the available data was used to determine the acrophase of the clock gene expression rhythms for each individual during each visit (Table 3.2).

For *PER1,* subjects 4 and 5 had significant rhythms of gene expression ($p \leq 0.05$) during visit 1 (at 13h42 and 17h55, respectively) while at visit 2, subjects 3, 4, 5, and 11 had significant rhythms of gene expression (at 5h14, 20h45, 2h46, and 23h22, respectively).

For *PER2,* subjects 4 and 11 had significant rhythms of gene expression ($p \leq 0.05$) during visit 1 (at 17h24 and 16h41, respectively) while at visit 2, only subject 11 had a significant rhythm of gene expression (at 18h25).

For *PER3,* subjects 4, 5, and 11 had significant rhythms of gene expression ($p \leq 0.05$) during visit 1 (at 17h17, 18h40, and 16h17, respectively) while at visit 2, subjects 2 and 10 had significant rhythms of gene expression (at 14h51 and 21h34, respectively).

For *BMAL1,* no subjects had significant rhythms of gene expression during visit 1 or visit 2.

For *REV-ERBα,* subjects 4 and 9 had significant rhythms of gene expression ($p \leq 0.05$) during visit 1 (at 14h24 and 22h42, respectively) while at visit 2, subjects 2, 3, 4, 9 and 11 had significant rhythms of gene expression (at 15h40, 3h40, 19h06, 9h01, and 20h36, respectively).

The rest of the subjects did not have significant rhythms of gene expression during either of their visits to the laboratory.

Since subjects 4, 5, 9, and 11 had significant rhythmic expression in certain clock genes during both visits, further analyses were conducted on their results (Table 3.2). Subject 4 had a shifted acrophase which was delayed by 7h03 for *PER1* expression and delayed by 4h42 for *REV-ERBα* expression in visit 2 vs. visit 1 (Table 3.2). Subject 5 had a shifted acrophase for *PER1* expression, which was delayed by 8h50 in visit 2 vs. visit 1. Subject 9 had a shifted acrophase for *REV-ERBα* expression, which was delayed by 10h19 in visit 2 vs. visit 1. Subject 11 had a shifted acrophase for *PER2* expression, which was delayed by 1h44 in visit 2 vs. visit 1 (Table 3.2).

Finally, since *PER1* had a significant rhythm of expression in both visits, the phase angle between its acrophase and that of urinary aMT6s', were determined, as seen in Table 3.3.

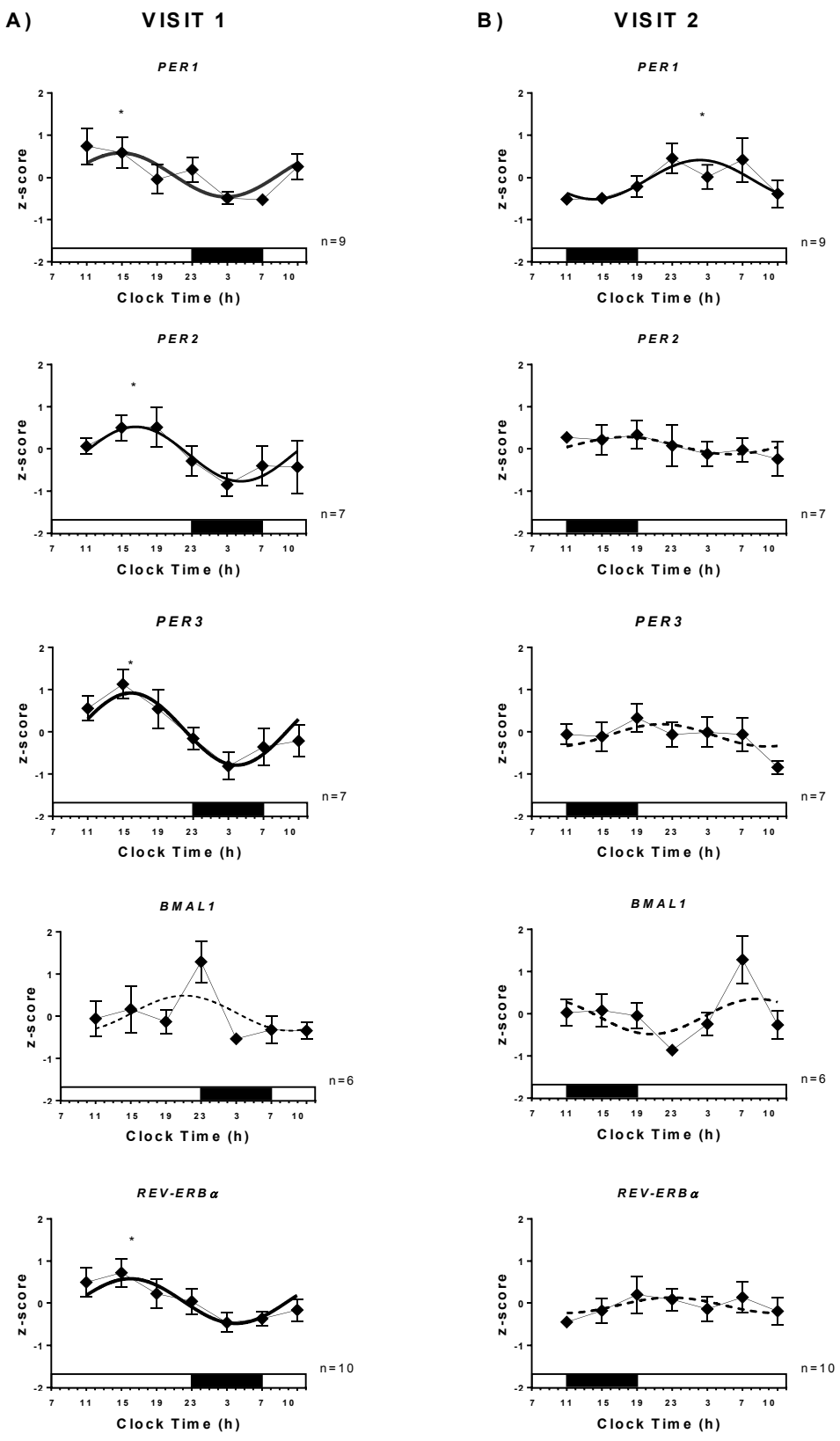

A)
VISIT 1
B)
VISIT 2
PER1
PER2
PER3
BMAL1
REV-ERBα
z-score
Clock Time (h)
n=9
n=7
n=7
n=6
n=10
*

Figure 5.1: Single-harmonic regression on group data of gene expression in oral mucosa cells

The single-harmonic regressions for the 24-h rhythms of clock gene expression for genes *PER1, PER2, PER3, BMAL1,* and *REV-ERBα* of the group during visit 1 are shown in (A) whereas that of visit 2 are shown in (B). The regressions that are significant are indicated by a solid, black line while the regressions that are non significant are indicated by a dotted, black line. The relative gene expression is indicated as z-score values of the raw data. The black bars indicate the subjects' sleep episodes at the laboratory and the white bars indicate their waking periods at the laboratory.

Analysis: Single-harmonic regression for 24-h. The group averages are mean ± SEM. *$*p \leq 0.05$.*

