MELATONIN, CORTISOL, AND PERCEIVED ADAPTATION
AFTER WORKING ONE NIGHT SHIFT

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Degree of Master of Science

by

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Chapter 1. Introduction

1.1 Background

Early death, cancer, and endocrine, cardiovascular, metabolic, reproductive, gastrointestinal, neurological, bone, immune, cognitive/affective, and sleep disorders are associated with shift work (Niu et al., 2011; Gu et al., 2015). These diseases result partly from shift work’s disruption of daily physiological rhythms, including the rhythmic release of hormones. Circulating levels of melatonin and cortisol normally rise and fall in a circadian rhythm that is disrupted during shift work, for example, by exposure to light at night (Kim et al., 2015). The multiple disease states associated with shift work result from disturbance in the molecular mechanisms that control levels of melatonin and cortisol during disruption of the sleep cycle and circadian rhythm. Do these cellular and physiological cascades also affect individuals’ perception of wellbeing after working just one night shift? This question is important where adaptation to a shift schedule affects work performance and can have life-and-death consequences, e.g., in a hospital setting (Berger and Hobbs, 2006).

The worldwide prevalence of shift work is 15-20% of workers (Wright et al., 2013). Traveling across time zones is common, and the United States Centers for Disease Control and Prevention estimate that 50-70 million Americans have a sleep or wakefulness disorder, so research in this area also has broad relevance (CDC, 2015). The health risks of shift work are also important for people dependent on shift workers, e.g., in hospitals and emergency services, and for drivers sharing the road. In the United States, 15-20% of motor vehicle crashes result from sleepiness; in one survey, 43% of all drivers who crashed had just worked a night shift or overtime (McCartt et al., 1996; Ftouni et al., 2013). An estimated 60% of US nurses work rotating schedules including night shifts, and their work performance is affected by their
schedule (Smith and Eastman, 2008; Pan et al., 2011). With more than 100,000 deaths in US hospitals per year due to medical error alone, and a higher rate of non-lethal medical errors, work performance of hospital staff including nurses is critically important (Kohn et al., 1999; Sharek, 2012).

When a shift work schedule consists of a series of consecutive night shifts, some individuals’ circadian rhythms can phase shift to synchronize to the new schedule. If melatonin rhythm does fully shift, it takes at least several days (Crowley et al., 2004). The circadian rhythm of cortisol also requires more than one night to align to night work (James et al., 2007). In most circadian rhythm studies of shift work, melatonin and cortisol have been measured after two or more consecutive night shifts (Grundy et al., 2011; Niu et al., 2011). However in rotating shift schedules, it is common to work a single night shift followed by a day or days off (Michaels, 1984; Petrovic, 2012). In the study described in this thesis, nurses’ melatonin, cortisol, and self-rated adaptation to their schedule are compared after a single night shift to their melatonin, cortisol, and adaptation after working a day shift. This study is unique in assessing perceived adaptation and circadian biomarkers melatonin and cortisol after only one night shift.

1.2 Biological rhythms and the circadian system

From single-celled bacteria to humans, living organisms must control the timing of physiological processes at multiple levels, e.g., reproduction vs. hibernation, sleep vs. alertness to prey, glycolysis vs. gluconeogenesis. The master molecular clock in mammals is the hypothalamic suprachiasmatic nucleus (SCN). The SCN is sensitive to environmental cues or zeitgebers (from German, synchronizer). Zeitgebers cue entrainment of the SCN’s coordination of rhythms in the body, including the daily circadian rhythm. Entrainment of this endogenous clock occurs primarily via direct projections from the retina, transmitting light signals, described in this section below. Non-photic direct projections entraining the SCN include the brain stem dorsal and median raphe nuclei, via serotonin (Glass et al., 2003). The intergeniculate leaflet of
the thalamic lateral geniculate complex integrates photic (retinal) and non-photic (raphe) input to
the SCN via NPY and GABAergic transmission of the geniculohypothalamic tract (Lall and
Biello, 2003). The SCN also receives many signals affecting circadian rhythm via other routes
including endocrine and paracrine (Vaze and Sharma, 2013).

Most organisms' inherent circadian rhythms (free-running periods) are not exactly 24-
hours, so entrainment by the daily light/dark cycle is necessary to keep rhythms synchronized.
This occurs via a non-parametric phase-shifting response, the phase-response curve (PRC).
During the day, light does not phase shift the clock. During the early night, light exposure phase-
delays the clock; during late night, light exposure phase-advances the clock. Other zeitgebers
besides light also produce their effects according to phase response curves, including
exogenous melatonin (Lewy et al., 1992). Phase shifts due to zeitgebers can also be inhibited;
light phase-shifts are modulated by activation of SCN GABA receptors (Mintz et al., 2002). In
addition to the phase response curve, dose-response curves also apply to some entrainers, for
example, light intensity (Fonken and Nelson, 2014).

The SCN clock is controlled by two interlocking transcription/translation feedback loops.
The primary loop is driven by proteins: two activators and two repressors. Activators brain and
muscle aryl hydrocarbon receptor nuclear translocator-like 1 (BMAL1) and circadian locomotor
output cycles kaput (CLOCK) heterodimerize to form a basic helix-loop-helix-PAS (PER-ARNT-
SIM) transcription factor that binds the enhancer box (E-box) promoter region for transcription of
period genes (Per1, Per2, and Per3) and cryptochrome genes (Cry1 and Cry2) for repressor
proteins PER and CRY. PER-CRY heterodimers and CRY alone inhibit transcription of their own
genes by binding to and inhibiting the CLOCK-BMAL1 E-box promoter (Ye et al., 2014).
Approximate 24-hour periodicity is maintained as PER and CRY are degraded via ubiquitination,
relieving their suppression of CLOCK-BMAL1 for the cycle to begin again. The rates of PER and
CRY ubiquitinylation or nuclear translocation are controlled by casein kinases (CSNK1δ and
CSK1ε) with protein phosphatases (PP1 and PP5). Both PER and CRY are degraded by the
26S proteasome complex, PER via Skp1-cullin-F-box protein (SCF) E3 ubiquitin ligase complex with β-TrCP, and CRY via adenosine monophosphate-activated protein kinase (AMPK), FBXL3, and SCF E3 ubiquitin ligase (Mohawk et al., 2012).

The primary CLOCK-BMAL1/PER-CRY loop interlocks with a secondary feedback loop activated by retinoid-related orphan receptor proteins (RORa, b, c) and repressed by REV-ERBα proteins transcribed by the reverse to erythroblastic leukemia nuclear receptor gene (Rev-erbα). Along with Per and Cry, CLOCK-BMAL1 activates transcription of Rev-erbα and many other clock-controlled target genes downstream. REV-ERBα competes with RORa for the ROR response element (RRE) promoter of Bmal1 and Cry transcription, leading to an antiphase oscillation in Bmal1 and Cry expression. The delay in Cry expression induced by REV-ERBα competition with RORa at the RRE is necessary for correct circadian period (Partch et al., 2014).

The same gene-protein feedback loops that control the SCN are also found in cells throughout the body, including in kidney, liver, heart, lungs, muscle, and intestines. The peripheral clocks are synchronized by SCN outputs via paracrine, endocrine, and direct neural signaling to multiple brain regions and organs including heart, pancreas, liver, and pineal gland (Bonmati-Carrion et al., 2014). Tissue-specific and system-specific circadian rhythms provide local regulation; for example, REV-ERBα and AMPK are important in metabolic rhythms (Duez and Staels, 2008; Nader et al., 2010b). In addition to the SCN’s output determining the circadian rhythm of the peripheral clocks, peripheral signals feed back to the SCN to influence its circadian rhythm.

The feedback loops of the SCN are primarily entrained by light. Light activates the intrinsically photoreceptive retinal ganglion cells whose signal travels through the retinohypothalamic tract to the SCN core. Glutamate (Reiter et al., 2014) acts on N-methyl-D-aspartate (NMDA) and other receptors to trigger action potentials in the SCN neurons, increasing intracellular calcium (Hastings et al., 2014). Calcium activates calcium/cAMP-
dependent transcription factor CREB to bind to calcium/cAMP-dependent response element (CRE), increasing Per transcription (Sakamoto et al., 2013). The PER surge resets oscillation of the primary feedback loop, contributing to the phase response curve of the effect of light on the SCN (Field et al., 2000; Hastings et al., 2014).

1.3 Sleep Regulation

Two regulatory systems control sleep—the circadian sleep drive and the homeostatic sleep drive (Borbely, 1982). The circadian sleep drive is strongest at the end of the night, and weakest at the end of the day, and is primarily entrained by the light dark cycle, but also by meals, activity, and social interaction (Morris et al., 2012). The homeostatic drive for sleep increases with time spent awake, and decreases during sleep. The homeostatic drive is hypothesized to result from accumulation of somnogenics in the brain (Landolt, 2008) or the need to balance synaptic plasticity and potentiation with synaptic depression (Tononi, 2009). These drives counterbalance each other, so sleepiness and sleep propensity become significant only after a normal 16-h day (Dijk and Czeisler, 1994; Postnova et al., 2013). Besides the homeostatic and circadian sleep drives, light—particularly blue light—has been described as a third, independent sleep regulator (Lockley et al., 2006; Hubbard et al., 2013).

The normally opposite peaks of circadian sleep drive and homeostatic sleep drive are no longer counterbalanced after a night of shift work. When the worker goes to sleep at sunrise, circadian sleep drive has just passed its high and is starting to decrease, but the normal increase in homeostatic sleep drive that occurs only during waking is alleviated as the person sleeps. As circadian sleep drive continues to decrease to its low at sunset and the homeostatic drive does not increase, it becomes more difficult for the worker to remain asleep (Morris et al., 2012).
1.4 Circadian rhythm disruption

When photic or non-photic entrainers of the circadian rhythm are mistimed, circadian rhythm disruption occurs, with extensive detrimental effects. These effects may be conflated with the effects of sleep deprivation or sleep cycle disruption in shiftwork studies outside the laboratory; within the laboratory, circadian disruption effects can be isolated. For example, circadian misalignment raises insulin resistance and inflammation markers independently of sleep loss. However, illustrating the multiplicity of pathways that produce the same effect at a systems level, sleep loss without circadian misalignment also increases these markers (Leproult et al., 2014). Mechanisms and pathways in metabolism, cardiovascular and immune function, and cancer relate the most to the health risks of shiftwork listed in section 1.1 above. Circadian rhythm disruption also affects melatonin and cortisol levels, as described in sections 1.6 and 1.7 below.

Metabolism is affected by circadian disruption also because energy and food-reward homeostasis are dependent on circadian alignment and sleep quality (Ferreira and Huijberts, 2013). Disrupted substrate oxidation and glucose-insulin metabolism affect energy balance and body weight. Irregular meal frequency/changed food intake timing increases blood pressure and levels of cortisol and catecholamines, as well as glucose, insulin, leptin, ghrelin, and glucagon-like peptide-1 (Ferreira and Huijberts, 2013). Contributing to metabolic effects of circadian disruption, muscle oxidative capacity is modulated by Rev-erba regulation of mitochondrial biogenesis and autophagy (Woldt et al., 2013).

Vulnerability to circadian disruption varies among individuals; gene-environment interactions affect circadian characteristics. Gamble (2011) studied nurses on a rotating shift schedule, an environment that switches frequently between different light-dark cycles and social/workplace times. Nurses’ phenotype and genotype affected their circadian system’s ability to adjust to their shift schedule, i.e. their adaptation: chronotype and sleep strategy were associated with adaptation (Gamble et al., 2011). Phenotype was associated with genotype:
several sleep characteristics and alcohol/caffeine consumption associated with single and multi-
locus polymorphisms in CLOCK, NPAS2, PER2, and PER3. Some associations were specific to
shift type, suggesting an interaction between environment (shift schedule) and genotype
(Gamble et al., 2011).

1.5 Sleep Deprivation

Acute sleep deprivation causes death more quickly than food deprivation in rats and Drosophila (Rechtschaffen et al., 1983; Shaw et al., 2002). Early death in humans is associated with chronic short sleep duration (less than 6 hours per night). Chronic short sleep duration is also associated with obesity, type 2 diabetes, cardiovascular disease, cancer, and immune and cognitive/affective dysfunction (Lo et al., 2014; Dashti et al., 2015; Furihata et al., 2015). In humans, short-term sleep deprivation affects insulin, blood glucose, appetite, and energy expenditure, cardiovascular autonomic response, immune modulation, cognitive function, and mood (Grandner et al., 2010; Tobaldini et al., 2013).

Models explaining the danger of sleep deprivation include the energy model: neurons use more energy during waking activity and increased ATP expenditure increases adenosine buildup, promoting sleep (Benington and Heller, 1995). In the synaptic homeostasis model, sleep, particularly slow wave, requires downregulation of synaptic potentiation, a necessary recovery period (Tononi and Cirelli, 2003). In the macromolecular biosynthesis model, sleep is necessary for biosynthesis of macromolecules required for membrane stability and signal transduction (Mackiewicz et al., 2007). Sleep deprivation also imbalances allostatic mediators, increasing the wear and tear on brain and body that comprises allostatic load. For example, secretion of allostatic mediator cortisol is increased by proinflammatory cytokines that are themselves suppressed by cortisol (McEwen, 2006).

Mechanisms and pathways of sleep deprivation-induced damage include acute changes in expression of PER2, BMAL1, and CRY1, causing circadian rhythm disruption (Kavcic et al.,
Besides affecting expression of clock genes, other gene expression patterns respond to the cellular stress of sleep deprivation, including increased free radical production and oxidative stress (McEwen, 2006). Misfolded and unfolded protein buildup triggers the unfolded protein response to protect the endoplasmic reticulum (Elliott et al., 2014). Chronic short sleep is associated with shorter telomeres (Cribbet et al., 2014). Sleep deprivation also affects melatonin and cortisol levels, as described in the next sections, 1.6 and 1.7. Sleep deprivation was an expected factor in the current study. When starting a series of night shifts, most nurses have remained awake since the previous night. They then continue to stay awake through their first night shift, going to sleep soon after their first night shift ends (Gamble et al., 2011). When nurses who work a rotating shift schedule have time off from work, most nurses spend more time awake during the day, and more time asleep during the night (Smith and Eastman, 2012). It was expected that nurses in the current study would typically be similarly sleep deprived when working their first night shift. The current study was conducted as a substudy within a larger primary study that started earlier. Sleep diaries from the primary study’s first ten nurses were available before this study began, and their sleep habits were consistent with this expectation, described in more detail in section 1.8 below. While it was expected that this study’s participants would experience some degree of acute sleep deprivation, as rotating shift workers they were also expected to be chronically sleep deprived (Guo et al., 2013).

