MONASH University

Developing biomarkers of alertness: Biological determinants of vulnerability to alertness failure and biomarkers of alertness state

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Abstract

In order to reduce the impact of alertness failure on safety and productivity, markers of enhanced vulnerability to alertness failure and associated performance impairment need to be identified. In addition to being able to determine 'who' may be more vulnerable in the future, it is also necessary to be able to determine 'when' and individual is vulnerable, and for this purpose biomarkers of alertness state are required. This thesis, therefore, aimed to examine a genetic marker of vulnerability to alertness failure and, using a targeted and untargeted approach, identify novel endocrine and metabolic markers of current alertness state.

In the first study, the Brain Derived Neurotrophic Factor (*BDNF*) Val66Met polymorphism is shown to be a genetic marker of vulnerability to executive dysfunction during acute sleep loss. It was found that carriers of the functional polymorphism, those with the Val/Met genotype, made more inhibitory errors on a Stroop task following 20-h of wakefulness, and also took longer to respond when inhibiting the prepotent response, particularly during the biological night. These findings have important implications for individuals who work extended duration shifts, particularly at night.

In the second study, menstrual phase, as previously shown, was a marker of vulnerability to cognitive impairment during sleep deprivation, with women in the follicular phase of the menstrual cycle displaying enhanced vulnerability to alertness mediated performance impairment. Furthermore, it was found that light exposure sufficient to suppress melatonin (i.e. >30% suppression) was an effective countermeasure, such that it improved night time performance in both phases of the menstrual cycle. This effect was greatest, however, for women in the more vulnerable follicular phase. Investigation of the reproductive hormones that change across the menstrual cycle, however, did not lead to the identification of viable biomarkers of the performance differences observed between the two phases of the menstrual cycle. Core body temperature demonstrated the required characteristics of a biomarker of alertness in that it differed between the vulnerable (follicular phase) and more resilient (luteal phase) women, showed a relationship with performance

and was altered by light exposure, but it is not a practical solution for continuous monitoring in non-laboratory settings. Countermeasures designed to manipulate temperature, however, may be effective for reducing the heightened vulnerability for alertness failure in the follicular phase.

In the final study, circadian and wake-dependent control of plasma polar metabolites was demonstrated in individuals across 40-h of acute sleep deprivation for the purpose of identifying metabolite biomarkers of the circadian and homeostatic systems, which are the two primary factors that govern alertness. Both circadian and wake-dependent changes in plasma polar metabolites were observed at the group-level. Analysis of individual participant metabolite profiles, however, showed large inter-individual differences in the timing of rhythmic metabolites and in the magnitude and direction of linear change in wake-dependent metabolites. These findings have implications for the use of metabolomics in future biomarker development research and may also inform our understanding of the etiology of metabolic disease associated with sleep and circadian disruption. Furthermore, these data have informed the future development of biomarkers of alertness state with the identification of 18 candidates for more targeted investigation.

Overall, this thesis has explored different approaches to developing biomarkers of alertness and associated cognitive impairment including potential genetic endocrine and metabolic biomarkers. The *BDNF* Val66Met polymorphism was shown to be a candidate biomarker of enhanced susceptibility to executive dysfunction during sleep loss. Based on their changes throughout the menstrual cycle, which was shown to modulate vulnerability to sleep loss, female reproductive hormones were targeted as biomarker of alertness state, although they did not show significant associations with performance impairment. Untargeted analysis of how polar metabolites change with acute sleep deprivation led to the identification of multiple plasma metabolites that show promise as putative biomarkers of alertness. Identifying biological determinants of vulnerability to alertness failure and biomarkers of alertness state will contribute to the development and application of more valuable alertness management strategies suited to both the environment and the individual.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis.