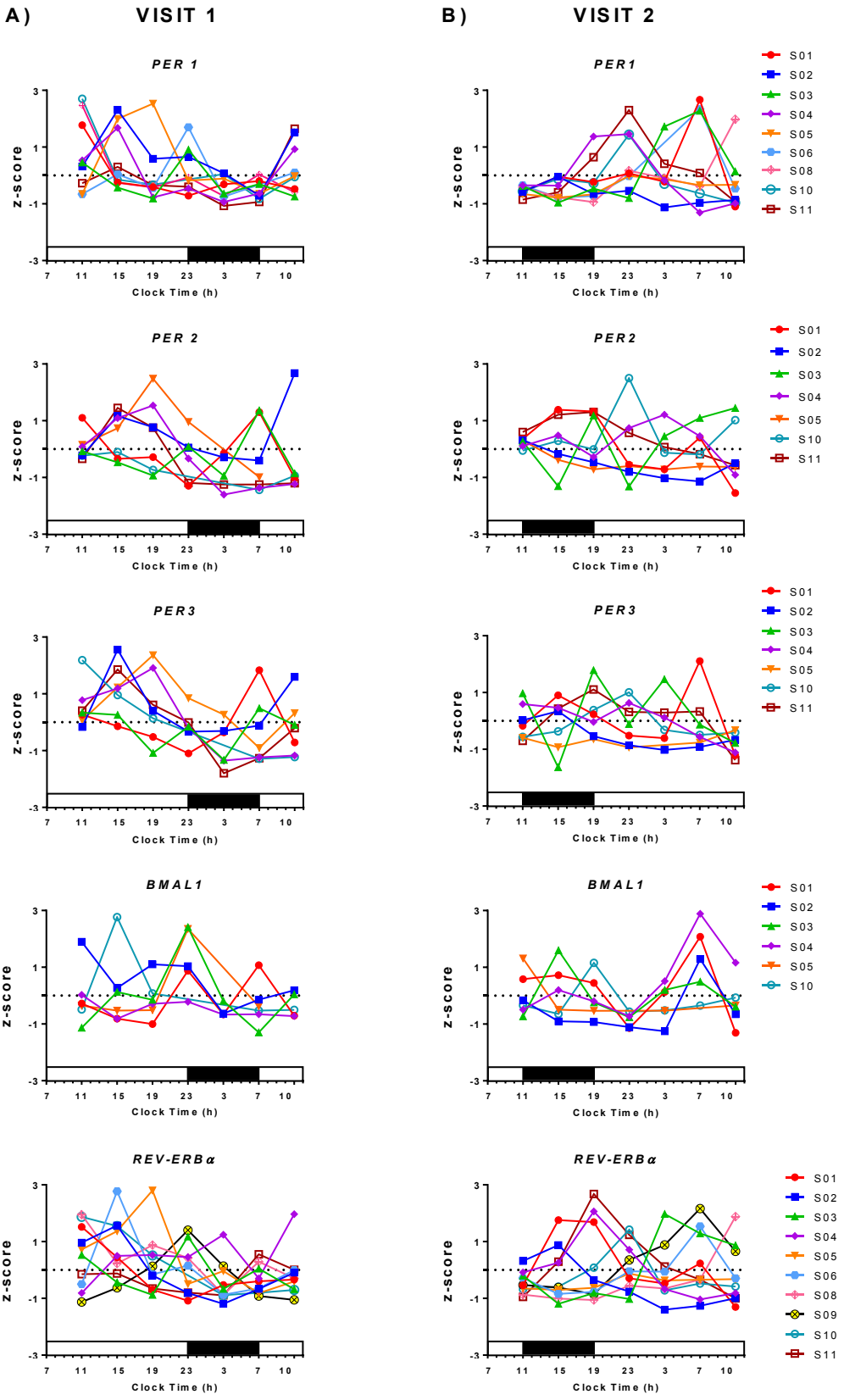

A)
VISIT 1
B)
VISIT 2
PER 1
PER1
PER 2
PER2
PER3
BMAL1
REV-ERBα
Z-score
Clock Time (h)
7 11 15 19 23 3 7 10
3 1 -1 -3
S01
S02
S03
S04
S05
S06
S08
S09
S10
S11

Figure 5.2: Single-harmonic regression on individuals' data of gene expression in oral mucosa cells

The 24-h rhythms of clock gene expression for genes *PER1, PER2, PER3, BMAL1,* and *REV-ERBα* for each individual during visit 1 is shown in (A) whereas those during visit 2 are shown in (B) (see side legend with subjects' colour codes). The relative gene expression is indicated as z-score values of the raw data. The black bars indicate the subjects' sleep episodes at the laboratory and the white bars indicate their waking periods at the laboratory.

Analysis: Single-harmonic regression for 24-h. *$p \leq 0.05$.

Table 3.1 A: Group data of clock gene expression in oral mucosa cells

Clock Gene	Visit	Phase±SD (hh:min)	Phase Shift†
PER1	Visit 1	**14:50 ± 1:13***	**11:18 (delayed)**
	Visit 2	**2:08 ± 1:21***	
PER2	Visit 1	16:34 ± 1:12*	---
	Visit 2	17:38 ± 3:10	
PER3	Visit 1	15:55 ± 0:51*	---
	Visit 2	21:45 ± 2:36	
BMAL1	Visit 1	21:05 ± 2:13	---
	Visit 2	8:36 ± 1:57	
REV-ERBα	Visit 1	16:00 ± 1:04*	---
	Visit 2	22:51 ± 3:43	

The group average phases of the clock gene expression regressions, before and after 7 consecutive night shifts. The rhythms that were significant are indicated by an asterisk.

† The phase shift was determined for rhythms that were significant during both visits.

Analysis: Single-harmonic regression for 24-h. The group averages are mean ± SD. *$p \leq 0.05$.

Table 3.1 B: Individuals' data of clock gene expression in oral mucosa cells

Clock Gene	Visit	Individual regressions (n significant/n total)
PER1	Visit 1	2/9 (22.22%)
	Visit 2	4/9 (44.44%)
PER2	Visit 1	2/7 (28.57%)
	Visit 2	1/7 (14.29%)
PER3	Visit 1	3/7 (42.86%)
	Visit 2	2/7 (28.57%)
BMAL1	Visit 1	0/6 (0%)
	Visit 2	0/6 (0%)
REV-ERBα	Visit 1	2/10 (20%)
	Visit 2	5/10 (50%)

The total number of individual significant regressions for each clock gene, per visit.

Analysis: Single-harmonic regression for 24-h. *$p \leq 0.05$.

Table 3.2: Individuals' acrophase data of clock gene expression in oral mucosa cells

	VISIT 1				
Subject ID	**PER1 Acrophase**	**PER2 Acrophase**	**PER3 Acrophase**	**BMAL1 Acrophase**	**REV-ERBα Acrophase**
Sx1	10:43	8:10	7:42	03:58	11:55
Sx2	16:05	14:42	14:49	16:27	22:26
Sx3	0:54	06:08	11:01	21:29	02:24
Sx4	**13:42***	17:24*	17:17*	20:06	**14:24***
Sx5	**17:55***	18:51	18:40*	00:48	17:30
Sx6	21:17				16:19
Sx7					
Sx8	11:01				14:56
Sx9					**22:42***
Sx10	11:26	14:38	14:30	15:49	15:17
Sx11	13:53	**16:41***	16:17*		10:04
	VISIT 2				
	PER1 Acrophase	**PER2 Acrophase**	**PER3 Acrophase**	**BMAL1 Acrophase**	**REV-ERBα Acrophase**
Sx1	4:38	17:10	09:02	08:14	18:10
Sx2	16:34	14:34	14:51*	08:01	15:40*
Sx3	**5:14**	07:59	00:54	14:06	03:40*
Sx4	**20:45**	01:48	20:29	07:05	**19:06***
Sx5	**2:46**	12:02	11:05	09:46	07:06
Sx6	4:30				02:53
Sx7					
Sx8	8:02				05:22
Sx9					**09:01***
Sx10	22:12	21:42	21:34*	18:08	21:55
Sx11	23:22*	**18:25***	21:38		20:36*
	PHASE SHIFTS†				
Sx4	7:03 delayed				4:42 delayed
Sx5	8:50 delayed				
Sx9					10:19 delayed
Sx11		1:44 delayed			

The phases of clock gene expression rhythms for each subject, before and after 7 consecutive night shifts. The asterisk indicates the acrophases of those rhythms which were signifcant.

† The phase shift was determined for gene expression rhythms that were significant during both visits. The bolded/asterisk values indicate such rhythms.

Analysis: Single-harmonic regression for 24-h. *$p \leq 0.05$.

Table 3.3: Phase angle analysis on group data of *PER1* gene expression and aMT6s rhythms

Visit	aMT6s Phase (hh:min)	*PER1* Phase (hh:min)	Phase Angle†
Visit 1	4:21	14:50	10h29 (delayed)
Visit 2	11h58	2:08	9h50 (advanced)

The phase angle between *PER1* gene expression and the 24-h component of the urinary aMT6s rhythms, before and after 7 consecutive night shifts.

† The phase angle was determined for gene expression rhythms and urinary aMT6s rhythms that were significant during both visits.

Analysis: Single-harmonic regression for 24-h (*PER1* gene expression). Dual-harmonic regression with 12-h and 24-h components (urinary aMT6s). *$p \leq 0.05$.

4 SLEEP TIME AND DURATION

The data collected from individual sleep-wake logs during the ambulatory periods were verified by wrist actigraphy monitoring. When inspecting each individual's sleep-wake logs, we realized that there was much variability in bedtimes and waketimes, day-to-day, both within individuals themselves and between different individuals, during the ambulatory periods. Thus, we show the average bedtime and waketime data of each individual (mean ±

SD), in order to demonstrate the high degree of variability within subjects, as well as the grouped average bedtimes and waketimes (mean ± SD) in order to demonstrate the degree of variability between subjects (Appendix: Table 5).