1.6 Melatonin

Melatonin is found in plants, bacteria, invertebrates, and vertebrates including humans. In both diurnal and nocturnal animals, melatonin levels are high in darkness, and low in light. In humans, melatonin controls circadian rhythm and has many effects including antioxidant and anti-inflammatory (Hardeland et al., 2006). Melatonin is produced when SCN projections to the hypothalamic paraventricular nucleus (PVN) cause pre-autonomic PVN neurons to produce sympathetic input to the pineal gland (Reiter et al., 2014).
The SCN’s signal to produce melatonin reaches the pineal gland via a series of synapses: SCN shell neurons synapse on the dorsal parvocellular PVN, whose presympathetic axons contact sympathetic preganglionic neurons of the intermediolateral cell column of the upper thoracic spinal cord (IML) (Kalsbeek et al., 2012). IML sympathetic preganglionic neurons synapse on superior cervical ganglion neurons whose postganglionic nerve fibers release norepinephrine to act on beta-adrenergic \(\beta_1G_3\) receptors on the pinealocyte. These receptors trigger the adenylate cyclase/cyclic adenosine monophosphate (cAMP)/phosphokinase A (PKA) second messenger pathway into the nucleus, to the phosphorylated cAMP response element-binding protein (CREB), causing transcription of the gene for arylalkylamine N-acetyltransferase (AANAT) in pinealocyte cytosol (Reiter et al., 2014).

In the pinealocyte, tryptophan enters from the capillaries and tryptophan 5-hydroxylase converts it to 5-hydroxryptophan, that is converted by L-tryptophan decarboxylase to serotonin. Serotonin is converted by AANAT to N-acetylserotonin that is converted by acetylserotonin methyl transferase (ASMT) to melatonin (Reiter et al., 2014). Posttranslational activation of constitutive AANAT enables fast synthesis of melatonin in humans (Stehle et al., 2011). From the pinealocyte, melatonin enters the capillaries, cerebrospinal fluid, and saliva. In the blood and CSF, melatonin circulates throughout the body and at the SCN, it binds MT1 and MT2 receptors whose signaling pathway inhibits the cAMP to PKA action on DNA, downregulating melatonin production (Reiter et al., 2014). MT1 and MT2 are class A, G protein-coupled receptors (GPCRs); MT3 is a high-affinity binding site on the cytosolic enzyme quinone reductase 2 that acts in cellular detoxification/antioxidant pathways (Kandel et al., 2013; Logez et al., 2014).

Melatonin binding and expression of melatonin receptors differ among species. In the human brain, MT2 receptors are expressed on arginine vasopressin (AVP) neurons in the hypothalamic SCN, supraoptic nucleus (SON), and paraventricular nucleus (PVN) (Wu et al., 2013). MT1 is found in the same nuclei as MT2, plus cerebellum, hippocampus, substantia nigra, and ventral tegmental area (Slominski et al., 2012). Outside the brain, MT1 is expressed
in the cardiovascular, immune, and reproductive systems, and many other tissues, including adrenal cortex, liver, kidney, skin and retina. MT2 is also in the immune system, mammary glands, retina, skin, kidney, blood vessels, and other tissues, plus pituitary, gastrointestinal tract, and adipose tissue (Slominski et al., 2012). Distribution of MT3 in humans is not yet characterized (Cernyiov et al., 2015). Inside the cell, melatonin reaches all cellular compartments (Venegas et al., 2012), performing many functions including antioxidation and free radical scavenging (Hevia et al., 2010). Melatonin is metabolized throughout the body, mainly via two indolic pathways: the classic hepatic cytochrome P450 pathway to 6-hydroxymelatonin (excreted in urine after sulfation) and a second indolic pathway to metabolites of 5-methoxytryptamine. Melatonin is also metabolized in kynuric pathways including enzymatic and nonenzymatic transformation to N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK). Melatonin can also be demethylated back to its precursor, N-acetylsertotonin (NAS) by CYP enzymes as needed (Kim et al., 2013).

Melatonin level is high during darkness and low during light exposure in all mammals, including both nocturnal and diurnal species (Challet, 2007). In humans, normal melatonin levels and rhythms have been established in labs using dim light and constant routine protocols. In humans, under dim light/constant darkness conditions, circulating melatonin normally starts to increase around 2 hours before an individual’s usual bedtime. When this increase is measured under dim lighting, it is a powerful marker of the phase of circadian rhythm, called the dim light melatonin onset (DLMO) (Lewy et al., 1999). Melatonin level continues to rise after the DLMO, peaking around 4 hours after bedtime, then decreasing again to a low level around 2 hours later—for example DLMO at 10 pm before a midnight bedtime, peaking around 4 am then decreasing to below DLMO threshold around 6 am. The decrease below DLMO threshold is called dim light melatonin offset (DLMOffset) and the lowest level of melatonin is called acrophase (Burgess and Fogg, 2008). Melatonin remains low throughout the day until DLMO. Outside the lab, normal circadian expression of melatonin is altered in healthy humans who are not under
dim light or in constant darkness: normal room light (or brighter light) suppresses melatonin levels. If healthy humans sleep with normal room lights on, melatonin expression typically decreases by 70% throughout most or all of the sleep period (Gooley et al., 2011). If healthy humans keep room lights on until going to sleep and then sleep in darkness, their melatonin remains at low daytime levels until the individual is in darkness. This real life scenario differs from the classical normal melatonin rhythm that is seen when healthy humans wake and sleep under dim light/constant routines in a lab, where dim light melatonin onset is expected about two hours before the individual’s usual bedtime. As soon as an individual is sleeping in darkness (and free of melatonin suppression due to other effectors such as posture), circulating melatonin level starts to rise and it remains high for 6-8 hours after lights out. If darkness and sleep persist for longer than 6-8 hours, the duration of high melatonin level varies among healthy individuals, with melatonin either decreasing toward daytime levels or remaining high until lights on, depending on the individual (Gooley et al., 2011). Individuals’ melatonin secretion levels vary highly even after controlling for conditions such as acute/chronic light/dark exposure and posture. Other factors associated with differences include age, sex (and menstrual cycle phase), ethnicity, BMI, posture/activity/exercise, intake of certain foods/drinks/medication, season, etc. This variation is attributed to differences at the genome level as well as epigenetics (Burgess and Fogg, 2008).

Acute sleep deprivation reduces melatonin levels during the deprivation period and the following day (Faraut et al., 2012). However in shift workers, the effects of sleep disruption on melatonin are secondary to the powerful effect of light exposure described above (Haim and Zubidat, 2015). After starting a series of night shifts, some individuals’ melatonin rhythm may fully phase shift after several days, but some individuals will not fully shift even with interventions such as timed bright light and dark during sleep (Crowley et al., 2004). Melatonin suppression is associated with metabolic and immune disorders, and cancer (Mantele et al., 2012; Fonken and Nelson, 2014). Exogenous melatonin improves sleep for healthy people.
1.7 Cortisol

Cortisol is known as the “stress hormone.” It is a steroid and glucocorticoid that contributes to the stress response and to regulation of growth, immunity, metabolism, and brain functions such as alertness and memory in a circadian rhythm (Chrousos, 2009). In the stress response, cortisol is produced when the stress system’s hypothalamic pituitary adrenal (HPA) axis is activated. The stress system also includes the autonomic locus ceruleus-norepinephrine/sympathetic nervous system (LC-NE/SNS). The LC-NE/SNS produces the “fight or flight” response of faster breathing and heart rate, and increased blood flow to muscles due to the release of catecholamines norepinephrine (NE) and epinephrine by the adrenal medulla and neurons (Chrousos, 2009). Cortisol’s role in the stress response is to promote carbohydrate and lipid metabolism and increase blood pressure to provide energy and oxygen to the organs crucial to surviving a threat—brain and muscle. Cortisol temporarily suppresses unnecessary energy-consuming functions including the immune and reproductive systems (Chung et al., 2011). Cortisol increases blood glucose via hepatic gluconeogenesis and peripheral insulin resistance, also increasing protein catabolism and lipolysis/circulating free fatty acids, although long-term cortisol excess causes central/visceral adiposity. Cortisol decreases secretion of growth hormone and release of luteinizing hormone, follicle-stimulating hormone, and thyroid stimulating hormone (Melmed et al., 2012).

Cortisol is secreted in response to stress when the PVN is stimulated by systemic or physiologic stressors, e.g. brain stem catecholaminergic input, or by peripheral signals such as immune cytokines responding to infection (Silverman et al., 2005). Neurogenic, emotional, or
psychological stressors signal the PVN additionally via nociceptive and somatosensory pathways and cognitive/affective brain centers including the cortex and amygdala. Increased circulating cortisol results in reaction and adaptation to stress in target tissues (Melmed et al., 2012). Acute cortisol action improves aversive memory formation, so that stressful stimuli are better retained than neutral ones (Tsigos and Chrousos, 2002). Hypothalamic PVN secretion of peptides arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH) into portal blood to the anterior pituitary gland’s CRH receptors, primarily CRH-R1, activate adenylyl cyclase. In the anterior pituitary, CRH increases tissue cAMP concentration and the rate of transcription of proopiомelanocortin (POMC) mRNA. The pituitary converts POMC into adrenocorticotropic hormone (ACTH) that is secreted into the circulation. ACTH stimulates the adrenal cortex to secrete cortisol (Melmed et al., 2012). In the adrenal cortex, cortisol is produced from cholesterol. Cholesterol is transported into the mitochondria by steroidogenic acute regulatory (STAR) protein. In the mitochondria and endoplasmic reticulum of the adrenal cortex fasciculata and reticularis zones, cytochrome P450 and hydroxysteroid dehydrogenases convert intermediates—pregnenolone, progesterone, 17a-hydroxypregnenolone, 17a-hydroxyprogesterone, 11-deoxycortisol/11-deoxycorticosterone—into cortisol (humans) or corticosterone (rodents) (Payne and Hales, 2004). Cortisol biosynthesis is rate-limited by transcriptional control of STAR (Son et al., 2011).

Cortisol regulation occurs in the brain and peripheral tissues. CRH and NE stimulate each other’s release at low levels, but inhibit release at higher levels (Tsigos and Chrousos, 2002). In the pituitary, glucocorticoids inhibit ACTH secretion and POMC mRNA synthesis. In the hypothalamus, glucocorticoids regulate synthesis of CRH and AVP mRNAs and direct synthesis of intracellular proteins increasing neuron membrane excitability and ion transport properties (Melmed et al., 2012). The HPA axis is reset and homeostasis is restored by cortisol’s negative feedback on both PVN and the pituitary gland (Nader et al., 2010a). However, SCN neurons do not express glucocorticoid receptors (Nicolaides et al., 2014).
Glucocorticoids are lipid soluble and freely cross the blood-brain barrier. In the brain, the mineralocorticoid receptor, Type 1 (MR), binds aldosterone and glucocorticoids with high affinity; Type 2 (GR), the glucocorticoid receptor, binds only glucocorticoids with high affinity. MRs saturate at basal corticoid concentration levels, mediating basal HPA activity, while GRs saturate only during peak phases of the circadian rhythm or during stress, mediating stress response (Melmed et al., 2012). The steroid-receptor complex interacts with transcription factors AP-1, COUP-TF1, NFkB, and the STATs, and binds regulator sequences in the genome (Chrousos, 2009). Glucocorticoids also exert rapid signaling events in neurons including endocannabinoid-mediated suppression of synaptic excitation via membrane-associated complexes (Melmed et al., 2012). Multiple GR isoforms arising from the single human GR gene enable tissue- and time-differential effects throughout the body (Nicolaides et al., 2014).

In addition to the stress response, cortisol is also secreted in a circadian rhythm. Like melatonin, cortisol’s circadian rhythm is synchronized by the SCN; SCN vasopressinergic output to PVN inhibits CRH output and the medial parvocellular PVN neurons that synthesize CRH (Kalsbeek et al., 2012). While melatonin level rises during darkness in both nocturnal and diurnal animals, cortisol peaks immediately after the sleep period, resulting in opposite cortisol rhythms in nocturnal and diurnal animals (Karatsoreos and McEwen, 2011). SCN activation of the autonomic nervous system via the PVN-IML-sympathetic chain ganglia/splanchnic nerves/supra-renal ganglia series of synapses controls adrenal cortex sensitivity to ACTH and diurnal variation of cortisol synthesis (Engeland and Arnhold, 2005; Nader et al., 2010a). The postganglionic sympathetic neurons modified by the adrenal medulla are also important entrainers of cortisol circadian rhythm (Hardeland et al., 2011). Cortisol circadian rhythm is produced by changes in the amplitude of the ultradian pulsatility of cortisol, due to the secretion of ACTH from the anterior pituitary in brief episodic bursts every 1-2 hours. Increasing amplitudes of ACTH bursts cause a corresponding rapid rise in cortisol, followed by a rapid fall in plasma ACTH and a slower fall in cortisol due to the slower clearance of cortisol from plasma.
Ultradian ACTH pulse amplitude is regulated by feed-forward/feedback between the anterior pituitary and adrenal gland (Kassi and Chrousos, 2013).

Glucocorticoid secretion influences peripheral clocks in liver, kidney, and heart (Balsalobre et al., 2000). In the adrenal medulla, splanchnic nerves from the SCN-autonomic nervous system axis reset the medulla’s local clock. Epinephrine from the adrenal medulla modulates adrenal sensitivity to ACTH. Glucocorticoids secreted by the adrenal cortex reset and phase delay the circadian rhythm CLOCK genes of other peripheral tissues by stimulating the expression of CLOCK-related genes, temporally adjusting the body’s reactivity to stress. Peripheral CLOCK genes also control counter-regulatory feedback against the effect of the SCN central clock on the HPA axis by CLOCK-BMAL1 and glucocorticoid receptor interaction (Nader et al., 2010a). The effects of glucocorticoids are regulated in peripheral tissues by CLOCK-BMAL1. ACTH increases adrenal protein levels of PER1, BMAL1, and STAR; circulating melatonin inhibits the effect of ACTH on the adrenal gland (Campino et al., 2011).