Signature: 

Print name: Leilah Grant

Date: 10/04/2018

List of publications and presentations during candidacy

Peer reviewed journal articles

Grant, L.K., Cain, S.W., Chang, A-M., Saxena, R., Czeisler, C.A., & Anderson, C. (2018). Impaired cognitive flexibility during sleep deprivation among carriers of the Brain Derived Neurotrophic Factor (*BDNF*) Val66Met allele. *Behavioural Brain Research, 338: p51-55.*

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Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer reviewed journals or submitted/prepared for publication. The core theme of the thesis is to explore different approaches to developing alertness biomarkers including potential genetic, endocrine and metabolic biomarkers. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Sleep and Circadian Medicine Laboratory, Monash Institute of Cognitive and Clinical Neurosciences and School of Psychological Sciences, Monash University under the supervision of Associate Professor Clare Anderson, Professor Steven W. Lockley, and Professor Shantha M.W. Rajaratnam.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. Declarations regarding the contribution of co-authors to published works are provided in each experimental chapter.

In the case of Chapters 3, 4 and 5 my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
Chapter 3	Impaired cognitive flexibility during sleep deprivation among carriers of the Brain Derived Neurotrophic Factor (*BDNF*) Val66Met allele	*Published*	65%. Initiation and formulation of experimental analysis, data analysis and interpretation, and preparation of manuscript for publication	1. Sean W Cain. Consultation and formulation in data analysis design, consultation in data analysis, discussion of ideas expressed in manuscript, and critical review of manuscript: 15% 2. Anne-Marie Chang. Formulation of original experimental design, data collection, and critical review of manuscript: 2% 3. Richa Saxena. Analysis of genetic samples, and critical review of manuscript: 1% 4. Charles A Czeisler. Formulation of original experimental design, and critical review of manuscript: 2% 5. Clare Anderson. Formulation of original experimental design, data collection, consultation and formulation in data analysis design, consultation in data analysis, discussion of ideas expressed in manuscript, and critical review of manuscript: 15%	*No*
Chapter 4	Differences in neurobehavioural performance due to menstrual phase: The role of temperature, progesterone and the	*Submitted*	65%. Initiation and formulation of experimental analysis, data analysis and interpretation,	1. Joshua J Gooley. Formulation of original experimental design, data collection, and critical review of manuscript: 2% 2. Melissa A St Hilaire. Data processing and critical review of the manuscript: 0.5% 3. Shantha M W Rajaratnam. Data collection, and critical review of manuscript: 0.5%	*No*

	progesterone/estradiol ratio		and preparation of manuscript for publication	4. George C Brainard. Formulation of original experimental design and critical review of manuscript: 1% 5. Charles A Czeisler. Formulation of original experimental design and critical review of manuscript: 1% 6. Steven W Lockley. Formulation of original experimental design, data collection, consultation and formulation in data analysis design, discussion of ideas expressed in manuscript, and critical review of manuscript: 15% 7. Shadab A Rahman. Consultation and formulation in data analysis design, consultation in data analysis, discussion of ideas expressed in manuscript, and critical review of manuscript: 15%	
Chapter 5	Circadian and wake-dependent changes in the human plasma polar metabolome during prolonged wakefulness	*Submitted*	80%. Formulation of original metabolomics experimental design, data collection, metabolomics analysis, formulation of experimental analysis, data analysis and interpretation, preparation of manuscript for publication.	1. Suzanne Ftouni. Formulation of original experimental design, data collection, and critical review of manuscript: 2% 2. Brunda Nijagal. Formulation of original metabolomics experimental design, metabolomics analysis, data processing, and critical review of manuscript: 1% 3. David P De Souza. Formulation of original metabolomics experimental design, and critical review of manuscript: 0.5% 4. Dedreia Tull. Formulation of original metabolomics experimental design, and critical review of manuscript: 0.5% 5. Malcolm J. McConville. Formulation of original metabolomics experimental design, and critical review of manuscript: 0.5% 6. Shantha M W Rajaratnam. Formulation of original experimental design, and critical review of manuscript: 0.5% 7. Steven W Lockley. Formulation of original experimental design, consultation and formulation in data analysis design, consultation in data analysis, discussion of ideas expressed in manuscript, and critical review of manuscript: 7.5% 8. Clare Anderson. Formulation of original experimental design, consultation and formulation in data analysis design, consultation in data analysis, discussion of ideas expressed in manuscript, and critical review of manuscript: 7.5%	*No*

Candidate's signature:  **Date:** 10/04/2018

Main supervisor's signature:  **Date:** 11/04/2018

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It is a truth universally acknowledged that a PhD student in possession of no fortune must be in want of a life…