Total sleep time of each subject was also determined and the results are shown in Appendix: Table 6. During ambulatory period 1 and 2, subjects did not have significantly different amounts of total sleep time (6:55 ± 0:55 hours and 6:27 ± 0:46 hours, respectively). Subjects also did not have significantly different amounts of total sleep time between their 2 laboratory sleep periods. During the scheduled sleep period of visit 1, which occurred between 23h00 and 7h00, subjects had an overall total sleep time of 7:22 ± 0:17 hours, and during the scheduled sleep period of visit 2, which occurred between 11h00 and 19h00, subjects had an overall total sleep time of 7:18 ± 0:19 hours.

5 LIGHT INTENSITY EXPOSURE

We obtained data regarding the total average amounts of light intensities that subjects exposed themselves to during their ambulatory periods using the Actiwatches that they wore during this period. The mean (±S.E.M.) hourly light exposure, that the group of subjects exposed themselves to over an average 24-hour period, during ambulatory periods 1 and 2 are plotted in Figure 6. A 2-way ANOVA for repeated measures was conducted with factors: condition and time-of-sample. The interaction between the 2 factors were significant with a $p \leq 0.0001$, a main effect of condition that was non-significant, a main effect of time-of-sample with $p=0.0005$. Multiple comparisons using Sidak's post-hoc test yielded that at sample time point 11h00-12h00, ambulatory period 1 vs. ambulatory period 2 had a $p \leq 0.01$; at sample time point 14h00-15h00, ambulatory period 1 vs. ambulatory period 2 had a

p≤0.0001; at sample time point 15h00-16h00, ambulatory period 1 vs. ambulatory period 2 had a p≤0.01; and at sample time point 16h00-17h00, ambulatory period 1 vs. ambulatory period 2 had a p≤0.01. The results from the multiple comparisons are shown in Figure 6. During laboratory visits 1 and 2, the subjects stayed in a time-isolated room and were exposed to light intensities of 121.13±38.12 lux and 104.21±18.60 lux, respectively (Appendix: Table 8). During their sleep periods, the light intensity was at 0 lux.

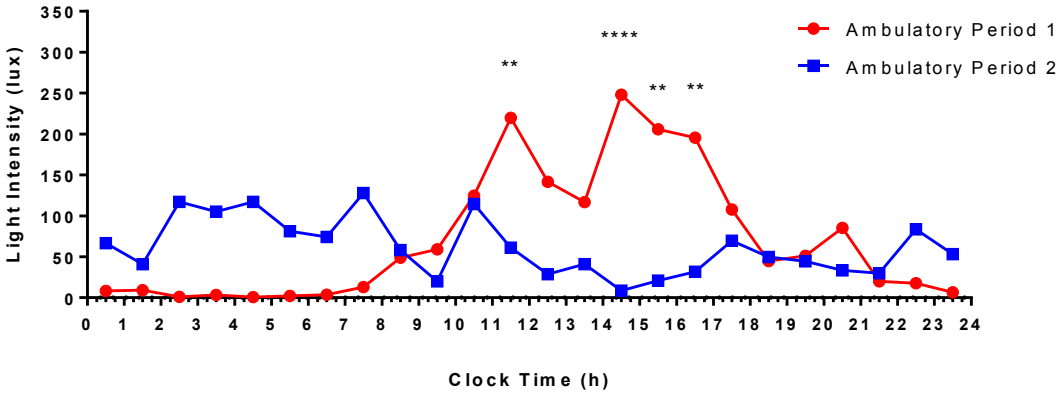


Figure 6: Group data of light exposure levels during ambulatory periods

The average daily light intensity levels (lux) per hour, is shown during ambulatory period 1 (blue squares) and ambulatory period 2 (red squares). The time points at which there was a significant difference in light intensity levels between ambulatory periods 1 and 2 are indicated by asterisks.

Analysis: Sidak's post-hoc test results. 2-way ANOVA, repeated measures, factors: condition vs. time-of-sample. The group averages are mean ± SEM. ***p≤0.01; ****p≤0.0001.*

DISCUSSION

1 DISCUSSION OF RESULTS:

1.1 Salivary Cortisol

Salivary cortisol is known to have its peak secretion immediately upon waking, which then decreases throughout the day, eventually reaching its lowest point of secretion before bedtime [94]. Cortisol is a hormone that is strongly regulated by the SCN as shown in studies that portray the endogenous circadian behaviour of cortisol. As such, we expected that cortisol levels would be higher upon awakening during visit 1, compared to before bedtime. Indeed, both the group and individual representation (Figure 2.1) of the data demonstrate that subjects secreted cortisol at higher levels at waketime than at bedtime, during visit 1, after at least a week of day-oriented schedule. Of note, since we did not have a 24-hour profile for salivary cortisol, we cannot make any precise conclusions regarding the adaptation of the cortisol secretion but the samples we have collected at waketime and bedtime will help demonstrate the possibilities of adaptation in these individuals.

During visit 2, we expected individuals to undergo varying levels of circadian adaptation, according to a previous study that assessed another central clock marker, salivary melatonin, in police officers after they worked 7 consecutive night shifts. We expected subjects who have adapted to their shifted schedules to continue secreting higher levels of cortisol upon waking during visit 2, even though their waketime was shifted by 8:02 ± 0:54 in visit 2 vs. visit 1. This would indicate that their circadian system adapted well to their shifted schedule.

On the other hand, lower levels of cortisol secretion upon waking during visit 2 compared to levels at bedtime would indicate that the individual's central clock remains

adapted to their day-oriented schedule, even though their work and sleep schedule has shifted by about 12 hours. This was what was seen with our group average results of cortisol levels. There was a significant reduction in cortisol levels at waketime in visit 2, compared with waketime at visit 1 ($p \leq 0.05$). This observation was also supported by previous studies in which nurses have shown reduced salivary cortisol levels at waketime, after shiftwork conditions, compared to after non-shiftwork conditions [62, 74]; however, these studies were conducted over 24-hour periods and thus, the results from our study are not completely representative of these prior studies.

What we can conclude is that the group reduction that is observed at waketime in visit 2, compared to at waketime in visit 1, is probably due to the reduction of salivary cortisol levels at an individual level (as seen in all individuals except subjects 5 and 6). Another explanation for the reduction in average levels that we observed could be due to the variability in phases of individuals' salivary cortisol rhythms; however, we did not assess this information and cannot comment further.

The reductions in salivary cortisol levels can be explained by the disrupted sleep schedules that these individuals have, leading to the non-adaptation of their central clock marker, which results in less cortisol being released at the expected time of awakening. Studies have also shown that high amounts of slow wave sleep could have an inhibitory effect on cortisol release [65]. As subjects do not sleep sufficiently during their week of night shifts, this could lead to an increase in their slow wave sleep, since slow wave sleep is known to be present at a higher than usual level following a sleep deprivation period. This could then subsequently lead to the reduction of cortisol levels [65]. Moreover, cortisol represents glucocorticoids in the body – important molecules that send signalling factors from the SCN

to peripheral tissues in order to entrain them. Lower levels of this hormone can thus lead to the desynchrony observed in peripheral clock markers.

To assess the adaptation of this central clock marker, we calculated the ratio of cortisol at waketime over bedtime for each individual, during each of the visits. We would expect this ratio to be greater than 1 during visit 1 since levels of cortisol secretion would be higher at waketime than at bedtime. During visit 2, based on whether we observe ratios greater than 1 (indicating adaptation to the new shifted sleep schedule) or whether ratios are closer to 1 or lower than 1 (indicating incomplete or lack of adaptation to the shifted sleep schedule), we can conclude about the different degrees of adaptation that an individual underwent. We observed that the cortisol secretion of 2 individuals, subjects 1 and 2, clearly indicated that their central clock did not adapt to their shifted sleep schedule, with ratios of cortisol at waketime/bedtime being lower than 1 during visit 2. However, before we conclude that the other 9 out of 11 subjects all adapted to the shifted schedule, we analyzed the individual ratios more closely.

Certain individuals, such as subjects 4, 7, and 11 had largely reduced ratios during visit 2 compared to visit 1, which were all close to the value of 1, and could thus indicate that they were probably non-adapted or only partially adapted to their shifted sleep schedules.

Subjects 3, 5, 9, and 10 had ratios that were similar between visits 1 and 2, with values being greater than 1, indicating that these individuals were adapted to their shifted sleep schedule.

Subject 8 had a reduced ratio during visit 2 compared to visit 1, but both ratios were greater than 1, indicating that he was also probably adapted to his shifted sleep schedule.

Of note, subject 6 had a ratio close to 1 at his first visit, indicating that he was unadapted at baseline, but had a very large ratio at his second visit, which indicates that he could have become adapted after working 1 week of night shifts. All of these individuals' results, as discussed, are shown visually in the appendix: Figure 8.

However, since there are only 2 time points for which we have salivary cortisol concentrations, we have no information on how subjects' 24-hour rhythms are behaving.