Cortisol circadian rhythm in healthy humans consists of a 50-75% rise immediately upon awakening that peaks 30-45 minutes later, the cortisol awakening response (CAR). Cortisol level then drops back to awakening levels or lower within the next one or two hours, and continues to decline throughout the day, reaching a nadir around normal bedtime. During the night, a slow increase occurs to return to normal awakening levels (Melmed et al., 2012). The CAR is an important characteristic of normal cortisol rhythm, but cortisol levels and rhythm throughout the day and night have a more significant effect on health. If it is not feasible to repeatedly measure cortisol across 24 hours, a measurement of cortisol at bedtime is the best data point to reflect area under the cortisol curve, representative of total cortisol exposure (Golden et al., 2013). Measurement at bedtime and upon waking provides diurnal cortisol slope (Adam and Kumari, 2009). Cortisol slope may not adequately represent cortisol amplitude or rhythm for many reasons, for example, a phase-shifted cortisol circadian rhythm. However
cortisol slope is a physiologically significant measure: flattened cortisol slope is associated with several pathologies (see below) (Zeitzer et al., 2014).

In addition to cortisol’s circadian rhythm and response to stress, cortisol levels are affected by the sleep/wake cycle, light exposure, activity, and exercise (Faraut et al., 2013). Bright light acutely suppresses cortisol (Jung et al., 2010). These factors can disrupt cortisol circadian rhythm; cortisol dysfunction also occurs in many disease states. Excess cortisol’s effects include insulin resistance in peripheral tissues, accumulation of visceral fat, metabolic syndrome, and osteopenia/osteoporosis. Decreased HPA activity occurs in chronic fatigue syndrome, and autoimmune disease e.g. Sjogren’s syndrome and fibromyalgia (Kassi and Chrousos, 2013). Circadian rhythm disruption induced in lab conditions lowers total cortisol (Wright et al., 2015); long-term night shift work is also associated with lower cortisol awakening response (CAR) and overall cortisol (Lac and Chamoux, 2004; Kudielka et al., 2007). When a series of night shifts is started, cortisol gradually phase shifts, reaching an individual’s norm for subsequent night shifts after approximately 5 days; rhythmic amplitude is smaller compared to day shift (Griefahn and Robens, 2010). Short-term sleep deprivation temporarily raises cortisol due to the absence of sleep permitting cortisol pulsatility, since sleep decreases cortisol pulse amplitudes and levels (Wright et al., 2015). Chronic stress has varying effects on cortisol, and can cause dysregulation of the HPA axis (Wolfram et al., 2013). Dysregulation of cortisol rhythm may be the key to the deleterious health effects of disrupted cortisol. Flatter cortisol slopes are associated with reduced survival from breast cancer, increased risk of cardiovascular disease mortality, aging, and pathologies including chronic and acute psychosocial stress, cancer (breast, ovarian, lung), type 2 diabetes, coronary artery disease, fibromyalgia, depression, early-life abuse, and disrupted sleep (Adam and Kumari, 2009; Zeitzer et al., 2014).
1.8 Effects of working a single night shift

Shift work is defined as any work shift including hours outside 9 am to 5 pm, and/or days other than Monday through Friday (Vyas et al., 2012). In this study, night shifts always included several hours of work after midnight, typically 7 pm to 7 am.

The first shift in a series of night shifts is when any circadian adaptation begins, but without special intervention such as bright light exposure, most nurses’ circadian rhythms will not continue to adapt to a night schedule even if they work multiple consecutive night shifts (Smith and Eastman, 2012). Instead, the nurses’ circadian rhythms will remain misaligned during their night shifts. A study of nearly 2000 Norwegian nurses found more than one-third suffered from shift work disorder (SWD) (Flo et al., 2012). Still, many nurses handle circadian misalignment and sleep deprivation well, and consider themselves adapted to their rotating shift schedule (Burch et al., 2009). For a few people working only night shifts, melatonin and cortisol rhythms can reverse completely to a night schedule, becoming fully entrained (Folkard, 2008). However, with melatonin and cortisol rhythms even partially adapted to their night-awake schedule, night shift workers will sleep better and perform better at work, than if their melatonin and cortisol rhythms remain in a day-awake pattern (Crowley et al., 2004).

In the current study, instead of measuring melatonin and cortisol after two or more consecutive night shifts, the measurements took place earlier—after the first night shift that followed a shift schedule rotation. Prior to the night shift, all of the participants had worked day shift schedules for at least a week, including at least one day off from work immediately preceding the night shift used in the current study. Samples collected after a single night shift were compared to samples collected after a single day shift. Day shifts in the current study were also preceded by at least a week on day shift schedule, that could also include days off from work. Since even small shifts in melatonin and cortisol levels that occur after just one night shift may affect cognitive performance and alertness (Crowley et al., 2004), and since published literature does not address this time point in relation to schedule adaptation (described in the
next section), a single night shift following a shift schedule rotation was chosen for the shift condition of interest in the current study.

This thesis research was conducted as a substudy within a larger primary study of nurses’ sleep and activity. The primary study had started before the substudy design was finalized, and sleep diaries recorded by the first ten nurses in the primary study were used to design the substudy. On their day(s) off before a schedule rotation to night shift, all ten nurses slept on a day-activity/night-sleep schedule, although one nurse delayed going to sleep by three hours on the night before working the night shift. Six of the ten nurses also napped during the day before the first night shift. By the time they finished working the night shift, according to their sleep diaries, most of the nurses were sleep deprived by at least 12 hours, and all had experienced disrupted sleep schedules.

Since most nurses return to sleeping at night and staying awake for most of the day as soon as they have time off from night shifts, adaptation to shift schedule rotation in the night-to-day direction is less of a health problem than the day-to-night direction (Lee et al., 2006). By the time most nurses start a series of day shifts, their circadian rhythms and levels of melatonin and cortisol will already have started to re-align with a normal day-awake schedule during their time off (James et al., 2007).

1.9 Adaptation

In most circadian and shiftwork studies, adaptation refers to the degree to which a subject's circadian physiology has shifted to align with a new environment. Melatonin, cortisol, and other measures are assessed as adaptation surrogates/markers, although the participant being assessed may also be asked about many aspects of their own perception of adaptation including energy, fatigue, and mood (Postnova et al., 2013). In the current study, melatonin and cortisol are also considered as variables with their own potential effects on self-rated schedule adaptation; adaptation is included as the third measure, or result.
In the current study, adaptation scores were determined using a previously published Likert scale question from Gamble’s study of nurses in rotating shiftwork (Gamble et al., 2011). Participants in the current study completed questionnaires every time that they collected a saliva sample. Questionnaires are shown in Appendices A and B. The adaptation question included in the current study’s questionnaires is also shown in Figure 1 below.

![Schedule adaptation question](image)

**Figure 1. Schedule adaptation question, modified from open access article (Gamble et al., 2011).**

Every time a participant collected a saliva sample, they also answered this question as part of the questionnaire (shown in Appendices A and B) that accompanied each saliva sample.

In the current study, participants rated their adaptation to their shift schedule after working a single night shift, and after working a day shift. The night shift was always preceded by a schedule rotation, including at least a week on a day shift schedule and at least a day off from work before the night shift. In the current study, all of the nurses were expected to maintain a nighttime sleep schedule during days off, as well as while working day shifts. This expectation was based on several studies of nurses and other shift workers’ sleep habits (Gamble et al., 2011; Smith and Eastman, 2012), and on the sleep diaries from the first ten nurses in the
primary study under which the current study was conducted as a substudy as described in the previous section, 1.8 above. All of the nurses in the current study were currently working a rotating shift schedule that included both night shifts and day shifts. Therefore, after each series of night shifts, as soon as the nurses returned to a nighttime sleep schedule during their days off and subsequent day shifts, they immediately began to (re)adapt to a day-awake schedule. Shift workers’ circadian rhythms typically return to normal (day-awake) timing, within 2 days off from work, or 5 days of working day shifts (Niu et al., 2015). Since all of the day shifts worked in the current study were preceded by at least a week of day shifts that could include days off from work, by the time the nurses worked the day shift that was used in the current study, they were expected to have already adapted to their day-awake schedule. In the current study, it was expected that the responses to the adaptation question after working a day shift would provide a baseline of relatively good adaptation, since the nurses were expected to have already adapted to their day shift schedule before the adaptation question was asked. When the adaptation question was also asked after the nurses had worked a single night shift in the current study, responses were expected to reflect each nurse’s adaptation (or lack of adaptation) to their first night of their new night shift schedule. As discussed in sections 2.12 and 0 below, three of the thirteen participants in the current study unexpectedly rated their shift schedule adaptation as better for the night shift, and worse for the day shift. This unanticipated result was taken into account as described in section 2.12 below.

1.10 Purpose of Study

The purpose of this study was to assess changes in melatonin and cortisol levels and rhythms after a first night shift, and to investigate if there is a difference in melatonin/cortisol of nurses who adapt well to a shift schedule rotation compared to nurses who have difficulty adapting. A melatonin/cortisol-adaptation relationship would provide insight about how schedule
adaptation occurs physiologically, perhaps suggestive of interventions to improve adaptation to a shift schedule rotation.

The current study also assessed melatonin and cortisol levels and perceived adaptation at an earlier time point than in any existing published study: after a single night shift preceded by a schedule rotation.

1.11 Research design

The current study—this thesis—was conducted as a substudy within a larger primary study of nurses’ physical activity and health that included diaries of sleep/wake hours. Before the current substudy design was finalized, 10 nurses in the primary study had already completed sleep diaries for a day-to-night shift schedule rotation. These 10 nurses were from the same population that was recruited for the current study, so the sleep habits of the 10 nurses were taken into consideration when designing the current study. Consistent with published studies (Gamble et al., 2011), these nurses reverted to a day wake/night sleep schedule on days off. On their days off before a schedule rotation to night shift, all ten nurses still slept on a day-activity/night-sleep schedule. Six of the nurses also napped during the day before the first night shift. Therefore, the current study was designed to comprise a daytime work shift plus the nighttime sleep period that followed the day shift, to be compared to a nighttime work shift plus the daytime sleep period that followed that night shift. Saliva samples would be collected and questionnaires answered, before and after both the nighttime and the daytime sleep periods by the same individuals, as within-subjects repeated measures. A total of four saliva samples would be collected by each participant at the pre-sleep and awakening time points for each shift condition (day/night), to be analyzed for melatonin and cortisol concentrations. The night shift would be preceded by a schedule rotation including at least a week on a day shift schedule and at least a day off from work before the night shift; the day shift would be preceded by at least a week on a day shift schedule, that could include days off from
work. In other words, two saliva samples were self-collected by nurses after a day shift, and two samples were collected after working just one night shift. To assess melatonin and cortisol rhythm, saliva samples were collected just before sleeping and immediately after waking. These collection times were selected because in healthy humans in lab conditions, melatonin peaks closer to the end of nighttime sleep, and a lower value before sleep and a higher value after waking would reflect this normal rhythm (Burgess and Fogg, 2008). Cortisol normally peaks soon after awakening and a lower value is expected before sleep (Kudielka et al., 2012).

Together, the pre-sleep and awakening samples represented the circadian rhythms of both melatonin and cortisol. There is also greater variation in melatonin and cortisol levels between individuals compared to variation across repeated measurements from the same individual (Burgess and Fogg, 2008; Griefahn and Robens, 2010). Therefore, in addition to the pair of samples collected after working the night shift, each nurse also collected a pair of samples before sleep and at awakening after a day shift for intra-individual comparisons.

1.12 Research questions

Melatonin levels are lower and pre-sleep/awakening values differ less—lower melatonin rhythm amplitude—when measured after multiple consecutive night shifts (Grundy et al., 2011; Dijk et al., 2012). After multiple night shifts, cortisol has a lower diurnal slope (Mirick et al., 2013). I predicted that the melatonin and cortisol effects that have been documented after multiple night shifts would already be measurable after just one night shift. I also predicted that while all individuals’ melatonin and cortisol levels would be affected by rotating to a night shift schedule, that individuals who perceived they were well adapted to the schedule rotation would show significantly less detrimental changes in melatonin and cortisol levels and rhythms. As an outcome or result to be assessed in relation to the melatonin and cortisol data, adaptation was measured using a modified version of a previously published question (Gamble et al., 2011)
about sleep problems and feeling tired or energetic; see section 2.2 below (Gamble et al., 2011).

If the predicted effects were found, it would suggest a relationship between melatonin and cortisol levels and/or rhythms with perceived adaptation to a shift schedule rotation at an early time point following a shift schedule rotation. This would provide insight into the physiological basis of adaptation to a rotating shift schedule and have implications about the health effects of a rotating shift schedule, particularly of interest at this earlier time point in a series of night shifts than has previously been published.

1.13 Predictions

1. Melatonin and cortisol levels will differ significantly between day and night shifts for most of the nurses.

2. Melatonin and cortisol levels and rhythms will be significantly less detrimentally changed from day to night shift conditions in saliva samples from individuals who perceive they are well adapted to the night shift schedule rotation.
Chapter 2. Methods

2.1 Research design

To capture saliva samples representative of melatonin and cortisol circadian rhythms, requirements were established for collection times. After working either a day or a night shift, nurses collected the pre-sleep sample before their intended longest sleep session—not before a nap. The night shift was preceded by at least a week on a day shift schedule, including at least a day off from work immediately before the night shift; the day shift was preceded by at least a week on a day shift schedule that could include days off from work. The awakening sample was collected immediately on waking up, if the nurse was going to get up and stay awake, but not if it was only a brief awakening followed by going back to sleep. It was emphasized that since cortisol begins to rise very quickly as soon as an individual wakes up, that the awakening sample must be collected immediately upon awakening, before moving around out of bed. Each sample’s collection date and time were recorded on a corresponding questionnaire.

2.2 Questionnaires

Questionnaires are shown in Appendices A and B. Each questionnaire corresponded to a particular saliva sample, and the nurses were directed to complete each questionnaire soon after they collected each saliva sample. Questions addressed consumption of melatonin supplements, certain foods and drinks, and medications that directly affect melatonin and cortisol levels. For example, 2 hours after eating a banana, a 2-fold increase in urinary melatonin occurs in healthy humans (Johns et al., 2013). Ramelteon (Rozerem) (Erman et al., 2006), hydrocortisone (skin cream, pill, eye drops, nasal spray) (Ekman et al., 2012), NSAID non-steroidal anti-inflammatory drugs (Murphy et al., 1996), steroids/anti-inflammatory
medication (Coutinho and Chapman, 2011), beta-blockers (Potter et al., 1984), hormone treatment / oral contraceptives (Davis et al., 2012), and antidepressants (Carvalho et al., 2009) were included in the questionnaires. The questionnaires also asked about stress and perceived adaptation to schedule rotation, and if applicable, date of first day of last menstrual period (Baker and Driver, 2007).