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List of abbreviations

ADA:	Adenosine deaminase
ADORA2A:	Adenosine A2a receptor
aPVT:	Auditory Psychomotor Vigilance Task
AUC:	Area under the curve
BDI:	Beck Depression Index
BDNF:	Brain Derived Neurotrophic Factor
BMI:	Body mass index
CBT:	Core body temperature
COMT:	Catechol-O-Methyltransferase
CR:	Constant routine
CSF:	Cerebrospinal fluid
DASS:	Depression Anxiety Stress Scales
DAT1:	Dopamine transporter 1
DLMO:	Dim light melatonin onset
DSM	Diagnostic and Statistical Manual of Mental Disorders
DSST:	Digit Symbol Substitution Task
ECG:	Electrocardiogram
EDTA:	Ethylenediaminetetraacetate
EEG:	Electroencephalography
ESS:	Epworth Sleepiness Scale
FD:	Forced desynchrony
FSH:	Follicle stimulating hormone
FSS:	Fatigue Severity Scale
GAD-7:	Generalised Anxiety Disorder-7

GC-MS:	Gas chromatography-mass spectrometry
GWAS:	Genome wide association study
HILIC:	Hydrophilic interaction liquid chromatography
ipRGCs:	Intrinsically photosensitive retinal ganglion cells
ISI:	Insomnia Severity Index
KSS:	Karolinska Sleepiness Scale
LC-MS:	Liquid chromatography-mass spectrometry
LH:	Luteinizing hormone
MAP:	Multivariable Apnoea Prediction
MMPI-2:	Minnesota Multiphasic Personality Inventory-2
mRNA:	Messenger ribonucleic acid
MS/MS:	Tandem mass spectrometry
MS:	Mass spectrometry
NMR spectroscopy:	Nuclear magnetic resonance spectroscopy
OC:	Oral contraception
PBQC:	Pooled biological quality control
PER3:	Period 3
PHQ-9:	Patient Health Questionnaire-9
PQC:	Plasma quality control
PSG:	Polysomnography
PSQI:	Pittsburgh Sleep Quality Index
PVT:	Psychomotor Vigilance Task
RCGs:	Retinal ganglion cells
RLS:	Restless legs syndrome
RP:	Reverse phase (chromatography)
SAS:	State Anxiety Scale

SCID-5	Structured Clinical Interview for DSM-V
SCID-R	Structured Clinical Interview for DSM-III-R
SCL-90-R	Symptom Checklist 90-Revised
SCN:	Suprachiasmatic nuclei
SDQ:	Sleep Disorders Questionnaire
SHBG:	Sex hormone binding globulin
SNP:	Single nucleotide polymorphism
SRS:	Sleep regulatory substance
SWA:	Slow wave activity
SWS:	Slow wave sleep
TNFα G3084:	Tumor necrosis factor alpha G3084
WMZ:	Wake maintenance zone

Chapter 1

Introduction and review of the literature

1.1. The cost of inadequate sleep

Four in 10 Australian adults are not getting enough sleep (Sleep Health Foundation, 2017). In the long term, insufficient sleep may result in a wide range of health problems, including increased risk of cardiovascular disease, metabolic disorders, depression, anxiety, cancer, and early mortality (Ayas et al., 2003; Breslau, Roth, Rosenthal, & Andreski, 1996; Cappuccio et al., 2008; Gangwisch et al., 2007; Heslop, Smith, Metcalfe, Macleod, & Hart, 2002; Knutson et al., 2009; Nieto et al., 2012; Vorona et al., 2005; Xu et al., 2010). While long-term health is important, the more immediate consequences of not getting enough sleep have implications for safety and productivity in the workplace. Insufficient sleep compromises core behavioural and cognitive functions, such as the capacity to remain awake and responsive to the environment. This impairment results in an increased risk of errors, accidents and injuries and impaired performance and productivity (Mitler et al., 1988; Rajaratnam & Arendt, 2001).