The reason as to why these subjects' salivary cortisol concentrations are higher during their evening waketime (19h00) than during their morning bedtime (11h00), at visit 2, could be either due to adaptation, or a shift in phase of the cortisol rhythm indicating a partial adaptation, or another other type of disturbance that could be a result of working shifts. Therefore, no conclusive statements on adaptation can be made without analyzing a 24-h rhythm. However, what these results suggest is a possibility of adaptation in 6 out of 11 individuals (approximately 54.55%) and a possibility of non-adaptation in the remaining five (approximately 45.45%). These results can be verified by comparing these results with another central clock marker that we studied, the aMT6s excretion rhythm of subjects over 24 hours. Nevertheless, even with just two key time points of analyses and a relatively small population of night shift working police officers, we observed that individuals showed various degrees of adaptation to a shifted sleep schedule. This observation was also demonstrated in the Boudreau et al. study in 2013, with a 44% adaptation rate in police officers who worked 7 consecutive night shifts while 56% of the officers remained non-adapted [17]. The police officers in this 2013 study had a work roster that very closely matches that of the police officers who participated in the current study. This could imply that the design of the work roster itself leads to spontaneous adaptation due to the numerous, continuous night shifts that

subjects work. The adaptation rate could also be explained due to spontaneous adaptation that occurs in certain shift workers who have good, regular sleeping habits, such as sleeping in total darkness, maintaining regular sleep schedules, and exposing themselves to bright light during their night shifts [61, 62]. It is important to note that the bedtimes of subjects 3, 5, 8, and 9, individuals who have possibly adapted, all had very small standard deviations (i.e. of less than 1 hour) during ambulatory period 2, which were also reduced compared to the standard deviations of their bedtimes during ambulatory period 1. Subjects 3, 5, 8, 9, and 10 (another individual who supposedly adapted), also had reduced standards deviation of their bedtimes during ambulatory period 2 compared to their standard deviations of bedtimes during ambulatory period 1. Finally, subject 6, the individual who looks to be unadapted during visit 1 but became adapted during visit 2, had a very large standard deviation of his bedtimes during ambulatory period 1 which was much reduced during ambulatory period 2. Thus, these individuals' probable adaptation could be due to maintenance of regular bedtimes during their week of night shifts (i.e. ambulatory period 2).

In conclusion, the central clock marker of salivary cortisol seems to show an overall maladaptation to the shifted sleep schedules of these police officers. Even though the levels of salivary cortisol at waketime are higher than at bedtime, before and after 7 consecutive night shifts, there is still a significant reduction in total cortisol levels in this population.

1.2 Urinary aMT6s

The averaged group results of the urinary aMT6s excretion rhythm showed that the peak excretion rate of melatonin, before the week of night shifts, occurs during the nighttime, at 4h20, with a peak excretion rate of 2032.06 ng/hr. This result was expected since melatonin is a hormone that is secreted by the pineal gland during the dark period, starting a few hours

before bedtime and peaking around the middle of the sleep period in healthy day-active individuals [95]. In visit 1, we observed that the group average aMT6s excretion rate during the sleep period was 1673.9 ng/h. This value corresponded with the average aMT6s excretion rate reported in a previous study, recorded in police officers during their sleep episode at baseline, of 2131.8 ng/h [96]. The excretion rate values from this prior study and our study did not differ significantly ($p=0.124$).

In recent studies by Bracci et al., conducted in nurses, urinary aMT6s levels as well as salivary melatonin levels did not significantly differ between rotating-shift working nurses and day-working nurses [74, 97]. However, in another study conducted by Boivin et al. in 2012, that assessed salivary melatonin levels in shift working nurses, it was shown that there were reduced levels of salivary melatonin during these nurses' shifted sleep episodes, while during their daytime sleep episodes, there were significantly higher levels [60]. Our observed results seem to suggest that, as a group, police officers do adapt partially to their shifted sleep schedule, as seen by a significant phase shift in the group-averaged aMT6s regression (Figure 3.1). When the subjects are assessed after working 7 consecutive night shifts during visit 2, the 24-h component of their regression has a phase at 11h58, which occurs during their shifted sleep period, with an amplitude of 454.61 ng/hr, and a peak level of 1234.01 ng/hr. This is a significant reduction from their peak level at baseline and thus, is a very important result. This reduction in peak aMT6s levels during visit 2, compared to visit 1, could be explained due to subjects not being able to adapt sufficiently to the shifted sleep schedules. Subjects are prone to exposing themselves to higher amounts of light intensities during their daytime sleep periods and the hours preceding their sleep. This can lead to the suppression of the natural production of melatonin as well as cause a change in the timing of the melatonin rhythms.

Furthermore, our study also showed non-significant increases in aMT6s levels during the night period of visit 2.

The observation of a lower peak excretion rate of the group regression during visit 2 compared to visit 1, could be explained either by a reduction of urinary aMT6s excretion rate during the sleep period at an individual level, or due to phase variability in the individual regressions. In line with the first possibility, a reduction in excretion rate during visit 2, compared to visit 1, was observed in all individuals except subjects 3, 8, and 9, during the sleep episode (appendix: Table 7). In accordance with the second possibility, the 24-h regression components that were significant during visit 1 all had acrophases which occurred within the range of 5h00 to 5h55 whereas the 24-h regression components that were significant during visit 2 had acrophases which ranged from 9h33 to 15h45, signifying the presence of a much larger range of variability. Thus, since there is evidence of both possibilities taking place, the reduction could probably be due to a combination of these 2 different explanations.

A limited number of studies have shown significant differences in urinary aMT6s excretion levels between shift-work and non-shiftwork conditions. Thus, the results we demonstrate in this study are very relevant since it shows that this central clock marker becomes significantly reduced after working 7 consecutive night shifts. Also, at a group level, the central clock marker of urinary aMT6s does seem to show partial adaptation after 7 consecutive night shifts. This is based on the result that their 24-h regression component was significant during both visits, with peak levels of expression occurring during the respective sleep periods. It is important to note, however, that at an individual level, not all subjects had adapted aMT6s rhythms. Only 2 out of 11 subjects (18.18%) adapted their urinary aMT6s

rhythms to their shifted individuals. The studied subjects were exposed to different intensities of light and had varied sleep-wake schedules, which could lead to the large variability observed between subjects' rhythms.

The most interesting observation was that, both within the individual data and the amongst the grouped data, there seems to be a bimodality in the rhythm of aMT6s excreted and, to our knowledge, this result has never clearly been demonstrated in shift-working populations. One study in humans have suggested that females who experience seasonal affective disorders have demonstrated bimodal melatonin rhythms [98]. However, these results have not been replicated in other, more recent scientific studies. Even though this second peak in our study was significant during visit 1, a second peak was absent during visit 2. The observation of bimodal rhythms in 10 out of 11 subjects led us to pay particular attention to why this might be. The acrophase of this second peak during visit 1 was at 16h52 with an amplitude of 73.74 ng/hr, with a peak metabolite level of 925.42 ng/hr. It occurred during the evening, well before the expected melatonin onset, with a much lower amplitude and peak level of melatonin than at nighttime. This second peak, observed in several subjects during both visits, suggests that there is some maladaptation of aMT6s rhythms in these shift workers, after - and more surprisingly - before, a week of working night shifts.

Reasons for this maladaptation could be due to the masking effect of light that we used in the laboratory during these subjects' visits. Studies that assess circadian phase using melatonin rhythms are usually conducted in dim light conditions in order to minimize the masking effects of light on the endogenous melatonin rhythms [99]. A light intensity of 121.13±38.12 lux and 104.21±18.60 lux, used during the waking periods at the laboratory, during visits 1 and 2, respectively, could also lead to the masking of the expression of urinary

aMT6s. This could possibly have created a dip in the aMT6s rhythm which led to a bimodal curve in the overall rhythm of aMT6s excreted. Also, as seen from the variations in light intensities and sleep-wake schedules of our subjects during the ambulatory periods that preceded the laboratory visits (Figure 6 and Appendix: Table 5), the lack of regularity in sleep behaviours and exposure to varying light intensities at inappropriate times could also lead to disturbed aMT6s rhythms in these police officers.