2.3 Population

Any nurse at the University Hospitals Case Medical Center in Cleveland whose shift rotation schedule included both day and night shifts was eligible to participate, apart from the ineligibility criteria listed below in section 2.4. Nurses were recruited to the primary study that included the current study as a substudy, using posters and emails. Recruitment of nurses into only the primary study (not the current study) was maintained for several months, and then nurses who enrolled into the primary study were also invited to participate in the substudy. For each individual, substudy participation was conducted during the same two-week period as that individual's participation in the primary study.

2.4 Participant selection, consent process, and financial information

Participants were eligible for inclusion if participating in the primary study, therefore, each participant was healthy and had both day and night shifts preceded by days off, within the two-week study period.

Participants were excluded if they had any condition incompatible with collection of saliva samples (bleeding/infections inside mouth or unable to hold small swab under tongue for 3 minutes). They were also excluded if they were not willing to answer questionnaires about intake of foods, drinks, supplements and medications that affect cortisol and melatonin levels: alcohol, caffeine, chocolate, banana/orange/pineapple fruit or juice, melatonin supplements, ramelteon (Rozerem), hydrocortisone (skin cream, pill, eye drops, nasal spray), NSAID non-
steroidal anti-inflammatory drugs, steroid/anti-inflammatory medication, hormone treatment / oral contraceptive, beta-blockers, and antidepressants. Participants were also excluded if they were not willing to answer yes/no questions on the questionnaires asking if they had an unusually stressful event in the last 24 hours, (if applicable) the date of start of last menstrual period, and how adapted to their shift schedule did they feel at the time of sample collection.

All participants who met the criteria were invited to participate in the substudy and all who were invited chose to participate. All participants provided four saliva samples and four questionnaires. Due to the limited number of recruits to the main study during the time in which the substudy was conducted, there were only 13 participants: women (n=10), men (n=3).

The primary study and substudy were approved by the Institutional Review Boards of both University Hospitals and Kent State University, including the informed consent documents. Before signing, all participants reviewed the informed consent document with a member of study staff, and asked any questions.

When a nurse consented to participate in the primary study, the nurse was then asked if they were interested in participating in the substudy. If so, the nurse was asked screening questions to determine eligibility per the above listed inclusion/exclusion criteria. If the nurse was eligible, they reviewed the informed consent form for the substudy in detail, including the check box where the nurse indicated if they did/did not want to have their melatonin and cortisol results mailed to them, and any questions were answered. If the nurse wished to participate, he or she signed the substudy consent form and proceeded to scheduling collection times and training on collecting the saliva samples.

There was no cost to the participant for participation in this study. Participants received $25 for completing all four saliva samples and their questionnaires per protocols. A check was mailed to participants from Kent State University within four weeks after participation was completed. To receive payment participants agreed to complete a Kent State Research Participant Receipt-2 Form to provide payment to research participants providing an address.
and social security number to the accounting department because the payment may qualify as taxable income by the IRS.

2.5 Confidentiality and results dissemination

Confidentiality was maintained using non-identifying participant ID numbers and locked storage of the participant name/ID number list, kept in a locked cabinet in a locked room at University Hospitals Wearn 102. Only the Primary Investigator and research staff had access to this information. All personal information was stored separately from de-identified research data in a locked cabinet.

Saliva samples and questionnaires were labeled only with the de-identified study ID numbers; therefore all data collected in the melatonin and cortisol substudy were de-identified. For nurses who requested to receive their melatonin and cortisol results, Dr. Anthony re-identified each nurse’s data using the locked study ID numbers / name list, and printed out a mailer to each individual nurse who requested their results. Electronic data were stored on a password-protected server at Kent State University and on a flash drive stored in a locked file in the lab of Dr. Colleen Novak.

Information in this substudy was collected for research purposes and may be presented or published, however names or other identifying information may not be used in any publication or presentation. Participants were informed that substudy findings would be written up to meet requirements for the Master’s Thesis for Lydia Heemstra.

2.6 Data collection: saliva samples

Saliva was collected using kits containing Salimetrics Oral Swabs, stored in Salimetrics Swab Storage Tubes. Participants were given detailed instructions, shown in Appendices A and B. Questionnaires to be completed for each sample included questions to confirm that collection protocols were followed. Participants were instructed to rinse their mouth with water at least 10
minutes before collecting the before-sleep sample, and to consume nothing else until the sample was collected. Participants were instructed that the wake-up sample must be collected immediately after waking without pre-rinsing, because cortisol and melatonin change rapidly after awakening. Because of the immediate onset of the cortisol awakening response and melatonin suppression by light, it was emphasized repeatedly to participants that the wake-up sample must be collected as soon as the nurse wakes up and turns on any lights.

Collecting a sample occurred in about 5 minutes: the swab package was opened, the swab was transferred directly from the package into the mouth, the swab was held under the tongue for at least 3 minutes, then the swab was spit directly into its storage tube, and the storage tube was sealed with a screw cap. Both sleep and wake samples were refrigerated in the nurse’s home refrigerator. Saliva samples of melatonin (Romsing et al., 2006) and cortisol (Aardal and Holm, 1995) are stable for at least 24 hours at room temperature. Home refrigeration of the samples is used to reduce bacterial growth, although bacterial growth during the first 24 hours after collection does not affect melatonin or cortisol levels (Whembolua et al., 2006).

Samples were only to be refrigerated in the nurse’s home refrigerator, before being brought to UH for pick-up by the research team. Saliva from healthy human subjects is not a biohazard classified material, it is an “exempt human specimen” (EHS, 2012). Saliva samples were hand carried in packaging that met Case Western Reserve University requirements for Exempt Human Specimens. Primary, secondary, and outer packaging comprised a plastic vial with water-tight cap and an absorbent paper towel, inside a biohazard-labeled zipper lock bag, inside an adhesive-strip sealed kraft paper envelope (EHS, 2012).

When a nurse consented to participate in the substudy, pick-up and drop-off times for the kits were arranged according to each nurse’s shift schedule. The kit for the first two samples was provided (along with items related to the main study) during the first visit. Each nurse was emailed or texted per their preference, before they were scheduled to collect their saliva
samples. The kit containing the first two samples was picked up during the work shift that immediately followed the scheduled collection, and the nurse was given the kit for their third and fourth samples’ collection. Each nurse was reminded again before scheduled collection of his or her third and fourth samples, and their kit was picked up during the next work shift.

Faculty oversight and student thesis on substudy: saliva samples for melatonin and cortisol levels were analyzed under the supervision of Dr. Colleen Novak, Co-Investigator of the primary study. Dr. Novak’s laboratory at Kent State University where assays were performed is a biological sciences laboratory and not part of a healthcare institution, therefore it is not subject to CLIA, JACHO, TJC, or Ohio Department of Health regulations that govern human testing for assessment or treatment of disease or health.

The saliva testing in the substudy was for research purposes only, and not for any treatment or assessment of disease or health. Assays were performed according to strict research policy requirements of Kent State University Biological Sciences department following U.S. National Lab Standard Operating Procedures, however since KSU is not a healthcare institution, results from the assays are not valid for any healthcare purpose. Since participants in the already ongoing primary study had said they would like to know their results if they participated in the substudy, results were provided as a product of their participation, but not for any diagnostic/healthcare purpose.

The informed consent form for the substudy included clear language stating that results from the tests are not provided for any assessment/treatment of health/disease, because the assays were performed in a biology research lab rather than a clinical testing/healthcare lab. The consent form also stated that since the tests were not collected nor analyzed in a certified clinical testing setting, it was expected that some errors may occur, and an error result would have no reflection on a participant’s actual melatonin or cortisol levels.

There was potential for error at several stages of the protocol, for example, contamination of a sample due to a participant not rinsing their mouth well enough, or with blood
from micro tears from recent tooth brushing. “Too low” levels for melatonin and cortisol could not occur with the assays used in the substudy, because a value of zero or “too low to detect” is within the normal range for these assays, for some individuals at some points in their circadian cycle (Salimetrics, 2012). This is also true for commercial laboratory saliva tests supported by health insurers for melatonin and cortisol. Participants with levels that assayed as higher than physiological normal were reported a result of “error” for that sample; for statistical analysis, excessively high results were imputed to normal published high concentrations as described in 2.10 below.

Participants were advised in the consent form and in their sample collection instructions that some error results were expected due to uncontrolled factors, e.g. home collection, and that an “error” result is not cause for any concern and has no connection to a participant’s actual melatonin or cortisol level. Likewise, no interpretation of results was provided. Individual melatonin and cortisol results were mailed to participants who chose this option. Included in the mailing were the ranges for the tests, as performed in Dr. Novak’s laboratory with this study’s participants.

In Dr. Novak’s laboratory, saliva samples were handled per CDC Standard Precautions that define all body fluids and substances as infectious—hand washing, and appropriate personal protective equipment such as gloves, gowns, masks, were used (OSHA, 2013). Saliva used in the assays was destroyed chemically, and as promised in the consent documents any remaining saliva was disposed per Kent State University Biological Sciences policy that complies with or exceeds CDC and OSHA regulations: household bleach was added, the bleach solution was diluted at least 1:10, then disposed into the waste water system.

### 2.7 Study Instruments

The questionnaires that were completed with each saliva sample are shown in Appendices A and B. A modified version of a previously published question about sleep
problems and feeling tired or energetic was used for individual self-rating of adaptation on a 1-10 Likert scale (Gamble et al., 2011). Additional questions asked about factors that could affect the saliva results, e.g. intake of certain medications.

2.8 **Saliva tests’ validity**

Measures of melatonin and cortisol in saliva are well validated (Aardal and Holm, 1995; Voultsios et al., 1997). The assays used are well represented in the literature: the Alpco Buhlmann Enzyme Linked Immunosorbent Assay (ELISA) (Middleton, 2006) and the Salimetrics Cortisol Enzyme Immuno Assay (EIA) (Gozansky et al., 2005).

External validity for each EIA was established by the manufacturer. Quality Control Certificates included with each assay kit showed that the included standards fell within specified ranges of absorbance units or optical density (OD), and that control samples’ calculated results fell within a specified range of concentrations (in pg/ml for melatonin and ng/ml for cortisol). The manufacturers also provided reliability data for the EIA’s by publishing test results for sensitivity, specificity/cross-reactivity, intra-assay precision, inter-assay precision, recovery of a pre-tested standard, and linearity of series of diluted samples. The reliability tests were shown for one EIA and specified to be representative for all EIA’s of that catalog number.

Internal validity for each EIA was established in this lab for every assay by confirming that all standards fell within the OD ranges specified, and that all control samples fell within the specified range of concentrations (in pg/ml for melatonin and ng/ml for cortisol) listed on the Quality Control Certificate provided with each assay kit.

2.9 **Data analysis: laboratory procedures**

Saliva samples were analyzed using the Alpco Buhlmann Melatonin Enzyme Linked Immunosorbent Assay (ELISA) EK-DSM (Alpco, Salem, NH) (Middleton, 2006) and the Salimetrics Expanded Range High Sensitivity Cortisol Enzyme Immuno Assay (EIA) 1-3002
Samples were stored at \(-80^\circ\)C for fewer than 6 months. On the day of their assay, samples were thawed and collection tubes were centrifuged for 10 minutes at 1500 x g. The collection swabs were discarded according to protocol and each saliva sample was aliquotted for use in the melatonin and cortisol assays.

The melatonin ELISA required pretreatment of the sample with included kit reagents. Melatonin and cortisol samples were assayed in duplicate with standards including high and low controls assayed simultaneously. Analyte concentrations were quantified by colorimetric absorbance analysis at 450 nm on a Biotek Synergy 2 Multi-Mode Reader using BioTek Gen5 Reader Control and Data Analysis Software. Standard curves were graphed in SigmaPlot and sample concentrations calculated per kit instructions. Intra-assay mean coefficients of variance were 13.28% and 16.48% for cortisol and melatonin, respectively.

2.10 Statistical analysis—melatonin and cortisol outliers and imputation

Each EIA assay included standards and control samples, provided by the manufacturer. Results for all standards and controls for higher concentration values were within manufacturer-specified ranges. Samples were assayed in duplicate and if a sample’s coefficient of variation exceeded 20%, another aliquot of that sample was assayed in the next EIA, although initial sample volumes were not sufficient to permit repetition of every sample. The duplicate with the lowest coefficient of variation was used in analysis.

Two participants used melatonin supplements. All participants noted if they took any melatonin supplement within 24 hours before collecting a saliva sample. If a participant did take a melatonin supplement within 24 hours before collecting a saliva sample, they noted the time it was consumed and the dosage. Melatonin-supplemented samples’ melatonin levels greatly exceeded physiological normal maximum values; there also appeared to be significant carry-over effect of melatonin supplementation beyond 24 hours (Braam et al., 2009) (see Appendix D). Since SPSS repeated measures ANOVA drops entire cases that are missing any values, all
Two melatonin samples from participants who did not take melatonin supplements, and three cortisol samples were identified that had higher concentrations than published ranges for normal, healthy adults. Participants in the current study noted in the questionnaires shown in Appendices A and B if they consumed any medications, foods, or beverages that affect melatonin or cortisol levels; none of the supraphysiological results were attributable to these sources. Other possible causes for excessively high results are discussed in section 4.5 below. All study participants were self-reported to be healthy and had their vital signs and blood tests including C reactive protein checked within 2 weeks of the data collection, as required by the primary study that included the substudy that comprises this thesis. Therefore, unsupplemented but excessively high melatonin and cortisol results were imputed to the maximum values from published ranges for healthy adults: 84.0 pg/ml for melatonin (Burgess and Fogg, 2008) and 22.0 ng/ml for cortisol (Alpco, 2012). Imputed, non-supplemented melatonin and cortisol results were used in all analyses below; the original pre-imputation values for the high melatonin and cortisol levels are shown in Appendices D and E. No melatonin results from the two participants who took melatonin supplements were used in statistical analyses, but the melatonin concentrations of these two participants’ saliva samples are also shown as separate series in Figure 5.