The cost of inadequate sleep places a considerable burden on the Australian economy, with nearly $20 billion a year spent on lost productivity and healthcare costs, and over $40 billion attributed to the loss of healthy life (Sleep Health Foundation, 2017). The prevalence of inadequate sleep resulting from sleep disorders, insufficient sleep and other sources of excessive daytime sleepiness is particularly high in adults of working age between 20 and 60 years of age, and is a contributing factor to almost 65,000 serious workplace injuries and more than 66,000 serious injuries from road accidents each year in Australia (Sleep Health Foundation, 2017). These figures highlight the importance of managing alertness failure resulting from inadequate sleep, particularly in high-risk, safety sensitive environments in order to reduce the economic and human cost of the sleepiness epidemic.

The risk of a workplace or motor vehicle accident or injury due to alertness failure is principally determined by the fundamental properties that govern alertness (Barger, Lockley, Rajaratnam, & Landrigan, 2009). These include 1) how long an individual has been awake (Borbély, 1982); 2) habitual sleep duration, where recurrent nights of inadequate sleep result in an

accumulation of alertness and performance decrements (Van Dongen, Maislin, Mullington, & Dinges, 2003); 3) the timing of the endogenous circadian clock, which modulates alertness throughout the 24-h day (Dijk, Duffy, & Czeisler, 1992); 4) impairment of alertness due to recently waking, known as sleep inertia (Jewett et al., 1999); and 5) the existence of a sleep disorder (Barger et al., 2015). Often due to a combination of these factors, shift workers are over represented in fatalities caused, in part or wholly, by reduced alertness (Safe Work Australia, 2015). This increased risk in the shift worker population has significant implications for the estimated 1.5 million Australians, or 16% of all workers, who engage in some form of shift work (Australian Bureau of Statistics, 2012).

While the consequences of an alertness failure can be serious, individuals are often not aware of their level of impairment, such that subjective ratings of alertness do not reflect observed changes in performance [e.g. (St Hilaire et al., 2017; Van Dongen et al., 2003)]. Therefore, in order to minimise the adverse consequences of impaired alertness and performance, development of objective measures of impairment, or 'alertness biomarkers', is essential. There are three ways that biomarkers of alertness could be used to protect against accident and injury risk. The first relates to identifying innate biomarkers of enhanced susceptibility which indicates one individual's vulnerability relative to another, the second relates to identifying biomarkers of alertness state which indicate whether an individual is currently impaired, and finally identifying biomarkers that can predict the likelihood of an impending alertness failure, for example at the end of an extended duration shift. Together, knowing 'who' may be more or less vulnerable, and 'when' that vulnerability may be apparent is an important step forward in managing the risk of alertness failure. A biomarker of alertness would need to be sensitive to the properties that govern alertness and must also have operational utility, a clear threshold for impairment and be sensitive to individual differences. Development of biomarkers to measure and predict alertness would inform the development of tools to reduce errors, accidents, injuries, and associated costs, and improve performance and productivity. Development of alertness biomarkers, therefore, is an important aspect of improving workplace safety and productivity, not only in Australia, but worldwide.

Currently, however, the capacity to accurately measure, monitor and predict alertness remains inadequate.

A significant barrier to the development of effective alertness management is a lack of practical and objective measures of alertness state. In laboratory settings, typical measures of alertness include neurobehavioural function (Banks, Van Dongen, Maislin, & Dinges, 2010; Basner & Dinges, 2011; Doran, Van Dongen, & Dinges, 2001; Shekleton et al., 2013; Wyatt, Ritz-De Cecco, Czeisler, & Dijk, 1999), changes in ocular electrophysiology (Anderson, Chang, Sullivan, Ronda, & Czeisler, 2013; Cajochen, Khalsa, Wyatt, Czeisler, & Dijk, 1999; Chua et al., 2014; Ftouni et al., 2013), and neurophysiological signals generated from the brain (Aeschbach et al., 1999; Cajochen, Brunner, Kräuchi, Graw, & Wirz-Justice, 1995; Finelli, Baumann, Borbély, & Achermann, 2000). While these 'biomarkers' are considered gold-standard in laboratory environments, they are limited in their capacity to measure alertness in real-world settings due to large inter-individual differences, substantial offline processing or environmental confounds when used outside of a highly controlled laboratory setting. For example, psychomotor performance, ocular measures of alertness and EEG are used in operational settings but are not optimal given the large inter-individual differences in these measures which may be further exacerbated in field settings. Therefore, there is a need to develop sensitive and accurate biomarkers of alertness that are not subject to such large inter-individual differences, and that are capable of detecting the risk of alertness vulnerability in a general population similar to that applied to blood alcohol concentrations.