Observing and drawing conclusions about 'adaptation' from the results at an individual level was made difficult by the lack of significance in a majority of the individual data sets. However, we had a few subjects who did have significant data and their results suggest that there are varying degrees of adaptation between these shift workers. Two individuals, subjects 5 and 6, had significant peak expression of urinary aMT6s excretion during their sleep periods of visit 1, and during their shifted sleep periods of visit 2. This indicates that they did adapt their aMT6s rhythms according to their shifted sleep schedule and thus, their central clock marker was adjusted to their shifted sleep-wake schedule. Three other individuals (subjects 1, 4, and 11) who had significant peak expression of aMT6s excretion rhythms during their sleep periods of visit 1 had lost this significance in aMT6s excretion rhythms during visit 2, suggesting that these individuals were maladapted during visit 2, either transitioning towards adaptation or remaining non-adapted to their shifted sleep schedules. Moreover, subject 3, 7, 8, and 9 had significant peak expression of aMT6s excretion rhythms during their sleep periods of visit 2 but not during their sleep periods of visit 1. Since this significant peak expression occurs during the shifted sleep periods for subjects 3, 8, and 9, it indicates that they were unadapted at baseline but became adapted after working a week of night shifts. It is important to note that subjects 3, 8, and 9 all had higher

urinary aMT6s levels during their visit 2 sleep period, compared to their visit 1 sleep period. This adaptation could also be explained by their potentially good habits of sleeping in total darkness, exposing themselves to high light intensities during their work periods, and maintaining good sleep schedules. Of note, subjects 5 and 6, who were the only 2 individuals that showed adaptation of their individual urinary aMT6s rhythms in visit 2, compared to visit 1, had very large standard deviations in their bedtimes during ambulatory period 1 which were reduced during ambulatory period 2 and very small (SD of less than 1 hour). Moreover, subjects 3, 8, and 9, the individuals who seemed to have been unadapted during visit 1 but became adapted during visit 2, seem to not only have very small standard deviations in their average bedtimes during ambulatory period 2 but also reduced bedtime standard deviations compared to during ambulatory period 1 (Table 5). Thus, maintenance of regular bedtimes during their week of night shifts seems to have helped these individuals adapt their urinary aMT6s rhythms.

Finally, we see that the amount of aMT6s excreted per hour is much higher during visit 1, compared with visit 2, and much higher during sleep periods, compared to wake periods. This significantly higher quantity of aMT6s excreted during visit 1than during visit 2, demonstrates that there is a disruption of aMT6s excretion occurring in these police officers when their sleep-wake cycle becomes shifted as a result of working 7 consecutive night shifts.

The significantly higher quantity of aMT6s excreted during the sleep periods compared to during wake periods is as expected in healthy, non-shift working individuals as melatonin is a hormone that is secreted during the dark period.

The reduction in total excreted amounts of melatonin during visit 2, compared with visit 1, has possible associated risks of developing cancers and inflammatory conditions.

Melatonin is known to have anti-inflammatory characteristics that have shown to inhibit the growth of tumors by inhibiting cell proliferation and thus, reduction of this very important hormone has important clinical implications [100, 101].

In conclusion, the central clock marker of urinary aMT6s seems to show an overall maladaptation to the shifted sleep schedules of these police officers. Even though the 24-h component of the regression shifted its acrophase to the new shifted sleep schedule, before and after 7 consecutive night shifts, there is still a significant reduction in total urinary aMT6s levels in this population.

1.3 PBMCs

Prior studies have shown that clock gene expression in PBMCs oscillate in a circadian manner in healthy individuals [48, 50, 89, 102]. Our main goal for this project was to test the impact of night shift work on this peripheral clock.

We observed that during visit 1, after 3 weeks of working on a day-oriented schedule, clock genes *PER1*, *PER2*, and *PER3* had significantly higher gene expression at 10h00 compared to 19h30. These results were as per our expectations as well as correlated well with prior studies [50, 102]. In fact, a recent study that followed a similar protocol as us in terms of collecting blood from nurses at 2 time points, showed that *PER2* expression showed similar results when assessed before shift work [73]. During visit 2, after a week of consecutive night shifts, all three of these clock genes lost their significant difference between 10h00 and 19h30. This suggests that these 3 genes were in transition while adapting spontaneously to the shifted schedule and that the 10h00 and 19h30 time points happened to occur during the rise and fall of the hypothetical rhythm of gene expression, respectively. The other hypothesis is that these genes lost its rhythmicity of expression and thus, there would be no significant

difference in amplitude throughout the 24-hour period, including during the 2 time points of interest. We cannot conclude which of the two situations may be happening since we only have 2 time points and cannot obtain this data without more frequent sampling. However, we do have results from a second peripheral clock, the oral mucosa cells, to verify these results (discussed below).

What we can conclude is that the loss of significant 10h00 vs. 19h30 difference in gene expression, at the group level, observed during visit 2, compared to during visit 1, is probably due to the loss of rhythmicity of regressions at an individual level or due to the phase variability of these genes' expression inter-individually. Since we see in Table 3.2 that several subjects had non-significant rhythms of clock genes and/or significant rhythms which had variable acrophases when compared to other significant rhythms, the actual explanation could be a combination of the 2 above explanations.

As for *BMAL1,* there was no significant difference found between 10h00 and 19h30 during either of the visits and this could possibly be because we missed the peak of *BMAL1* in these individuals in that they had *BMAL1* acrophases that occurred after 19h30 and so we do not see relatively higher gene expression between the 2 time points of interest. Another explanation could be that subjects had variability between each other but this is improbable since we see all the data points of subjects clumped together at 19h30 during visit 1.

For *REV-ERBα,* a significant decrease was observed between 10h00 and 19h30 only during visit 2 and not during visit 1. The lack of significance during visit 1 could also be explained by the possibility of missing the peak of *REV-ERBα* in these individuals in that they had *REV-ERBα* acrophases that occurred much earlier than at 10h00. This explanation could be verified by observing the rhythmic *REV-ERBα* gene expression results we have over a 24-h

period in oral mucosa cells and checking when the acrophase of *REV-ERBα* actually took place in this second peripheral clock. The significant difference we see between 10h00 and 19h30 for *REV-ERBα* expression levels during visit 2 could be explained by a potential phase delay shift that individuals are undergoing as their circadian system tries to adapt to the shifted work schedule during week 2.

In the Fang et al. study, the nurses actually showed an increase in *PER2* levels in the evening prior to the night shift than in the morning after the night shift, after working at least 1 week of night shifts [73]. The discrepancy between these results and ours could be explained by several differences between the 2 studies. The nurses in the prior study had different work schedules compared to each other, their blood samples were collected at inconsistent time points, and there were no measures used to control variability between subjects [73]. In our study, subjects had the same work schedules as each other, blood samples were collected at the same times for all subjects, and we used sleep logs and Actiwatches to control between subjects (although there was still variability observed in sleep times).

Overall, it is clear that just one week of night shifts has the potential to disturb clock gene expression in a peripheral clock, such as in PBMCs, namely in clock genes *PER1, PER2,* and *PER3*. To observe what is happening to clock gene expression at a rhythmic level, we needed to assess the expression of clock genes in oral mucosa cells, over a 24-hour period.

1.4 Oral Mucosa

From the group analyses, we observed that 4 out of the 5 clock genes that we assessed in oral mucosa cells had a significant gene expression rhythm during visit 1, which occurred immediately before the police officers worked their week of night shifts. Out of the 4 genes,

only *PER1* remained significant after the week of night shifts, indicating that it was adapted. This can be supported by results from previous studies that have shown *PER1* to be a gene that is acutely responsive to glucocorticoids [48]. Although our group average results of salivary cortisol indicate non-adaptation of the central clock, our group results of urinary aMT6s does indicate adaptation. Considering that we did not have the appropriate number of saliva samples to clearly indicate the behaviour of cortisol over a 24-h period, we can turn to our urinary aMT6s results to conclude that the central clock marker in our subjects did probably adapt to a week of night shifts. If so, this could mean that salivary cortisol levels were also adapted and thus, lead to the acute entrainment of the *PER1* gene expression rhythms. Of note, is that this observation was most likely due to the direct action of the central clock marker and not an endogenous adaptation of the peripheral clock in oral mucosa cells.

The results we observed in the remaining clock genes are in line with the expected pattern of expression of these clock genes at baseline levels, even if studied in a different peripheral clock than previous studies. Studies in PBMCs have shown that at baseline, *PER1, PER3,* and *REV-ERBα* all had significant peak expression in the early-morning to mid-morning times [48, 50, 89, 102]. Studies in oral mucosa and other peripheral clocks such as hair follicles have also shown that these clock genes have peak expressions in the morning [23, 103, 104]. It is important to note that we did obtain gene expression acrophases in oral mucosa cells that were slightly delayed than gene expression acrophases observed in previous studies that assessed clock gene expression in PBMCs. However, this could simply be explained by the reason that peripheral clocks themselves can behave differently from other peripheral clocks. Different tissues have different physiological functions that are timed differently and thus, clock genes in these tissues could also be playing their role at different

times. Moreover, in a recent study by Weissova et al. that also assessed clock gene expression rhythms in oral mucosa cells, it was shown that rhythms of *PER1* gene expression in healthy subjects peaked at around 12h00, a phase that was 2h50 advanced than the acrophase that we observed in this study [103]. Another study which assessed *PER3* and *REV-ERBα* expression in human hair follicles showed a variable range of acrophases that ranged from 2h34 to 9h71 in *PER3* and from 22h36 to 6h01 in *REV-ERBα* [23].