### 2.11 Statistical analysis—analysis of variance (ANOVA)

IBM SPSS Statistics 22 was used for two-way repeated measures ANOVA to analyze dependent variables melatonin, cortisol, and adaptation measured at two time points (pre-sleep
and awakening) after both shift conditions (day/night), controlling for age and sex. Two-tailed paired t-test was used to compare melatonin amplitudes for day shift vs. night shift.

2.12 Congruence among adaptation, melatonin, and cortisol

This analysis was designed to assess the hypothesis that individuals who rated themselves as well adapted to the night shift rotation schedule would also have significantly less disrupted melatonin and cortisol levels and rhythms. The analysis is diagrammed in Figure 2 below and was performed as follows.

For all participants as a group: First, preferred-shift means for all measures (adaptation melatonin, and cortisol) were calculated using results from all participants, substituting imputed values for supraphysiological results, and omitting the melatonin-supplemented results, as described later in this section.

For each participant individually: Second, each participant’s night shift results were compared only to that same individual’s day shift results, for each measure. The change from the day result to the night result for each measure was assessed in relation to known healthy rhythms and levels for that measure, as described in this section below. Third, the changes in each participant’s results from day shift to night shift for each measure were quantified according to specific equations described below. Fourth, using the assessment from step 2, and the significance from step 3, the change from day to night in each measure for each participant was described, for adaptation, as “better/worse/unchanged,” and for melatonin and cortisol, as “improved/disrupted/unchanged.” Fifth, congruence was assessed by comparing the step 4 result for each measure for each participant with that participant’s step 4 result for each of the other two measures. For example, if that participant’s night shift adaptation was “worse,” than that participant’s day shift adaptation and that participant’s night shift melatonin was “improved,” compared to that participant’s day shift melatonin, then that participant’s adaptation and melatonin are congruent.
For all participants as a group: Sixth, congruence was calculated as a percentage of participants whose results were congruent for adaptation/melatonin, adaptation/cortisol, and melatonin/cortisol.
Figure 2. Congruence analysis flowchart.
For the overall congruence analysis, group means for pre-sleep and awakening melatonin and cortisol were calculated for the thirteen participants, using the values from each individual’s preferred shift condition, shown in Figure 9 below. Preferred shift was determined using the mean of the pre-sleep and awakening adaptation scores for day shift, compared to the mean of the pre-sleep and awakening adaptation scores for night shift. As shown in Figure 1 above, adaptation score refers to the participant’s answer to the question “At the time you took this sample, how well adapted to your current shift schedule did you feel?” (Gamble et al., 2011). Participants circled a number from 1-10 with 1 being the worst adapted and 10 being the best adapted. The questionnaire listed examples for scores of 1, 5, and 10 as “1 = not well at all; I tended to feel tired all the time, cannot enjoy my days off, and my sleep cycles never seemed to be regulated. 5 = Middle of the road; I was okay with working this shift, but I still felt tired on my first day off, and my sleep patterns varied at times. 10 = Very well; I really enjoyed this shift, had no trouble getting my energy back on my first days off, and slept just as well when working as I did when not working."

Since higher adaptation scores correspond to feeling better, the preferred shift for each participant was the shift where that individual had a higher mean for pre-sleep and awakening adaptation scores. For the ten participants whose day shift adaptation scores were higher than their night shift adaptation scores, day shift results for melatonin and cortisol were used to calculate the preferred-shift means for the group. For the three participants whose adaptation scores were higher after a night shift and lower after a day shift, their night shift melatonin and cortisol levels were used in calculation of the preferred-shift means. Supraphysiological melatonin and cortisol results were changed to imputed high normal values as described in section 2.10 above. Supplemented melatonin results were omitted from the preferred-shift mean calculations.

Separate plots with triple vertical axes were graphed for each participant, to visualize adaptation, melatonin, and cortisol for each individual for comparing night shift to day shift,
shown in Figure 9 below. For each of the three measures, each individual’s results after a night shift were compared to that individual’s results after a day shift as follows.

For adaptation, a higher score corresponds to better adaptation. Since the 1-10 scale was illustrated by Gamble et al. (2011) to comprise three zones as shown in Figure 3, in the current study, mean adaptation scores for each shift that changed to, or across, the cutoff values of 4 and 7 were considered significantly different in the current study.

In the current study, for melatonin, the means of the pre-sleep and awakening melatonin levels were calculated for each participant for each shift. A reduction in mean was assessed as “disrupted,” because overall daily melatonin suppression is associated with cancer and obesity (Fonken and Nelson, 2014). In Kennaway’s (Kennaway et al., 1999) literature review of studies comparing total 24-hour excretion of melatonin metabolite 6-sulfatoxymelatonin (aMT6s) among age cohorts, a 32% decrease in aMT6s was the difference between the 20-35 years old and the 50-65 years old cohorts (Kennaway et al., 1999). In studies comparing peak plasma melatonin level among age cohorts, a 36% decrease in peak plasma melatonin level corresponds to the difference between the 20-35 years old and the 50-65 years old cohorts (Kennaway et al., 1999) in (Pandi-Perumal et al., 2005)). Therefore in the current study, a between shifts difference in the means of pre-sleep and awakening melatonin of 36% or more was considered significant.
Figure 3 is reproduced only to show the three adaptation zones—the day and night adaptation means in Figure 3 are from the 2011 Gamble study and not from the current study. The three adaptation zones are:

- Adaptation score < 4 = dark gray = “not well”
- 4 < Adaptation score < 7 = light gray = “middle-of-the-road”
- 7 < Adaptation score < 10 = white = “very well”

In the current study, the question (modified from Gamble et al., 2011 and shown in Figure 1 above) was asked with each sample collection, “At the time you took this sample, how well adapted to your current shift schedule did you feel?” participants circled a number from 1-10. 1 represented the worst adaptation and 10 represented the best adaptation. Example descriptions were included with the question used in the current study, unmodified from Gamble 2011, for scores of 1, 5, and 10. “1 = not well at all; I tended to feel tired all the time, cannot enjoy my days off, and my sleep cycles never seemed to be regulated. 5 = Middle of the road; I was okay with working this shift, but I still felt tired on my first day off, and my sleep patterns varied at times. 10 = Very well; I really enjoyed this shift, had no trouble getting my energy back on my
first days off, and slept just as well when working as I did when not working,” in supplementary file S1, page 2, question #4, (Gamble et al., 2011), also shown in Appendix C below.

For cortisol in the current study, two criteria were used to assess changes from day to night shift: diurnal slope and pre-sleep level. Cortisol slope is usually described as negative, because the first sample is collected on awakening (normally high) with the second sample collected pre-sleep, or 12 hours post-awakening (normally low) (Kraemer et al., 2006). In the current study, the pre-sleep sample (normally low) was collected before the awakening sample (normally high), so the healthy slope for diurnal cortisol is positive. Flatter cortisol slopes (corresponding to less positive slopes in the current study) are associated with reduced survival from breast cancer, increased risk of cardiovascular disease mortality, and pathologies including chronic and acute psychosocial stress, cancer (breast, ovarian, lung), type 2 diabetes, coronary artery disease, fibromyalgia, depression, early-life abuse, and disrupted sleep (Adam and Kumari, 2009; Hackett et al., 2014; Zeitzer et al., 2014). In a study comparing diurnal cortisol of healthy adults aged 18-22 and 65-88 years old, the slope of the older adults was 47% flatter than the younger adults’ (Heaney et al., 2012), so in the current study an intra-individual difference of 47% was considered significant. In the current study, cortisol slope was the primary criterion used to assess change in cortisol as improved/disrupted/unchanged. However, if the change in cortisol slope was less than 47% from day shift to night shift, the slope was assessed as unchanged. In the case of an unchanged slope, the second cortisol criterion—pre-sleep level—was examined, as follows.

Pre-sleep cortisol level is the second criterion assessed to designate cortisol change from day to night shift as improved/disrupted/unchanged. In healthy humans, restful sleep can only occur when there is a lower cortisol level at the beginning of the sleep period, relative to the rise that occurs shortly before awakening (Niu et al., 2011). Immediately after awakening, cortisol level rises further by 50-75%, in the cortisol awakening response that contributes to
alertness. While the awakening surge is necessary, it is the cortisol level throughout the rest of the waking period and the sleep period that has the greatest effect on health, and during those periods healthy cortisol levels are significantly lower than the awakening concentration, as described in section 1.7 above. Excess cortisol specifically before sleep may reflect impaired negative feedback sensitivity of brain mineralocorticoid receptors, keeping the HPA axis active for too long (Kumari et al., 2011). In a study comparing 20-25 year olds and 47-88 year olds, the older cohort had a pre-sleep cortisol level that was 40% higher than the younger cohort (Ceccato et al., 2015). In the current study, an intra-individual difference of greater than 40% in pre-sleep cortisol level was considered significant.

For all participants as a group: congruence among all three measures was assessed for each participant individually. The number of participants with congruence in each pair of measures was counted and is shown as a percentage in Table 4 below.
Chapter 3. Results

3.1 Demographics and time period of study

Thirteen nurses were enrolled into the study; demographics are shown in Table 1. Each subject provided all four saliva samples and completed a questionnaire regarding effectors of melatonin and cortisol (e.g. medications) with every sample.

Table 1. Participant demographics, Mean (SEM)

<table>
<thead>
<tr>
<th>Gender (n)</th>
<th>Age in years</th>
<th>Range (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (10)</td>
<td>28.9 (1.9)</td>
<td>23 – 41</td>
</tr>
<tr>
<td>Male (3)</td>
<td>32.0 (6.6)</td>
<td>24 – 45</td>
</tr>
</tbody>
</table>

Melatonin and cortisol levels are affected by season (Claustrat et al., 2005; Persson et al., 2008). While all samples were collected within a four-month period in spring/summer (4/24/14 - 8/9/14), seasonal effects were not controlled for in statistical analyses.

3.2 Melatonin and cortisol saliva concentration results

As described in section 2.10 above, two of the participants consumed melatonin supplements within 24 hours of collecting at least one of their saliva samples. Melatonin results from the two participants who used supplements were omitted from all analyses below; they are shown as separate series in Appendix D. Apart from the melatonin-supplemented samples, two melatonin samples and three cortisol samples were measured to have higher analyte concentrations than published ranges for normal, healthy adults. These supraphysiological values were imputed to the maximum values in published ranges for healthy adults: 84.0 pg/ml
for melatonin (Burgess and Fogg, 2008) and 22.0 ng/ml for cortisol (Alpco, 2012). Possible causes for excessively high results are discussed in section 4.5 below. Imputed concentrations are included in means, SEM, and ranges shown in Table 2.

Table 2. Melatonin and cortisol saliva concentrations: means, SEM, and ranges including 2 imputed maximums for melatonin and 3 imputed maximums for cortisol.¹

<table>
<thead>
<tr>
<th>Units (n*)</th>
<th>Shift</th>
<th>Collection Time</th>
<th>Mean (SEM)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin, pg/ml (11)</td>
<td>Day</td>
<td>Pre-sleep</td>
<td>21.16 (6.84)</td>
<td>0.81 – 71.71</td>
</tr>
<tr>
<td>Melatonin, pg/ml (11)</td>
<td>Awakening</td>
<td>38.23 (7.23)</td>
<td>9.41 – 84.00*</td>
<td></td>
</tr>
<tr>
<td>Melatonin, pg/ml (11)</td>
<td>Night</td>
<td>Pre-sleep</td>
<td>6.02 (1.22)</td>
<td>1.39 – 13.44</td>
</tr>
<tr>
<td>Melatonin, pg/ml (11)</td>
<td>Night</td>
<td>Awakening</td>
<td>19.58 (9.24)</td>
<td>2.09 – 84.00*</td>
</tr>
<tr>
<td>Melatonin, pg/ml (11)</td>
<td>Day</td>
<td>Pre-sleep</td>
<td>4.67 (1.08)</td>
<td>0.86 – 14.62</td>
</tr>
<tr>
<td>Melatonin, pg/ml (11)</td>
<td>Day</td>
<td>Awakening</td>
<td>11.88 (1.83)</td>
<td>3.03 – 22.00*</td>
</tr>
<tr>
<td>Cortisol, ng/ml (13)</td>
<td>Night</td>
<td>Pre-sleep</td>
<td>7.71 (1.84)</td>
<td>0.87 – 19.35</td>
</tr>
<tr>
<td>Cortisol, ng/ml (13)</td>
<td>Night</td>
<td>Awakening</td>
<td>9.48 (2.21)</td>
<td>1.04 – 22.00*</td>
</tr>
</tbody>
</table>

¹ Melatonin concentration results from samples collected by participants who used melatonin supplements were omitted from calculation of melatonin means, SEM, and ranges.

* Abnormally high results were imputed to published normal maximums for two melatonin and three cortisol results: 84.00 pg/ml for melatonin and 22.00 ng/ml for cortisol.

3.3 Adaptation results

Adaptation scores were self-rated at each of the four times that saliva was collected, using a modified version of a previously published questionnaire instrument (Gamble et al., 2011). As shown in Figure 1 above, adaptation score refers to the participant’s answer to the question “At the time you took this sample, how well adapted to your current shift schedule did you feel?” (Gamble et al., 2011). Participants circled a number from 1-10 with 1 being the worst adapted and 10 being the best adapted. The questionnaire listed examples for scores of 1, 5,
and 10 as “1 = not well at all; I tended to feel tired all the time, cannot enjoy my days off, and my sleep cycles never seemed to be regulated. 5 = Middle of the road; I was okay with working this shift, but I still felt tired on my first day off, and my sleep patterns varied at times. 10 = Very well; I really enjoyed this shift, had no trouble getting my energy back on my first days off, and slept just as well when working as I did when not working.” The same adaptation question was asked at all four times that the saliva samples were collected; answers are shown in Table 3.