Developing biomarkers of alertness is an emerging area of research in sleep and circadian science. While several different approaches have been used to identify putative biomarker candidates, there are currently no established biomarkers that can be used to measure and predict alertness. This thesis will therefore examine a number of previously employed biomarker discovery approaches and, given the range and scope of potentially viable biomarkers, will also examine a variety of possible candidates including genetic, endocrine and metabolic markers.

1.2. Defining alertness

The term alertness has been described with respect to the two-process model of sleep/wake regulation (reviewed in section 1.3). In the two-process model, alertness is thought to be modulated through interactions between pacemaker signals generated from the circadian system (Process C), and signals from the system that tracks duration of wakefulness referred to as the sleep homeostat [Process S; (Borbély, 1982)]. In the two-process model, alertness is thought to exist on a spectrum with sleepiness, where an individual's level of alertness or sleepiness depends on the time of day (circadian mechanism) and the time since waking (homeostatic mechanism).

The view that alertness is related to sleep-wake and circadian regulation can be compared to the construct of tonic alertness proposed by Posner and Rafal (1987), which they define as "how awake the organism is from one time of day to another" (p.183, Posner & Rafal, 1987). Within their model, alertness is described as an aspect of attention and consists of two components—tonic alertness and phasic alertness. Both tonic and phasic alertness are not subject to conscious control, but this basic level of central nervous system activation is thought to be a prerequisite for the more complex aspects of attention, such as focused or divided attention (Kraemer et al., 2000; Sturm et al., 1999). Tonic alertness is defined as general wakefulness or the capacity to respond to the environment at any moment, whereas phasic alertness refers to response readiness or short-term alertness triggered by warning stimuli (Posner & Rafal, 1987; Sturm et al., 1999). Tonic alertness has been shown to be influenced by both time awake and circadian phase (Kraemer et al., 2000; Valdez et al., 2005), suggesting that this aspect of alertness is modulated by both the circadian and homeostatic systems as described in the two-process model. Therefore, the construct of alertness in the current thesis, refers to alertness related to sleep/wake and circadian regulation, or tonic alertness in Posner and Rafal's model.

1.3. Two-process model of sleep regulation

The two-process model describes two opposing processes that modulate alertness or sleep propensity throughout the day— Process S and Process C (Borbély, 1982). Understanding these

two processes and how they interact is critical in the development of biomarkers of alertness. Process S refers to the homeostatic drive for sleep, or sleep propensity. Process S builds from the moment of awakening and dissipates during sleep. Process C, the circadian drive for alertness, opposes the homeostatic sleep pressure as it builds throughout the day, such that alert wakefulness can be maintained even when levels of sleep drive have increased. During the night, the circadian drive for alertness is reduced, particularly in the early morning hours so that a consolidated sleep episode can be achieved and the build-up of sleep pressure from the preceding day can be fully dissipated. While Process S is dependent on prior sleep/wake history, Process C is generated independently of prior sleep and wake by the hypothalamic circadian pacemaker which determines the optimum times for initiation and maintenance of sleep.

The way in which Process S and Process C interact means that the optimal time for sleep initiation and maintenance is during the biological night when the circadian drive for alertness is reduced, and the optimal time for wakefulness is during the day when the drive for alertness is highest. This is evident by shorter sleep latencies and increased total sleep time when sleep is initiated on the descending limb of the circadian drive for alertness (Dijk & Czeisler, 1994, 1995; Zulley, Wever, & Aschoff, 1981). Toward the end of a typical waking day when sleep pressure has accumulated, the circadian alerting signal is highest such that it can counteract the build-up in sleep pressure. The increased drive for alertness during this time, known as the Wake Maintenance Zone (WMZ), makes it difficult to initiate and maintain sleep irrespective of previous time awake (Strogatz, Kronauer, & Czeisler, 1987). The WMZ, which precedes sleep, typically lasts for about ~2-3 hours (Strogatz et al., 1987) prior to the onset of release of the pineal hormone melatonin (Lavie, 1997), discussed in more detail below (section 1.3.2.1.).