No studies thus far, to our knowledge, have shown gene expression rhythms in oral mucosa cells in actual shift working conditions and thus, the results we obtained are of great relevance. They show for the first time that there is significant reduction of rhythmicity of clock gene expression in a shift working population after just 7 days of working night shifts. This reduction could be explained by the fact that at an individual level, subjects either lost rhythmicity in their gene expression after 7 consecutive night shifts which led to an overall loss of significant rhythmicity in the group data or that individuals had varying phases of gene expression rhythms that cancelled each other out when averaged, resulting in an overall loss of significant rhythmicity in the group data. To verify which of these justifications was the appropriate reason, we had to assess the gene expression pattern at an individual level. Unfortunately, majority of the individual data that we have lacked significance and thus, was difficult to draw conclusions from. However, we did have a few subjects that did have significant data and their results suggest that the reason may be a mix of the 2 above justifications.

Certain individuals had significant rhythms of gene expression during both visits, such as subject 4 who had significant *PER1* and *REV-ERBα* expressions, subject 9, who had significant *REV-ERBα* expression, and subject 11, who had significant *PER2* expression.

Other individuals who had gene expression rhythms during visit 1 lost significance in their gene expression rhythms during visit 2. Therefore, this suggests that the loss of rhythmicity we observe in group data could be explained by some disturbance that is occurring in the circadian rhythms of these night shift workers – either a desynchrony of rhythms between different subjects and/or a loss of rhythmicity in individual subjects. Also, by visually inspecting the group *BMAL1* expression results, which did not have a significant rhythm during visit 1, it seems that with a greater sample size, these rhythms have the potential to become significant, at the level of the group. Since the rhythms do not look dampened or flat, the lack of significance may not be due to an actual lack of rhythmicity in these genes' expression levels. In fact, Weissova et al. showed that *BMAL1* in oral mucosa did have significant peak expression at approximately 23h00, in day-active subjects [103].

Overall, our results from oral mucosa cells suggest that shift work brings about a general disruption in clock gene expression, even at baseline, which is also supported by the observation of a general increase in expression of *BMAL1, PER1, PER2,* and *REV-ERBα* and an overall reduction of *PER3* expression in rotational shift-working nurses compared to day-working nurses in previous studies [72, 97]. In our study, we observed that all clock genes which were significantly rhythmic before a week of night shifts become arrhythmic, at a group level, after working consecutive night shifts. However, *PER1* expression adapted to the week of night shifts, probably via an acute effect of cortisol.

1.5 Comparisons between Sleep and Melatonin

Due to the large variability, both within and between subjects, in bedtimes and waketimes, comparison between melatonin acrophases and sleep times were not made. However, the fact that there was so much variability in these officers' rest-activity cycles

during the ambulatory periods could influence their circadian system and its inability to adapt perfectly to a shifted schedule. Thus, intra- and inter-individual variability of bedtimes and waketimes may be the reason why we observe a 2nd peak in the group results of these individuals, indicating some degree of maladaptation occurring. Also, the varying amounts of light intensities that these police officers were exposed to, with especially higher, more significant intensities during the week before their night shifts, implies that the individuals' aMT6s rhythms are highly impacted, especially during their visit 1, by the amount of light that they expose themselves to during their work periods.

1.6 Comparisons between Melatonin and Cortisol

Subjects 5 and 6 were the only 2 subjects that showed adaptation in their urinary aMT6s excretion, and thus we could compare their results between the two central clock markers. Both the aMT6s rhythms and salivary cortisol levels seem to have adapted in both these individuals of subject 5 and 6, suggesting that the 2 markers of the central clock seem to compliment each other.

The group average results of both the urinary aMT6s levels and salivary cortisol levels also seem to compliment each other, even though they both suggest a maladaptation of the central clock. Both markers, though they suggest partial shifting, show a significant reduction in their respective levels after a week of night shifts, and thus at a group level, the central clock seems to be maladapted in these police officers.

1.7 Comparisons between Oral Mucosa and PBMCs

Results of *PER1* and *PER3* gene expression rhythms in oral mucosa corresponds to the results seen in PBMCs in that these 2 genes showed a significantly higher expression at 10h00 than at 19h30, during visit 1 which was lost during visit 2. *PER2* expression was significantly

higher at 10h00 than at 19h30 during visit 1 in PBMCs which did not correspond to the non-significant results observed in oral mucosa cells but this could be explained by the small sample size and the lack of statistical power for this particular gene.

The lack of significance in *BMAL1* expression in PBMCs, between 10h00 and 19h30, during visit 1, could be explained by the fact that we just missed the time of the peak expression of *BMAL1* with our blood sample collection time of 19h30. In comparison, when observing the *BMAL1* expression in oral mucosa, it seems to have had an acrophase around 21h00 during visit 1, which suggests that we probably did in fact miss the time of peak expression of *BMAL1* in the PBMCs.

Additionally, the lack of significance in *REV-ERBα* expression between 10h00 and 19h30, during visit 1 can also be explained by the fact that we just missed the time of the peak expression of *REV-ERBα* with our blood sample collection time of 10h30. A closer observation of the oral mucosa results show that *REV-ERBα* expression seems to have had an acrophase around 16h35 during visit 1, and thus, the acrophase probably occurred in between our 2 blood sample collection times of 10h00 and 19h30 and we did in fact miss the time of peak expression of *REV-ERBα*.

For the most part, the 2 peripheral clocks seem to behave similarly in terms of clock gene expression in these tissues, although the precise acrophase can vary between peripheral clocks, as mentioned in the introduction [22, 47]. Thus, the 2 peripheral clocks that were studied seem to have been non-adapted to a week of night shifts.

1.8 Comparisons between Melatonin and Oral Mucosa

When comparing the two 24-h rhythms of aMT6s excretion and clock gene expression in oral mucosa cells, we observed that at a group level, aMT6s excretion seemed to have

adapted to a shifted sleep schedule in police officers who work 7 consecutive night shifts while clock gene expression seems to be disrupted after working 7 consecutive night shifts. In other words, the central clock marker seems to have adapted to a shifted sleep schedule after just 7 consecutive night shifts while the peripheral clock marker seems to be disturbed after just 7 consecutive night shifts.

1.9 Light intensity levels

The light intensity curves in Figure 6 shows the daily amounts of light intensities that the police officers expose themselves to during the ambulatory periods. The officers were exposed to a significantly higher intensity of light during their day-oriented work week than during their week of night shifts (main effect of condition yielding $p \leq 0.0001$), especially during the middle of the day (when subjects are sleeping during their night shift week). However, the lack of significant difference between the light intensities during the middle of the night (i.e. when subjects are working during their night shifts) is important to note. This indicates that subjects are probably exposing themselves to lower light levels than during their regular day/evening shifts, which could serve as a strong synchronizer of the circadian system and could lead to the adaptation of individuals' circadian rhythms to their shifted sleep schedules. Although, another explanation is that we do have a small sample size and this non-significant increase in light intensity during this night shift work period could potentially become significant with a larger n value.

Also, it can be seen that during the subjects' sleep periods in their day-oriented week, they are exposed to an intensity of approximately 0 lux of light, while during their sleep period in their week of night shifts, they are exposed to varying intensities of light, and this could influence the lack of adaptation seen in individuals' circadian rhythms.

2 STUDY LIMITATIONS:

Our 2 time points of blood sampling do not allow us to see the 24-h clock gene rhythms in PBMCs. We decided to limit collection of blood to 2 time points due to the invasiveness of indwelling catheters needed to do 24-h blood sampling procedures. Thus, as an alternative to using white blood cells for assessing 24-h clock gene expression rhythms, we developed a new, less invasive technique to assess rhythms of clock gene expression over a 24-h period – we collected oral mucosa cells, approximately every 4 hours, as an additional peripheral cell in which we can observe peripheral clock rhythms over a larger period of time. Moreover, this technique is promising because it could be used in field studies in the future.

Another limitation of analyzing clock gene expression is the variability in acrophases and amplitudes of rhythms of clock gene expression between subjects, especially in *BMAL1*, a gene that is known to have high inter-individual variability. To minimize the effects of inter-individual variability, we did a z-score analysis on the clock gene expression data in order to standardize and compare values from different officers.