<table>
<thead>
<tr>
<th>n</th>
<th>Shift</th>
<th>Collection Time</th>
<th>Mean (SEM)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Day</td>
<td>Pre-sleep</td>
<td>5.5 (0.7)</td>
<td>2 – 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Awakening</td>
<td>5.7 (0.7)</td>
<td>2 – 9</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>Pre-sleep</td>
<td>4.0 (0.5)</td>
<td>1 – 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Awakening</td>
<td>3.5 (0.4)</td>
<td>1 – 6</td>
</tr>
</tbody>
</table>

Table 3. Adaptation scores

1 Adaptation Scale 1-10: 1 = not adapted, 10 = well adapted.

3.4 Melatonin, cortisol, and adaptation ANOVA

For statistical analysis, melatonin concentration values were normalized with natural log transformation and two high values were imputed as described in section 3.2 above. Cortisol concentrations and adaptation scores were not transformed due to their normal variance of residuals; three cortisol values were imputed as described in section 3.2 above. Using imputed maximums, there were no outliers as assessed by examination of studentized residuals for values greater than 3 or less than -3 (Field, 2013). Two-way repeated measures ANOVA was used to analyze dependent variables melatonin, cortisol, and adaptation measured at two time points (pre-sleep/awakening) after both shift conditions (day/night), controlling for age and sex.
3.5 Interaction of shift (day/night) x time (pre-sleep/awakening) for cortisol

After accounting for variance due to age and sex, a two-way interaction was found between shift and time for cortisol, $p = 0.029$, but not for melatonin or adaptation. This interaction had been predicted for both melatonin and cortisol. The interaction in cortisol revealed that there was significant difference between the change in concentrations from pre-sleep to awakening after a day shift, compared to the change from pre-sleep to awakening after a night shift (see Figure 4).

![Figure 4. Interaction shift x time in saliva cortisol concentration (ng/ml); mean, SEM.](image)

** There was a significant interaction of shift x time for cortisol: after a day shift, the concentration change from pre-sleep to awakening differed significantly from the night shift concentration change from pre-sleep to awakening, $p = 0.029$.

* There was a significant effect of time of collection for cortisol after a day shift, $p = 0.044$.
3.6 Simple main effect of shift on melatonin

Simple main effects were examined for shift and time by repeated measures ANOVA, using Bonferroni adjustment for multiple comparisons (Field, 2013). There was a main effect of shift for melatonin on awakening, $p = 0.018$, shown in Figure 5 below. A significant main effect of shift had been predicted for both melatonin and cortisol, but was found only for melatonin.

![Figure 5. Saliva melatonin concentration (pg/ml) means and SEM](image)

* Melatonin was higher on awakening than before sleep, after a day shift, $p = 0.032$.

** Melatonin on awakening was higher after a day shift than after a night shift, $p = 0.018$.

1 Melatonin supplement users’ melatonin levels were excluded from means and SEM; imputed maximum values (see section 3.2 above) were included in means and SEM. The pre-imputation (original) supraphysiological values and the supplement users’ melatonin levels are shown in Appendix D.
Melatonin was also predicted to show a smaller difference between sleep/wake concentrations after a night shift, when compared with day shift sleep/wake concentration differences—i.e., a blunted melatonin rhythm amplitude, shown in Figure 6 below. However, no difference was found by two-tailed paired-samples t-test of day shift vs. night shift melatonin amplitudes.

![Figure 6. Amplitudes of saliva melatonin mean levels (pg/ml).](image)

1 Means include imputed maximums and exclude melatonin supplement users’ data.

- $D =$ amplitude of day shift rhythm $= 17.07$ pg/ml.
- $N =$ amplitude of night shift rhythm $= 13.56$ pg/ml

3.7 Simple main effect of shift on adaptation

In addition to melatonin, there was also a main effect of shift on perceived adaptation at awakening, $p = 0.019$, shown in Figure 7 below. This effect of shift was predicted.
It was noted that for three of the 13 participants, adaptation scores were higher after a night shift for both pre-sleep and on awakening, compared to both collection times after a day shift, shown in Figure 8. For the other 10 participants, adaptation means for day were higher than for night, or the same for both shifts.

* Adaptation on awakening was better after a day shift than after a night shift, $p = 0.019$. 

Figure 7. Adaptation Means (scale 1 = not adapted; 10 = well adapted), SEM.
Participants #336, 825, and 779 reported feeling better adapted to their shift schedule after a night shift compared to after a day shift.

3.8 Simple main effect of time on melatonin and cortisol

There was a simple main effect of time for melatonin after a day shift, $p = 0.032$, shown in Figure 5 above. There was also a simple main effect of time for cortisol after a day shift, $p = 0.044$, shown in Figure 4 above. These effects were predicted for both melatonin and cortisol.

3.9 Congruence of changes among melatonin, cortisol, and adaptation

As described in section 2.12 above, each participant’s preferred shift was determined from that participant’s mean adaptation scores for pre-sleep and awakening for each shift: the shift with the higher adaptation score mean was designated as the preferred shift for that individual. Means for adaptation scores, and for melatonin and cortisol saliva levels, were
calculated for the group of thirteen participants, during each participant’s preferred shift. Means for all three measures were also calculated for each participant's non-preferred shift. The group means for all three measures for the preferred shift and non-preferred shift are shown in Figure 9 below.

Separate plots of each individual’s adaptation, melatonin, and cortisol results are shown in Figure 10 and Figure 11 below. Calculations were used to determine significance of differences for each measure, as described in section 2.12 above and as shown in an example assessment diagrammed in Figure 12 below. Each participant’s adaptation, melatonin, and cortisol results after a night shift were compared to that individual’s results after a day shift. That individual’s night shift results for adaptation were designated as “better/worse/unchanged” and their melatonin and cortisol results were designated as “improved/disrupted/unchanged” compared to day shift.
Figure 9. Triple vertical axes: melatonin (pg/ml), cortisol (ng/ml), and adaptation (scale 1-10) means for all participants, for preferred* and non-preferred* shifts.

* Each participant's preferred shift was assessed by comparing the mean of pre-sleep and awakening adaptation scores after a day shift vs. the mean for both scores after a night shift; the shift with the higher adaptation score mean was designated as the preferred shift for that individual, as described in section 2.12 above.
Figure 10. Individual plots for participants #160-764 with triple axes:
adaptation (scale 1-10), melatonin (pg/ml), and cortisol (mg/ml).
<table>
<thead>
<tr>
<th>Participant</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>#779</td>
<td>Better: day mean (2) &lt; 4 = night mean (4)</td>
</tr>
<tr>
<td></td>
<td>M Improved: day mean (9.7) + 364% (&gt; 36%) = night mean (45.0)</td>
</tr>
<tr>
<td></td>
<td>C Improved: day slope (-56°) + 218% (&gt; 47%) = night slope (65°)</td>
</tr>
<tr>
<td>#798</td>
<td>Worse: day mean (5) &gt; 4 &gt; night mean (3)</td>
</tr>
<tr>
<td></td>
<td>M Disrupted: day mean (48.8) - 95% (&lt; - 36%) = night mean (2.4)</td>
</tr>
<tr>
<td></td>
<td>C Disrupted: day slope (74°) - 3% (&gt; - 47%) = night slope (72°)</td>
</tr>
<tr>
<td></td>
<td>C Disrupted: day pre-sleep (0.9) + 353% (&gt; 40%) = night pre-sleep (3.9)</td>
</tr>
<tr>
<td>#809</td>
<td>Worse: day mean (8) &gt; 7 &gt; night mean (5)</td>
</tr>
<tr>
<td></td>
<td>M N/A (supplemented night shift awakening sample not used)</td>
</tr>
<tr>
<td></td>
<td>C Disrupted: day slope (70°) - 157% (&lt; - 47%) = night slope (-40°)</td>
</tr>
<tr>
<td>#817</td>
<td>Worse: day mean (8) &gt; 7 &gt; night mean (5)</td>
</tr>
<tr>
<td></td>
<td>M Disrupted: day mean (14.5) - 66% (&lt; - 36%) = night mean (4.9)</td>
</tr>
<tr>
<td></td>
<td>C Disrupted: day slope (27°) - 318% (&lt; - 47%) = night slope (-59°)</td>
</tr>
<tr>
<td>#825</td>
<td>Better: day mean (2) &lt; 4 = night mean (4)</td>
</tr>
<tr>
<td></td>
<td>M Improved: day mean (25.7) + 68% (&gt; 36%) = night mean (43.2)</td>
</tr>
<tr>
<td></td>
<td>C Improved: day slope (-11°) + 310% (&gt; 47%) = night slope (24°)</td>
</tr>
<tr>
<td>#849</td>
<td>Worse: day mean (8) &gt; 7 &gt; night mean (5.5)</td>
</tr>
<tr>
<td></td>
<td>M Disrupted: day mean (35.9) - 89% (&lt; - 36%) = night mean (3.8)</td>
</tr>
<tr>
<td></td>
<td>C Disrupted: day slope (-25°) - 114% (&lt; - 47%) = night slope (-3°)</td>
</tr>
</tbody>
</table>

Figure 11. Individual plots for participants #779-849 with triple axes: adaptation (scale 1-10), melatonin (pg/ml), and cortisol (mg/ml).
Figure 12. Example of Congruence Assessment.
Frequencies and percentages of congruence of changes from day to night for melatonin/adaptation, cortisol/adaptation, and melatonin/cortisol are shown in Table 4 below. Omitting the 2 of 13 participants who took melatonin supplements (“N/A”), 100% of 11 individual melatonin changes from day to night were congruent with those 11 individuals’ adaptation changes from day to night. Cortisol was 85% congruent with adaptation for all 13 participants, with two participants’ adaptation and cortisol not congruent (15%). Comparing melatonin and cortisol for the 11 participants whose melatonin was unsupplemented, there was 91% congruence between melatonin and cortisol, with one participant’s melatonin and cortisol not congruent (9%). High congruence among all three measures was predicted.

Table 4. Congruence of changes from day/night for adaptation/melatonin, adaptation/cortisol, and melatonin/cortisol.

<table>
<thead>
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Chapter 4. Discussion

4.1 Introduction

The results of this study show for the first time that there is significant disruption of melatonin and cortisol that appears to be associated with self-rated adaptation to a shift change, after a person works a single night shift. Working fewer consecutive night shifts or working single night shifts does not fully protect a worker from the health risks of shift work. Nurses prefer to work more than one night shift in a row because the first night shift is perceived to be more difficult than subsequent night shifts in a series (Hakola and Kalliomäki-Levanto, 2010). This study shows significant disruption of melatonin and cortisol that corresponds to the perceived difficulty of a single night shift.

Disruption of melatonin or cortisol results in primary circadian rhythm disruption due to entrainment of the SCN from both melatonin and cortisol feedback (Hardeland et al., 2011) and melatonin and cortisol entrainment of peripheral cell clocks (Mavroudis et al., 2012). Circadian disruption has detrimental effects on multiple physiological systems. The interaction of melatonin and cortisol with the SCN, peripheral clocks, and epigenetics leading to these disease states is complex. Below, the most significant pathogenic roles of the specific changes in melatonin and cortisol that were found in this study are addressed.

4.2 Melatonin

Melatonin level was lower on awakening after a night shift than after a day shift. Before discussing the pathogenicity of this change, it is important to note that while the day shift melatonin levels recorded in this study were normal, the timing of those levels is opposite to the classical normal melatonin rhythm assessed under dim light or constant routine conditions. In
this study, melatonin levels measured after a day shift were low before sleep, and high on awakening. In dim light (as maintained in a sleep lab), melatonin level normally rises before sleep and falls before awakening. This study’s melatonin timing represents normal melatonin rhythm for most humans outside of a sleep lab—with normal room light on until they go to sleep, followed by sleeping in darkness. The low melatonin level pre-sleep is due to the suppression of melatonin by normal room light by an average of 71% (Gooley et al., 2011). The high melatonin level on awakening is due to two factors. First, the release from light suppression of melatonin allows the melatonin level to rise as soon as the individual is in darkness. Second, from the start of the rise of melatonin in darkness, if a healthy person sleeps for 8 hours, their melatonin level remains high normally for 7-8 hours (Gooley et al., 2011). The participants in this study slept an average of 6-6.5 hours after a day shift. They did not sleep long enough to reach the 7 hour point when the normal drop in melatonin level would have occurred in some individuals, while for others, melatonin would have remained high for 8 hours. This study’s mean day shift melatonin results for the thirteen participants were normal for healthy individuals in both level and rhythm.

The night shift melatonin pattern for the current study was an unchanged (from day shift) pre-sleep melatonin level and a significantly suppressed awakening melatonin level (19.58 pg/ml compared to day shift awakening mean = 38.23 pg/ml). The lower awakening melatonin level after a night shift may be attributed to several causes. First, participants were behaving in direct opposition to their own endogenous circadian rhythm while remaining awake during the night shift, and then when sleeping during the day after the night shift ended. Since circadian rhythm phase shifting occurs only after multiple night shifts are worked, and only in some individuals, none of the participants would have significantly phase shifted their circadian rhythm during the course of a single night shift. While melatonin levels of the awakening samples collected after a night of sleep following a day shift were high, the melatonin levels of the awakening samples collected after sleep during the day following a night shift were low. Low
melatonin during the late afternoon collection time of these samples is consistent with the participants’ endogenous circadian rhythms not shifting, or only slightly shifting, after working one night shift. A second possible cause of low melatonin levels for awakening samples after a night shift is the suppression of melatonin by light exposure during sleep. If the participants sleeping during the day did not sleep in a dark room, light suppression of melatonin during sleep may have kept their melatonin levels low; in a study of five participants sleeping in normal room light, melatonin suppression ranged from 29 - 93% (Gooley et al., 2011).

Another contributor to the low awakening melatonin levels after a night shift is sleep deprivation. It is expected that the nurses were sleep deprived, based on the sleep diaries recorded by the first 10 nurses in the primary study preceding this substudy, and typical sleep behavior by nurses as described in section 1.5 above. Nurses on a rotating shift schedule do not fully compensate in advance for the resulting sleep deprivation of working a first night shift, although some nurses take naps. However, all of the nurses in our study and in the 2011 Gamble study (Gamble et al., 2011) slept during the night before a first night shift, then remained awake through most of the day before work and through the night shift itself, incurring a sleep debt of several hours. Melatonin levels are suppressed during the day after a night of sleep deprivation (Faraut et al., 2012).