1.3.1. Marker of the sleep homeostat

The time course of the homeostatic drive for sleep in the two-process model is based on the dissipation of slow wave activity (SWA) during sleep, as measured by electroencephalography [EEG; (Borbély, 1982)]. Originally however, another candidate slow wave sleep (SWS), was

considered as a marker of the sleep homeostat. While SWS predominates the first part of the sleep period, declines as the sleep period continues (Dement & Kleitman, 1957; Feinberg, 1974; Webb & Agnew, 1971) and is enhanced by sleep deprivation (Åkerstedt & Gillberg, 1979; Kales et al., 1970; Webb & Agnew, 1971), the duration of recovery sleep is not proportional to the amount of sleep lost (Gulevich, Dement, & Johnson, 1966), suggesting that it is the intensity of the sleep, rather than the amount or duration, that regulates sleep drive. The intensity aspect of SWS, SWA, dominates SWS and is defined as EEG activity in the ~0.75-4.5 Hz range (Dijk, Shanahan, Duffy, Ronda, & Czeisler, 1997). During baseline sleep, SWA declines in each successive sleep cycle and is reduced following daytime napping, whereas following sleep deprivation and sleep restriction SWA is enhanced relative to baseline sleep (Borbély, Baumann, Brandeis, Strauch, & Lehmann, 1981; Brunner, Dijk, & Borbely, 1993; Dijk & Beersma, 1989; Werth, Dijk, Achermann, & Borbély, 1996). SWA, therefore, represents a gradual declining process during sleep and its starting point is governed by prior length of wakefulness (Dijk, Brunner, Beersma, & Borbely, 1990; Dijk et al., 1997).

The homeostatic modulation of SWA was confirmed by forced desynchrony (FD) protocols, which decouple, or forcibly desynchronise, the homeostatic and circadian processes by scheduling individuals to sleep/wake cycles outside the limits of entrainment of the human circadian pacemaker (e.g. 28-h day with 9.33-h sleep). FD protocols allow for measurement of sleep episodes that are distributed across all circadian phases without altering the duration of prior wakefulness (Dijk & Czeisler, 1994). These FD experiments showed that while many aspects of sleep timing, structure and continuity are influenced by both the circadian and homeostatic systems, SWA is more strongly regulated by the homeostatic process (Dijk & Czeisler, 1995; Dijk et al., 1997). SWA therefore has the expected qualities of Process S in the two-process model of sleep regulation and is considered to be a marker of the sleep homeostat. Biochemical markers of the sleep homeostat have also been proposed (see section 1.6 for more detail), but the molecular underpinnings of the sleep homeostat are currently not well understood.

1.3.2. Marker of the circadian pacemaker

The circadian drive for alertness is generated by a central pacemaker in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The SCN was identified as the region of the central clock as ablation of this area results in the loss of behavioural and physiological rhythms in rodents and transplantation of a donor SCN restores these rhythms in animals where the SCN has been ablated (Moore & Eichler, 1972; Ralph, Foster, Davis, & Menaker, 1990; Stephan & Zucker, 1972). The clock in the SCN drives a near 24-h rhythm in many behavioural and physiological processes, including alertness.

As the endogenous circadian period is slightly longer than 24-h on average (Czeisler et al., 1999; Wright, Hughes, Kronauer, Dijk, & Czeisler, 2001) the circadian system must be synchronised or entrained daily by external time cues in order to keep time with the 24-h solar day. Time cues that entrain circadian rhythms are called Zeitgebers, and for humans, light is the most potent of these (Czeisler, Richardson, Zimmerman, Moore-Ede, & Weitzman, 1981). Exposure to light in the early biological night phase delays the clock (shifts to a later time), whereas light exposure in the late biological night (early morning), phase advances the clock [shifts to an earlier time; (Czeisler et al., 1989; Jewett et al., 1997; Khalsa, Jewett, Cajochen, & Czeisler, 2003; Minors, Waterhouse, & Wirz-Justice, 1991; Rüger et al., 2013; St Hilaire et al., 2012)]. The magnitude of the phase shift is dependent on the intensity and duration of light exposure (Boivin, Duffy, Kronauer, & Czeisler, 1996; Chang et al., 2012; St Hilaire et al., 2012; Zeitzer, Dijk, Kronauer, Brown, & Czeisler, 2000).