Variability between the different peripheral clock markers was also a limitation in our study since we could not effectively compare our results from the 2 clocks we studied.

The subjects studied in this project were actual shift workers who were on patrol during their week of night shifts. Different individuals were exposed to different light intensities, different feeding and sleeping behaviours, and experienced different situations as part of their work week and thus, this was not a very controlled study in the field. However, these subjects used Actiwatches and filled out sleep-wake logs throughout their ambulatory periods, allowing us to monitor them as closely as possible. We also made sure that the

laboratory visits, during which we collect the biological samples, are very well controlled between the different subjects.

Another limitation was that we did not conduct any PSG recordings or document any slow wave sleep of these subjects. As discussed in the introduction, high amounts of slow wave sleep could have an inhibitory effect on cortisol release.

The subjects were exposed to different intensities and durations of light during their ambulatory periods that were not controlled for. Since light is a very strong synchronizer of the central clock, it would have potentially influenced the circadian rhythms that were observed in clock gene expression of oral mucosa and in aMT6s excretion, especially due to the masking effect of nocturnal melatonin levels by light.

Finally, the results thus far are obtained from 9 individuals and thus, the statistical power is small. The clock genes that seem to show low rhythmicity in expression could probably display enhanced rhythmicity with the inclusion of data from additional subjects.

3 IMPLICATIONS AND STATEMENT OF EXPECTED CONTRIBUTIONS TO ORIGINAL KNOWLEDGE:

To our knowledge, very few studies have addressed the different levels of desynchrony of circadian clocks on individuals living on a shifted sleep-wake cycle. This study considered the complexity of desynchronization and will thus significantly advance our knowledge in the field. This study also demonstrated the effects we reported in our prior studies, with simulated shift workers, in actual shift-working police officers.

The combined results we found from our simulated night shift work, in addition to the results from this police officers study, will serve as an investigational tool for other field studies and clinical interventions. It could eventually lead to the use of a translational

approach in order to help the general public population, including travelers who experience jet lag and insomniacs, with sleep-related issues such as increased risks of developing heart attacks, strokes, and even cancer.

We will also be able to test specific combinatory-therapies that act on both central and peripheral clocks, on shift workers. These therapies could align their circadian rhythms and sleep-wake cycles and thus, allow for better circadian adaptation to night shift work, which will in turn lead to reduced disturbances during sleep periods, decreased metabolic issues, and less risks of accidents in workers.

4 CONCLUSION:

Our observations after 7 consecutive night shifts demonstrate that circadian disruption does occur in peripheral clocks with an overall loss of rhythmicity when compared to baseline results. The central clock markers seem to show adaptation to the shifted work schedule at a group level but demonstrate varying levels of disruption when comparing between individuals. Significant influence on our circadian parameters from subjects' varying sleep-wake and rest-activity cycles as well as exposure to varying light intensities could also help explain the observed results and large inter-individual variability in the results. Overall, the findings from this study have important clinical implications for the occurrence of several shift-work associated health issues and disorders.

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APPENDIX

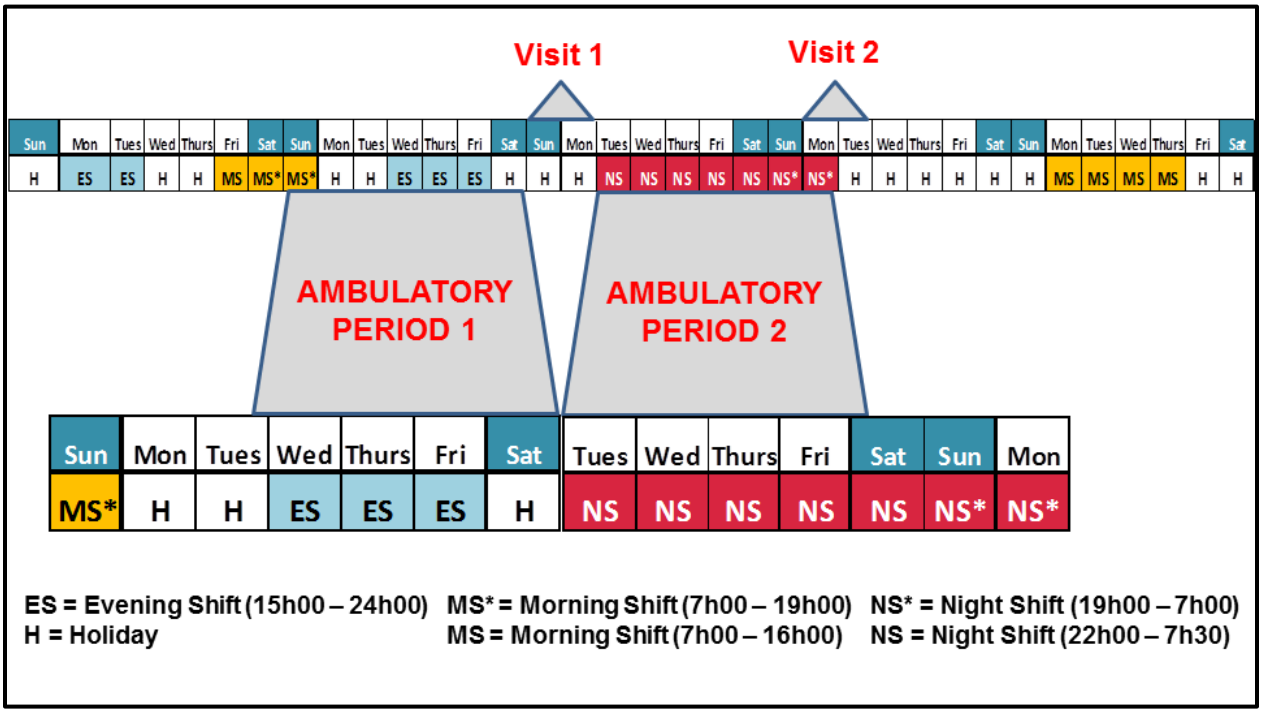

Figure 7: Subjects' 35-day work roster

Police officers who were studied in this study were recruited from a police department that has a 35-day work roster (shown above) which repeats throughout the year. During ambulatory period 1, which preceded the subjects' 1st visit to our laboratory, subjects worked a morning shift which was 12 hour long (MS*), followed by 2 days off (H), then 3 evening shifts which was 9 hours long (ES), and another final day off (H) on the day preceding their 1st laboratory visit. Their visit 1 always occurred on the Sunday and Monday which preceded their week of night shifts (ambulatory period 2). The last 2 night shifts that the police officers did consisted of a 12-hour shift as opposed to their regular 9.5-hour night shifts). Immediately after their last night shift, subjects re-entered the laboratory for their 2nd visit.

Table 4: Subjects' Chronotypes

Subject ID	Chronotype*
Sx1	Intermediate
Sx2	Intermediate
Sx3	Intermediate
Sx4	Moderately Morning
Sx5	Intermediate
Sx6	Moderately Evening
Sx7	Intermediate
Sx8	Intermediate
Sx9	Intermediate
Sx10	Moderately Evening
Sx11	Intermediate

Subjects 1-3, 5, 7-9, and 11 were intermediate chronotypes while subject 4 was a 'moderately morning' chronotype and subjects 6 and 10 were 'moderately evening' chronotypes.

*Chronotype was assessed by use of the Morningness-Eveningness Questionnaire (MEQ) [86].