Overall suppression of melatonin during the sleep period is acutely detrimental. As described in section 1.6 above, melatonin is found in most tissues and organs and in addition to binding its receptors, it moves through the cell membrane into the cytosol, nucleus, and mitochondria (Venegas et al., 2012). Melatonin and its metabolic derivatives are potent antioxidants against reactive oxygen and nitrogen species, directly scavenging radicals and radical products. Melatonin and its metabolites also induce expression of antioxidant enzymes, reduce activation of pro-oxidant enzymes, and maintain mitochondrial homeostasis, preventing mitochondrial cardiolipin oxidation and facilitating global DNA methylation (Paradies et al., 2010; Zhang and Zhang, 2014; Haim and Zubidat, 2015). Melatonin modulates the cell cycle and
induction of apoptosis, inhibiting telomerase activity and facilitating cellular aging-suppressor protein, silent information regulator 1 (SIRT1) (Mediavilla et al., 2010a; Hardeland, 2013). Overall suppression of melatonin results in suppression of the cellular, Th1 type immune response, and increased circulating interferon-gamma (Cutolo et al., 2006). Melatonin suppression also down-regulates GLUT4 glucose transporters in adipose and muscle tissue, reducing melatonin modulation of glucose uptake in human adipocytes via MT2 receptors (Cipolla-Neto et al., 2014). Lowered awakening melatonin as experienced by the participants in the current study after working a night shift and then sleeping is also acutely detrimental, because the correctly-timed increase in melatonin is a fundamental chronobiotic for central (direct SCN melatonin receptor feedback) and peripheral (control of transcription/translation of peripheral clock genes) circadian rhythms (Archer et al., 2014).

4.3 Cortisol

The combination of the shift by time interaction and the simple main effect of time after a day shift for cortisol revealed that cortisol level was higher before sleep and lower on awakening after a single night shift compared to after a day shift. The simple main effect of time showed that cortisol levels and rhythms were normal after the day shift: low before sleep and high on awakening. After the night shift, cortisol levels and rhythm were disturbed: the pre-sleep cortisol levels were higher and the awakening cortisol levels were lower, resulting in a flattened cortisol slope after the night shift. Flattening of diurnal cortisol slope is known to occur in extended periods of night shift work (Mirick et al., 2013) and is associated with cancer (breast, ovarian, lung) and reduced survival from breast cancer, coronary artery disease and increased risk of cardiovascular disease mortality, type 2 diabetes, fibromyalgia, chronic and acute psychosocial stress, depression, and disrupted sleep (Adam and Kumari, 2009; Hackett et al., 2014; Zeitzer et al., 2014). In a study comparing diurnal cortisol of healthy adults aged 18-22 and 65-88 years old, the slope of the older adults was 47% flatter than the younger adults’ (Heaney et al., 2012).
In the current study, the increased pre-sleep night shift cortisol levels not only contributed to the flattening of the night shift cortisol slopes, but they are also independently significant due to the shift by time interaction and in terms of their health effects. In a study comparing 20-25 year olds and 47-88 year olds, the older cohort had a pre-sleep cortisol level that was 40% higher than the younger cohort (Ceccato et al., 2015). Other studies have also shown higher pre-sleep cortisol levels associated with aging (Ferrari et al., 2001). Flattened diurnal cortisol slope due to high evening cortisol—associated with aging, but also occurring in many of the disease states listed above—is attributed to impaired negative-feedback control of the HPA axis (Bjorntorp, 2001).

In the current study, the participants’ night shift pre-sleep cortisol levels are only high relative to their healthy, lower, day shift pre-sleep levels. However, the inappropriate timing of the relatively high pre-sleep cortisol triggers detrimental mechanisms. Additionally, the sensitivity of glucocorticoid target tissues varies in a circadian rhythm due to CLOCK-mediated acetylation of glucocorticoid receptors, making the effects of inappropriately high evening cortisol more pathogenic (Nicolaides et al., 2014). High evening cortisol increases glucose at the same time as it decreases insulin secretion and sensitivity (Hackett et al., 2014), and the mistiming of this high cortisol causes oxidative stress and endothelial cell damage even in healthy subjects with normal glucose tolerance (Watanabe et al., 2011). Increased reactive oxygen species and activated complement trigger many detrimental pathways (polyol, hexosamine), increase membrane attack complexes, and activate transcription effectors protein kinase C and NFκβ, mechanisms of organ damage complications of diabetes (Ghosh et al., 2015). Mediated by glucocorticoid receptors, increased cortisol also increases activation of lipoprotein lipase, causing lipid accumulation in adipocytes. Intra-abdominal, visceral fat has the greatest density of GR, potentiating cortisol’s fat accumulating effects in this location, contributing to increased abdominal fat, a component of metabolic syndrome (Bjorntorp, 2001). Increased pre-sleep cortisol also extends the daytime anti-inflammatory humoral Th2-type
cytokine profile with high interleukin (IL)-10, into the sleep period, inhibiting expression of pro-
inflammatory cytokines IL-6, IL-1, and TNF-α necessary for immune function during sleep. This
reversed immune profile is a mechanism that triggers pathways that cause adverse cardiac
events and increased mortality in coronary bypass patients (Cutolo et al., 2006; Ronaldson et
al., 2015). In the current study, flattened cortisol rhythm after a night shift is also due to lower
awakening cortisol levels. Too low cortisol at the wrong circadian time correspondingly releases
inhibition of expression of IL-6, IL-1, TNF-α, and C-reactive protein—but when upregulated
during waking hours, these cytokines cause fatigue and sickness behavior (Sephton and
Spiegel, 2003; Irwin, 2011; Ronaldson et al., 2015). Glucocorticoid feedback is also necessary
for correct function of PER2 protein effects in the SCN and target organs, affecting other
circadian rhythms (Zhu et al., 2014). These are only the acute effects of the cortisol rhythm
disruption seen after a single night shift in the current study, mechanisms known to occur in
healthy individuals that are also the first steps in the pathogenesis of the disease and health
risks listed in this section as associated with the cortisol dysfunctions seen in the current study.

4.4 Adaptation

There was a main effect of shift on perceived adaptation at awakening. In other words,
the only time participants felt significantly better adapted to their shift schedule was on
awakening after a day shift, compared to awakening after a night shift, when they felt
significantly worse adapted to their shift schedule. The lack of a main effect of time shows that
participants rated their adaptation fairly similarly before sleep and on awakening, after each shift
type. Since the awakening adaptation score after a day shift was significantly better than the
awakening adaptation score after a night shift, and since the pre-sleep and awakening
adaptation scores for each shift type did not differ significantly, participants generally felt better
adapted to their day shift schedule than to their night shift schedule.
As shown in Figure 7 above, mean adaptation scores before sleep and on awakening after a day shift were only 5.5 and 5.7 respectively: even though participants rated their adaptation to their day shift schedule better than their adaptation to the night shift, they only rated their day shift adaptation as “middle-of-the-road.” Mean adaptation before sleep and on awakening after a night shift were rated as 4.0 and 3.5 respectively: 4.0 is the cut off score between the “middle-of-the-road” level and the “not well” adapted level.

While the ANOVA was calculated on the melatonin, cortisol, and adaptation results classified by shift (day or night), the adaptation scores revealed that the day shift was not the preferred shift for 3 of the 13 participants (at least, at the times that they rated their adaptation—the substudy questionnaire used in the current study asked participants to rate how they felt at specifically the times when the saliva samples were collected, not in general). Three of the participants in the current study rated their adaptation to their shift schedule more highly after a night shift, than after a day shift. However, these three night owls still rated their adaptation to their preferred shift only at a mean score of 4.0, the cut-off score between “middle-of-the-road” and “not well.” All three night owls rated their adaptation to their non-preferred shift, the day shift, at a score of 2.0 for both pre-sleep and on awakening. Recalculating the mean adaptation scores with all preferred-shift adaptation scores together and all non-preferred shift adaptation scores together, the preferred-shift pre-sleep and awakening adaptation score means are both 6.08: still “middle-of-the-road.” The non-preferred shift mean adaptation score before sleep is 3.46 and on awakening is 3.08—both scores correspond to being “not well” adapted.

4.5 Imputation of supra-normal results for melatonin and cortisol

Excessively high analyte concentrations were measured for two of the unsupplemented melatonin samples and three of the cortisol samples. For statistical analyses, these samples were imputed to the highest published normal values for melatonin (Burgess and Fogg, 2008).
and cortisol (Alpco, 2012). Graphs including supraphysiological melatonin and cortisol levels as separate series are shown in Appendices D and E.

Melatonin and cortisol levels can be raised above physiological norms by certain foods, drinks, and medications (Halpern et al., 2012; Johns et al., 2013), so these were listed and quantified in the questionnaires, shown in Appendices A and B. However, none of the participants with excessively high samples had consumed anything that would have increased their results. Therefore the high results were attributed to error that could have occurred during collection or handling by the participant. A participant might not have sufficiently rinsed his or her mouth before collecting the sample, or the saliva swab could have been dropped or otherwise contaminated, perhaps during transfer from the participant’s mouth into the plastic collection tube.

4.6 Congruence among measures’ changes from normal/healthy to disrupted/worse

The research question underlying the current study’s design asked if changes in adaptation after a single night shift compared to after a day shift would correspond to changes in melatonin and cortisol. For example, comparing night to day shift, if an individual’s mean melatonin levels decreased more than 36%—a change associated with aging and disease (Pandi-Perumal et al., 2005)—did that individual’s perceived adaptation also drop significantly, as predicted? Comparing day to night shift, if an individual’s diurnal cortisol slope flattened by more than 47%—an unhealthy change (Heaney et al., 2012)—did that individual’s perceived adaptation also drop significantly, as predicted? Since it was not possible to address this question using correlation analysis due to the low number of participants, separate graphs were plotted for each individual’s results for visual examination (see Figure 10 and Figure 11 above).

As described in section 2.12 above, preferred-shift means for pre-sleep and awakening melatonin and cortisol were calculated for the group of thirteen participants, using the values from each individual’s preferred shift condition (each individual’s shift with higher-rated
adaptation scores). Due to normal inter-individual variation in melatonin and cortisol measurements, each participant’s night shift melatonin and cortisol results were compared only to that individual’s day shift values (Smyth et al., 1997; Burgess and Fogg, 2008). Each participant’s night shift melatonin and cortisol levels and rhythms were assessed as improved/disrupted/unchanged in comparison to the day shift measures for that individual, and significance of any change was calculated. Adaptation was likewise assessed as better/worse/unchanged, with significance of any change calculated, as described in section 2.12 above. Individual plots with triple vertical axes were graphed for each participant, to visualize possible correspondence in improvement or deterioration among the three measures for each individual when comparing night shift to day shift, shown in Figure 10 and Figure 11 above).

There was a high rate of congruence of individual cortisol and melatonin day to night changes with individual adaptation day to night changes—85% for cortisol/adaptation and 100% for melatonin/adaptation. Melatonin/cortisol congruence was 91%. In other words, if a participant’s night shift melatonin was significantly suppressed, their night shift adaptation was significantly worse, for 100% of the current study’s participants. If a participant’s night shift diurnal cortisol slope was significantly flattened, their night shift adaptation was significantly worse, for 85% of participants. Suppressed melatonin and flattened cortisol were seen together in 91% of participants. This suggests that self-rated shift schedule adaptation scores may be affected by melatonin and cortisol, with worse adaptation scores occurring along with disrupted physiological measures. As seen in the congruence of measures for the three night owls, better adaptation may likewise occur along with improved melatonin and cortisol levels and rhythms. The high congruence of individual melatonin and cortisol changes with each other is consistent with these hormones’ reciprocal effects on each other.

There are several possible interpretations of the high degree of congruence among participants’ melatonin, cortisol, and adaptation. A person whose hormone levels and rhythms
are healthier is likely to also feel better adapted to their current shift schedule (important due to adaptation’s association with work performance, i.e. patient safety). A person who is better adapted to their shift schedule may improve their melatonin and cortisol levels and rhythms, e.g. by self-entraining effects such as sleeping when necessary and controlling light and dark exposure.

4.7 Limitations

The hypothesis that individuals who perceived they were well adapted to the night shift rotation schedule would show significantly less detrimental changes in melatonin and cortisol levels and rhythms is supported by the literature (Gamble et al., 2011; Boudreau et al., 2013). Due to the small N of this study a statistical analysis of association among the three measures—melatonin, cortisol, and adaptation—was not possible. While visual qualitative analysis of the data appears suggestive of relationships among the measures, this study’s congruence analysis is an exploratory venture rather than a statistical analysis of the data.

4.8 Conclusion

The specific changes in melatonin and cortisol levels and rhythms that occur after a single night shift, as shown in the current study, directly disrupt circadian rhythms throughout the body. The reduction in melatonin directly and acutely inhibits protective cellular and physiological mechanisms, thereby promoting pathways to metabolic disease and inhibiting melatonin’s anti-oxidant and anti-inflammatory effects against cancer, cardiovascular disease, and cellular aging (Mediavilla et al., 2010b; Paradies et al., 2010; Hardeland, 2013; Cipolla-Neto et al., 2014; Zhang and Zhang, 2014; Haim and Zubidat, 2015). The flattening of cortisol slope and the increase in evening cortisol levels directly and acutely impact mechanisms of organ damage in diabetes, inflammatory profiles that lead to adverse cardiac events, and intra-abdominal lipid accumulation, a metabolic syndrome component (Bjorntorp, 2001; Watanabe et
The current study shows that several of the mechanisms that directly increase health risks and diseases associated with long-term shiftwork are already fully engaged after even normal, healthy individuals have worked a single night shift.

As well as affecting shift workers’ health after a single night shift, the changes in melatonin and cortisol demonstrated in the current study appear to also affect or predict individuals’ perceived adaptation to their shift schedule. Since adaptation affects work performance, job satisfaction, and patient safety, it is important to support the health of shift workers so that working one or more night shifts is less disruptive. Several interventions have been shown to improve individuals’ melatonin and cortisol function, for example, appropriate melatonin supplementation, bright light exposure and caffeine upon awakening, timed physical exercise, stress reduction, and maintenance of sleep hygiene (Smith and Eastman, 2012). Beyond individual interventions, medical shift workers, their patients, and people sharing the road with shift workers would benefit if management/administrators of shift workers recognize that working a single night shift is detrimental to health—a single night shift is not safer or easier than a series of several night shifts. Nurses are already known to prefer to work rotations that include several night shifts, rather than a single night shift (Petrovic, 2012), and the current study provides physiological evidence supporting this preference.
REFERENCES


CDC, 2015. Insufficient Sleep Is a Public Health Problem | Features | CDC. Centers for Disease Control and Prevention.