Light entrains the circadian pacemaker in the SCN via a direct input from the retina. Projections from retinal ganglion cells (RGCs) input into the SCN via the retinohypothalamic tract (Moore & Klein, 1974; Moore & Lenn, 1972) and are responsible for non-visual responses to light, such as circadian entrainment and suppression of the pineal hormone melatonin. These non-visual responses remain intact in some totally blind individuals (Czeisler et al., 1995; Zaidi et al., 2007), suggesting that these responses to light may not be reliant on rod and cone mediated

photoreception. Studies of mice entirely lacking rods and cones identified a third type of photoreceptor in the mammalian eye located in intrinsically photosensitive RGCs (ipRGCs) that are responsible for the non-visual effects of light (Freedman et al., 1999; Lucas, Freedman, Muñoz, Garcia-Fernández, & Foster, 1999). ipRGCs are photoreceptive and contain a photopigment called melanopsin that is most sensitive to short wavelength (blue) light (Berson, Dunn, & Takao, 2002; Gooley, Lu, Chou, Scammell, & Saper, 2001; Hattar, Liao, Takao, Berson, & Yau, 2002; Hattar et al., 2003; Provencio et al., 2000). Therefore, the circadian system in humans is maximally responsive to blue light, such that exposure to blue light produces the greatest melatonin suppression and alerting effects compared to other wavelengths of light (Brainard et al., 2001; Cajochen et al., 2005; Gooley et al., 2010; Lockley, Brainard, & Czeisler, 2003; Lockley et al., 2006; Rahman et al., 2014; Revell, Arendt, Fogg, & Skene, 2006; Thapan, Arendt, & Skene, 2001).

The timing of the circadian pacemaker and the effects of light on the circadian system are typically measured using behavioural and physiological rhythms strongly under circadian control. The circadian system drives a strong 24-h rhythm in core body temperature [CBT; (Aschoff, 1965; Czeisler, Weitzman, Moore-Ede, Zimmerman, & Knauer, 1980; Kräuchi & Wirz-Justice, 1994)] and melatonin (Arendt, 1979; Weitzman et al., 1978), and these rhythms are the most commonly used markers of the circadian clock in humans. This review will focus only on melatonin, however, as it is the phase marker of the circadian system used in this thesis.

1.3.2.1 Melatonin

Melatonin is a hormone produced by the pineal gland and is considered the biochemical signal of darkness. The pineal gland receives input from the SCN (Teclemariam-Mesbah, Ter Horst, Postema, Wortel, & Buijs, 1999) which signals the production of melatonin on a 24-h rhythm, typically during darkness. Under normal conditions, therefore, melatonin production only occurs during the dark period at night and is suppressed by light exposure at night (Bojkowski et al., 1987; Lewy, Wehr, Goodwin, Newsome, & Markey, 1980; Zeitzer et al., 2000). Melatonin receptors in the SCN (Liu et al., 1997) allow melatonin to provide feedback to the central clock, thus allowing

melatonin to alter the timing of its own production. Administration of synthetic melatonin can therefore be used to entrain the circadian clock, as has been done in blind individuals who are not synchronised by light to the 24-h day (Lockley et al., 2000; Sack, Brandes, Kendall, & Lewy, 2000). As a phase marker of the circadian clock, melatonin can be used in several ways, the most common of which is to calculate dim light melatonin onset (DLMO), the time at which melatonin levels reach a certain threshold (Lewy, Cutler, & Sack, 1999). DLMO is commonly used as its calculation does not require a full night assessment and can usually be captured by taking samples leading up to (~5 hours) and shortly after (~2 hours) habitual bedtime. DLMO can be measured using either saliva or plasma samples collected under dim light conditions (Voultsios, Kennaway, & Dawson, 1997). Methods for calculating DLMO are discussed in **Chapter 2**.