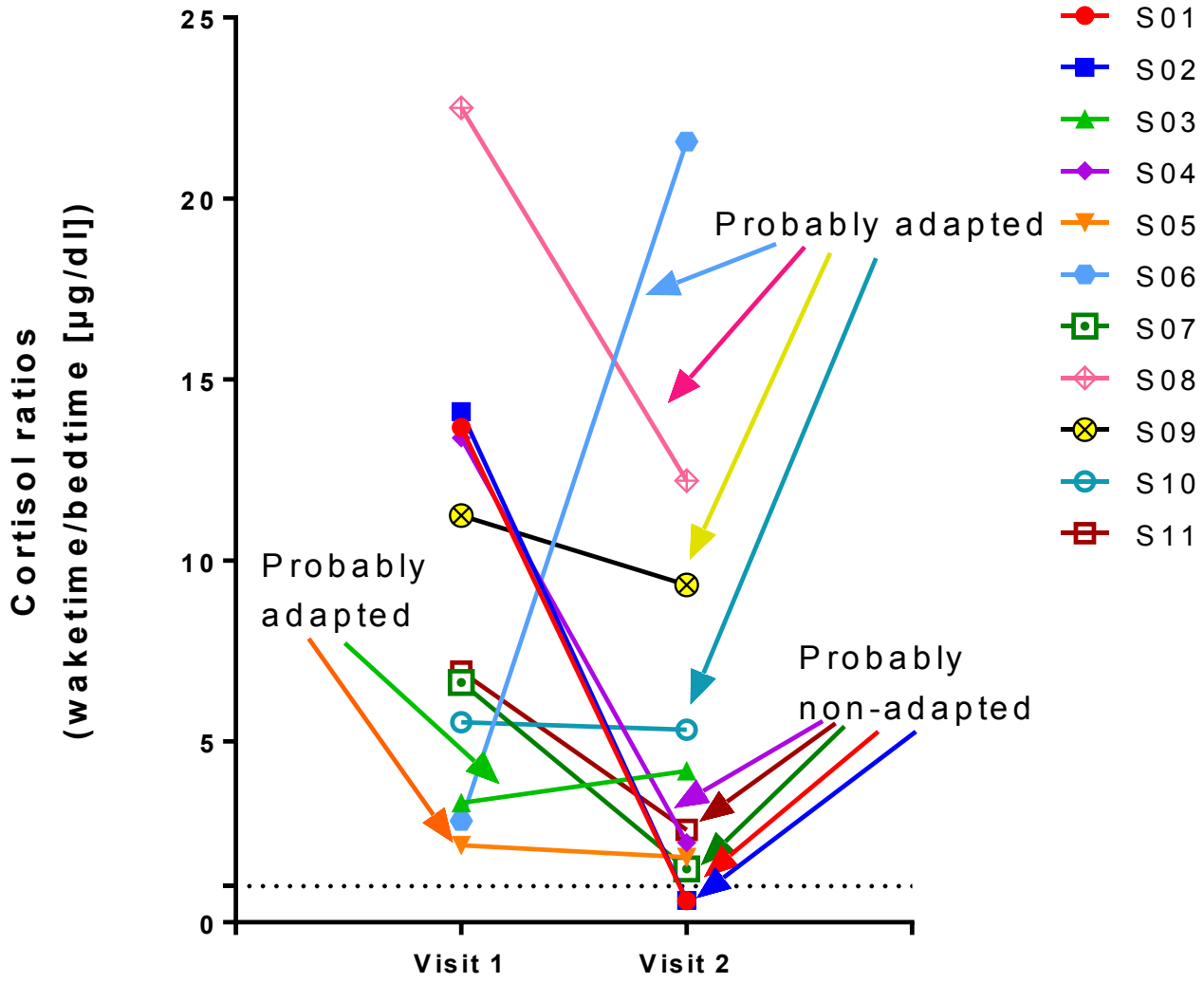

Figure 8: Individuals' probability of adaptation in salivary cortisol levels

The probability of adaptation in salivary cortisol levels, based on ratios of cortisol at waketime over bedtime, during visits 1 and 2.

Table 5: Individuals' bedtimes and waketimes during ambulatory periods

Subject ID	Bedtime (AP1) (hh:min)	Waketime (AP 1) (hh:min)	Bedtime (AP2) (hh:min)	Waketime (AP 2) (hh:min)	Shift in bedtime	Shift in waketime
Sx1	3:03±0:55	9:48±1:50	9:41±1:25	16:33±1:51	6:38	6:45
Sx2	23:47±1:15	7:34±1:54	9:48±1:01	16:24±2:20	10:01	8:49
Sx3	23:33±1:54	7:44±1:51	8:23±0:33	15:44±0:53	8:50	7:59
Sx4	23:56±1:31	8:13±1:18	8:01±0:28	15:35±1:05	8:05	7:21
Sx5	0:14±1:29	8:43±1:23	8:59±0:51	17:10±1:12	8:45	8:27
Sx6	1:28±2:34	10:29±1:56	9:39±0:58	17:23±1:27	8:10	6:53
Sx7	0:48±2:08	8:09±1:57	9:49±1:18	17:23±1:09	9:00	9:14
Sx8	0:19±1:31	8:49±1:02	8:19±0:48	16:10±0:12	7:59	7:21
Sx9	0:55±2:45	7:32±0:21	9:39±0:12	16:59±0:56	8:44	9:27
Sx10	2:30±3:30	8:21±2:51	9:22±1:42	16:22±0:58	6:52	8:01
Sx11	22:55±2:11	7:38±0:55	8:36±0:17	15:49±1:07	9:40	8:10
Average:	2:51	8:27	9:07	16:30	6:15	8:02
Std. Dev.:	1:58	1:35	0:52	1:12	6:53	0:54

The average bedtime and waketime values of each subject in hh:min (mean ± SD) obtained from their sleep-wake logs and verified by their Actiwatches are shown below for AP1 (ambulatory period 1) and AP2 (ambulatory period 2). The average bedtimes and waketimes for the group are also shown at the bottom with the group standard deviation.

Table 6: Individuals' total sleep time during ambulatory and laboratory sleep periods

Subject ID	Total Sleep Time (AP1) (hh:min)	Total Sleep Time (V1) (hh:min)	Total Sleep Time (AP 2) (hh:min)	Total Sleep Time (V2) (hh:min)
Sx1	6:12 ± 1:11	7:37	5:59 ± 1:52	7:02
Sx2	6:53 ± 1:34	7:14	4:32 ± 2:38	7:09
Sx3	7:13 ± 0:58	7:11	6:52 ± 0:46	7:20
Sx4	7:26 ± 1:12	7:21	6:52 ± 1:05	7:34
Sx5	7:45 ± 0:56	7:27	7:27 ±1:00	7:38
Sx6	7:57 ± 2:18	6:34	6:05 ± 1:42	7:23
Sx7	6:25 ± 1:19	7:29	6:56 ± 0:13	6:31
Sx8	7:37 ± 1:59	7:32	7:07 ± 0:45	7:24
Sx9	5:44 ± 2:05	7:27	6:39 ± 0:51	7:40
Sx10	5:06 ± 2:03	7:39	6:14 ± 1:19	7:27
Sx11	7:42 ± 1:35	7:26	6:20 ± 0:42	7:11
Average	6:55	7:22	6:27	7:18
Std. Dev.	0:55	0:17	0:46	0:19

The average total sleep time of each subject in hh:min (mean ± SD) obtained from their sleep-wake logs and verified by their Actiwatches are shown below for AP1 (ambulatory period 1) and AP2 (ambulatory period 2). The total sleep time in hh:min is also shown for each individual during their laboratory visit 1 (V1) and visit 2 (V2). The average total sleep time for the group is also shown at the bottom with the group standard deviation.

Table 7: Individuals' aMT6s excretion rates during laboratory sleep and wake periods

Subject ID	Visit 1 - Sleep aMT6s Excretion Rate (ng/h)	Visit 2 - Sleep aMT6s Excretion Rate (ng/h)	Visit 1 - Wake aMT6s Excretion Rate (ng/h)	Visit 2 - Wake aMT6s Excretion Rate (ng/h)
Sx1	1879.11	407.29	877.81	967.78
Sx2	1676.04	791.09	1272.85	836.19
Sx3	941.98	1656.95	532.18	656.38
Sx4	4008.70	3084.29	1687.10	1775.51
Sx5	2385.25	2113.47	779.62	544.44
Sx6	2662.16	2086.75	1303.34	748.77
Sx7	1156.06	850.05	859.15	1064.79
Sx8	580.69	1295.75	958.87	317.40
Sx9	120.62	506.95	609.82	277.78
Sx10	852.26	196.77	452.21	408.40
Sx11	2150.53	792.56	501.65	466.61
Average	1673.95	1252.90	894.06	733.09
Std. Dev:	1108.60	890.47	389.83	431.35

The aMT6s excretion rate of each subject in ng/h during the sleep and wake periods during visits 1 and 2 at the laboratory. The average excretion rate for the group is also shown at the bottom with the group standard deviation.

Table 8: Individuals' exposure to light intensity during laboratory visits

Subject ID	Light Intensity (lux)	Light Intensity (lux)
	Visit 1	Visit 2
Sx1	112±7.98	107.8±9.88
Sx2	88.92±7.10	102.80±19.69
Sx3	87.36±12.36	100.38±12.40
Sx4	88.48±13.92	82.70±18.74
Sx5	106.08±19.40	103.54±8.19
Sx6	87.96±15.19	95.52±22.22
Sx7	167.26±34.33	94.94±14.68
Sx8	164.14±39.82	91.42±25.09
Sx9	184.84±14.57	126.34±46.97
Sx10	92.4±15.33	91.96±10.36
Sx11	153.00±17.67	148.96±26.70
Average:	121.13	104.21
Std. Dev.:	38.12	18.60

The average measured light intensity that each subject was exposed to during their stay at the time isolation laboratory, during visits 1 and 2 (mean ± SD). The average total light intensity for the group is also shown at the bottom with the group standard deviation.