Cross, C.B. SALIVARY HORMONE LEVEL TESTING. Capital Blue Cross.


Gooley, J.J., Chamberlain, K., Smith, K.A., Khalsa, S.B., Rajaratnam, S.M., Van Reen, E.,
Zeitzer, J.M., Czeisler, C.A. and Lockley, S.W., 2011. Exposure to room light before
bedtime suppresses melatonin onset and shortens melatonin duration in humans. J Clin
Endocrinol Metab 96, E463-72.

determined by enzyme immunoassay is preferable to serum total cortisol for assessment

associated with short sleep: Bridging the gap between laboratory and epidemiological
studies. Sleep Medicine Reviews 14, 239-247.

Griefahn, B. and Robens, S., 2010. The normalization of the cortisol awakening response and of
the cortisol shift profile across consecutive night shifts--an experimental study.

of light at night exposure on melatonin levels among Canadian rotating shift nurses.
Cancer Epidemiol Biomarkers Prev 20, 2404-12.

Gu, F., Han, J., Laden, F., Pan, A., Caporaso, N.E., Stampfer, M.J., Kawachi, I., Rexrode, K.M.,
Willett, W.C., Hankinson, S.E., Speizer, F.E. and Schernhammer, E.S., 2015. Total and
W.H., 2013. The Effects of Shift Work on Sleeping Quality, Hypertension and Diabetes in
Retired Workers. Plos One 8, 6.

Cortisol With Type 2 Diabetes in the Whitehall II Study. Journal of Clinical Endocrinology
& Metabolism 99, 4625-4631.


Laboratory, A.L. Melatonin Unsupplemented Female & Male Melatonin Ranges pg/ml. Aeron LifeCycles Laboratory.
Laboratory, A.L. Unsupplemented Salivary Cortisol Ranges for Women and Men. Aeron LifeCycles Laboratory.


Salimetrics, 2012. High Sensitivity SALIVARY CORTISOL ENZYME IMMUNOASSAY KIT.


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Appendices

Appendix A. Pre-sleep instructions/questionnaire for both day and night shifts, page 1.

**How to Collect Your Before-Sleep Saliva Sample**

1. At least 20 minutes before collection...
   
   - Finish eating, drinking, chewing gum, brushing teeth, using mouthwash
   - Wipe off ALL lipstick / lip balm.

2. At least 15 minutes before collection...
   
   - Rinse your mouth with water - until your spit is colorless.

3. Open just one end of the swab pack.

4. Put swab in your mouth without touching swab with your hands.

5. Keep swab **under your tongue** (mouth closed)
   for **AT LEAST 3 MINUTES**...
   
   - Fill in questionnaire on the back of this page while you wait.

   Keep tube in your home refrigerator if possible.

   Put bag and this page inside envelope.
   Keep at room temperature at UH.

**PLEASE TURN THIS PAGE OVER FOR QUESTIONS...**
Appendix A (continued). Pre-sleep instructions/questionnaire for both day and night shifts, page 2.

### BEFORE-SLEEP SAMPLE

Please check the boxes and/or fill in the blanks below.

**Date when you collected this sample:**

**What was the EXACT TIME when you placed the swab into your mouth?**

A.M. □ / P.M. □

**Before this sample, when did you last eat/drink anything except water?**

A.M. □ / P.M. □

**Before this sample, when did you last eat a meal (not a snack)?**

A.M. □ / P.M. □

**In the 24 hours before this sample did you have any...**

- **alcohol drinks? 1 drink = 1 beer, 1 glass of wine, 1 shot, 1 mixed drink, etc.**
  - No □ Yes, I had ___ drink(s)

- **caffeine drinks and/or pills?**
  - 1 drink = 1 cup of coffee/tea, 1 can of energy drink/Cola, etc.
  - No □ Yes, I had ___ drink(s), ___ total mg pill(s)

- **chocolate food or drinks?**
  - 1 serving = 1 candy bar, 1 brownie, 1 piece of cake, 1 cup hot chocolate, etc.
  - No □ Yes, I had ___ servings

- **banana, orange or pineapple?**
  - 1 serving = 1 cup of juice, 1 banana/orange, 1 cup pineapple chunks, etc.
  - No □ Yes, I had ___ servings

**In the 24 hours before this sample, did you take any...**

- **Melatonin?**
  - Yes □ No □
  - If yes, how much did you take? ______ mg.
  - At what time(s)? _______ A.M. □ / P.M. □, _______ A.M. □ / P.M. □

- **Ramelteon (Rozerem)?**
  - Yes □ No □
  - If yes, how much did you take? ______ mg.
  - At what time(s)? _______ A.M. □ / P.M. □, _______ A.M. □ / P.M. □

- **Hydrocortisone (skin cream, pill, eye drops, nasal spray)?**
  - Yes □ No □
  - If yes, how much did you take? ______ mg.
  - At what time(s)? _______ A.M. □ / P.M. □, _______ A.M. □ / P.M. □

- **NSAID non-steroidal anti-inflammatory drug (do NOT include Tylenol/Excedrin)**
  - DO INCLUDE aspirin, ibuprofen, naproxen, Advil, Motrin, Aleve, Celebrex, etc.?
  - Yes □ No □

- **Steroid / anti-inflammatory medication?**
  - Yes □ No □

- **Hormone treatment / oral contraceptive?**
  - Yes □ No □

- **Beta-blockers?**
  - Yes □ No □

- **Antidepressants?**
  - Yes □ No □

**In the 24 hours before this sample, did you experience any unusually stressful events?**

Yes □ No □

**Menstrual period. Are you female?**

Yes □ No □

**If yes, do you have menstrual periods?**

Yes □ No □

If yes, when was the **first** day of your last menstrual period? Date ______

---

**Schedule adaptation.**

(Answer the following question by circling the appropriate number on the line below):

Examples: 1 = Not well at all. I tended to feel tired all the time, cannot enjoy my days off, and my sleep cycles never seemed to be regulated.

5 = Middle-of-the-road. I was okay with working this shift, but I still felt tired on my first day off, and my sleep patterns varied at times.

10 = Very well. I really enjoyed this shift, had no trouble getting my energy back on my first days off, and slept just as well when working as I did when not working.

**At the time you took this sample, how well adapted to your current shift schedule did you feel?**

1 2 3 4 5 6 7 8 9 10
Appendix B. Awakening instructions/questionnaire, for both day and night shifts, page 1.

HOW TO COLLECT YOUR WAKE-UP SALIVA SAMPLE

1. Take this sample IMmediately after you wake up!
   Wipe off any lip balm before taking sample.
   Do NOT rinse with water before taking sample!

2. Open just one end of the swab pack.

3. Put swab in your mouth without touching swab with your hands.

4. Keep swab under your tongue (mouth closed) for AT LEAST 3 MINUTES ...
   The swab must be soaked – if your mouth is too dry you can...
   ...move your jaw as if chewing (but keep swab under your tongue)
   ...think about your favorite foods.

5. Spit swab into tube. Cap tube tightly.
   Keep tube in your home refrigerator if possible.

   Put bag and this page inside envelope.
   Keep at room temperature at UH.

PLEASE TURN THIS PAGE OVER FOR QUESTIONS...
Appendix B (continued). Awakening instructions/questionnaire, for both day and night shifts, page 2.

**Wake-Up Sample**

Please check the boxes or fill in the blanks below.

<table>
<thead>
<tr>
<th>Date when you collected this sample</th>
<th>What was the EXACT TIME when you placed the swab into your mouth?</th>
<th>A.M. □ / P.M. □</th>
</tr>
</thead>
<tbody>
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</table>

**What was the EXACT TIME when you woke up?**

<table>
<thead>
<tr>
<th>A.M. □ / P.M. □</th>
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**Did you eat/drink anything except water after you collected your before-sleep sample?**

Yes □ No □

If yes, when was the last time you ate/drank anything except water?

<table>
<thead>
<tr>
<th>A.M. □ / P.M. □</th>
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</table>

**After you collected your before-sleep sample, did you have any...**

<table>
<thead>
<tr>
<th>Alcohol drinks?</th>
<th>1 drink = 1 beer, 1 glass of wine, 1 shot, 1 mixed drink, etc.</th>
<th>No □ Yes □ I had ___ drink(s), ___ total mg pill(s)</th>
</tr>
</thead>
<tbody>
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</table>

<table>
<thead>
<tr>
<th>Caffeine drinks and/or pills?</th>
<th>1 drink = 1 cup of coffee/tea, 1 can of energy drink/cola, etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No □ Yes □ I had ___ drink(s), ___ total mg pill(s)</td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chocolate food or drinks?</th>
<th>1 serving = 1 candy bar, 1 brownie, 1 piece of cake, 1 cup hot chocolate, etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No □ Yes □ I had ___ servings</td>
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<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Banana, orange or pineapple?</th>
<th>1 serving = 1 cup of juice, 1 banana, 1 orange, 1 cup pineapple chunks, etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No □ Yes □ I had ___ servings</td>
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</tbody>
</table>

**After you collected your before-sleep sample, did you take any...**

<table>
<thead>
<tr>
<th>Melatonin?</th>
<th>Yes □ If yes, how much did you take? ___ mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No □ At what time(s)?</td>
<td>A.M. □ / P.M. □</td>
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</tbody>
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<tr>
<th>Ramelteon (Rozerem)?</th>
<th>Yes □ If yes, how much did you take? ___ mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No □ At what time(s)?</td>
<td>A.M. □ / P.M. □</td>
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| Hydrocortisone (skin cream, ointment, eye drops, nasal spray)? | Yes □ If yes, how much did you take? ___ mg. |
|                                                              | No □                                             |
| No □ At what time(s)? | A.M. □ / P.M. □ |
|                  |                                             |

**NSAID non-steroidal anti-inflammatory drug (do NOT include Tylenol/Excedrin) DO INCLUDE aspirin, ibuprofen, naproxen, Advil, Motrin, Aleve, Celebrex, etc.?**

Yes □ No □

**Steroid/anti-inflammatory medication?**

Yes □ No □

**Hormone treatment/sexual contraceptive?**

Yes □ No □

**Beta-blockers?**

Yes □ No □

**Antidepressants?**

Yes □ No □

**After you collected your before-sleep sample, did you experience any unusually stressful events?**

Yes □ No □

**Schedule adaptation.**

(Answer the following question by circling the appropriate number on the line below):**

Examples: 1 = Not well at all; I tended to feel tired all the time, cannot enjoy my days off, and my sleep cycles never seemed to be regulated.

5 = Middle-of-the-road; I was okay with working this shift, but I still felt tired on my first day off, and my sleep patterns varied at times.

10 = Very well; I really enjoyed this shift, had no trouble getting my energy back on my first days off, and slept just as well when working as I did when not working.

**At the time you took this sample, how well adapted to your current shift schedule did you feel?**

<table>
<thead>
<tr>
<th>Not well</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>Middle-of-the-road</th>
<th>Very well</th>
</tr>
</thead>
<tbody>
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Appendix C. Original adaptation question from Gamble 2011.

Reproduced from open access article, supplementary file S001 (Survey), pg. 2, question #4 (Gamble et al., 2011). This is the original source of the adaptation question that was used in the current study, after modification, as shown in Appendices A and B.

1. Which hours do you currently work?
   - 7am-7pm
   - 7pm-7am
   - 7am-3pm
   - 3pm-11pm
   - 11pm-7am
   - Other “daytime” 12-hour shift (example: 4am-4pm)
   - Other “nighttime” 12-hour shift (example: 4am-12:00pm)
   - Other daytime 8-hour shift (example: 12:00 pm-8:00pm)
   - Other nighttime 8-hour shift (example: 12:00 pm-8:00pm)
   - Other hospital shift hours (please give shift times):
     I do not work in hospital shift hours; I work “normal” clinic/office hours

2. How many months/years have you worked these current hours?
   - 0-11 months
   - 1-2 years
   - 3-5 years
   - 6-10 years
   - more than 10 years

3. Do you routinely alternate the hours you work between daytime and nighttime hours (at least once every two weeks)?
   - yes
   - no

4. On a scale from 1-10, how well do you adapt to your current work hours?
   (Answer by circling the appropriate number on the grid below):
   Examples: 1 = not well at all; I tend to feel tired all the time, cannot enjoy my days off, and my sleep cycles never seem to be regulated
   5 = middle-of-the-road; I'm okay with working this shift, but I still feel tired on my first day off, and my sleep patterns vary at times
   10 = very well; I really enjoy this shift, have no trouble getting my energy back on my first days off, and sleep just as well when working as I do when not working

5. Which hours did you work? (choose only one option; if you’ve worked in more than one position as a shift worker, or more than one type of shift, please refer to your most recent position):
   - 7am-7pm
   - 7pm-7am
   - 7am-3pm
   - 3pm-11pm
   - 11pm-7am
   - Other daytime 12-hour shift (example: 4am-4pm)
   - Other nighttime 12-hour shift (example: 4pm-4am)
   - Other daytime 8-hour shift (example: 4am-12:00pm)
   - Other nighttime 8-hour shift (example: 12:00 pm-8:00pm)
   - Other hospital shift hours (please give shift times):

6. How many months/years did you work these shift hours?
   - 0-11 months
   - 1-2 years
   - 3-5 years
   - 6-10 years
   - more than 10 years
Appendix D. Melatonin (pg/ml) supplemented and pre-imputation saliva levels.

Shown with each ID number as a separate series: saliva melatonin levels for all samples collected by the two participants who used melatonin supplements (ID numbers 336 and 809); original (pre-imputation) saliva melatonin concentrations for all samples collected by the two participants whose samples included the 2 supraphysiological (> 84.0 pg/ml) cortisol levels (ID numbers 414 and 825). Also shown for reference are means that are calculated using the two imputed maximum concentrations and excluding melatonin data from supplement users.
Appendix E. Cortisol (ng/ml) original (pre-imputation) maximum saliva levels.

Shown with each ID number as a separate series: original saliva cortisol concentrations for all samples collected by the two participants whose samples included the three supraphysiological (> 22.0 ng/ml) cortisol levels (ID numbers 414 and 798). Also shown for reference are means and SEM that are calculated using the three imputed maximum concentrations and not the original maximum concentrations.

** Concentration change from pre-sleep to awakening differed significantly from the night shift concentration change from pre-sleep to awakening, $p = 0.029$.

* There was a significant effect of time of collection for cortisol after a day shift, $p = 0.044$. 