1.4. Genetic markers of vulnerability to alertness failure and associated cognitive impairment

It is well documented that some people respond more poorly to sleep loss than others, and this vulnerability has been shown to be trait like, such that across repeated exposures to sleep deprivation both cognitive and physiological responses to sleep loss remain stable (Chua et al., 2014; Rupp, Wesensten, & Balkin, 2012; Van Dongen, Baynard, Maislin, & Dinges, 2004). The trait-like nature of this individual vulnerability suggests that genetic factors may play a role in individual responses to sleep loss. In support of this hypothesis, a study of twins showed that a large proportion of variance in neurobehavioural performance during sleep deprivation could be explained by genetic factors (Kuna et al., 2012).

Several candidate genes have been identified as putative markers of vulnerability to sleep loss. Allelic variations in the *PER3* (Groeger et al., 2008; Maire, Reichert, Gabel, Viola, Krebs, et al., 2014; Maire, Reichert, Gabel, Viola, Strobel, et al., 2014; Rupp, Wesensten, Newman, & Balkin, 2013; Viola et al., 2007), *ADORA2A* (Bodenmann et al., 2012; Rupp et al., 2013), *ADA* (Bachmann, Klaus, et al., 2012; Reichert et al., 2014), *COMT* (Bodenmann et al., 2009; Satterfield, Wisor, Schmidt, & Van Dongen, 2017), *TNFα* G3084 (Satterfield, Wisor, Field, Schmidt, & Van

Dongen, 2015; Satterfield et al., 2017), *DAT1* (Holst et al., 2017; Satterfield et al., 2017) and *BDNF* (Bachmann, Klein, et al., 2012) genes have been associated with vulnerability to impaired neurobehavioural and/or executive functions during sleep deprivation. Associations between some of these genes (e.g. *PER3*, *COMT*) and cognitive performance, however, has not been shown following chronic sleep restriction in some studies (Goel, Banks, Lin, Mignot, & Dinges, 2011; Goel, Banks, Mignot, & Dinges, 2009), suggesting that the genes involved in modulating cognitive responses to sleep loss may be different for total sleep deprivation and partial sleep restriction. Alternatively, since differences in cognitive performance between some genotypes appears to be greatest during the biological night [e.g. for the *PER3* polymorphism (Groeger et al., 2008)], it may be that sleep restriction studies report no differences between genotypes because cognitive testing occurred mainly during the biological day and test scores were averaged across the day (Goel et al., 2011; Goel et al., 2009). Furthermore, genetic modulation of cognitive performance deficits in response to sleep loss may be task specific. For example, $PER3^{5/5}$ homozygotes, the reported vulnerable genotype, appear to show performance impairment following sleep restriction on a working memory task with a high executive load, whereas performance on sustained attention and working memory tasks with lower executive loads are not different between genotypes (Lo et al., 2012). This later finding is commensurate with previous research suggesting that the *PER3* polymorphism does not affect daytime neurobehavioural performance following partial sleep restriction (Goel et al., 2011; Goel et al., 2009), although more complex cognitive functions may be compromised (Lo et al., 2012).

The possibility that the genes associated with cognitive deficits during sleep deprivation differ from those associated with sleep restriction should be carefully considered. Chronic sleep restriction is more common in society than is total sleep deprivation, such that genetic markers of vulnerability to sleep restriction may have more utility for everyday application. Despite chronic sleep restriction being more common, however, genetic markers of vulnerability to sleep deprivation are still needed, particularly for safety sensitive professions where rotating shift work and extended duration shifts are common, such as transportation workers, health care providers,

police officers and firefighters. For example, shift workers rotating from day to night shift have often already been awake for several hours before the start of their first night shift (Knauth et al., 1980; Purnell, Feyer, & Herbison, 2002; Santhi, Horowitz, Duffy, & Czeisler, 2007), such that the interaction between prior wakefulness and circadian phase leads to marked performance impairment and a higher risk of errors, accidents and injuries. Therefore, a marker of vulnerability to alertness failure resulting from acute sleep deprivation would be useful in this population and would allow for application of countermeasures to reduce the impact of alertness failure on safety and productivity.

While several different genes have been investigated as biomarkers of vulnerability to neurobehavioural and executive function impairment during sleep loss, this thesis will focus on a single genetic polymorphism, the Brain Derived Neurotrophic Factor (*BDNF*) Val66Met polymorphism. This functional polymorphism in the *BDNF* gene has been associated with increased cognitive dysfunction, particularly in individuals where cognitive function is already compromised, for example, in some psychiatric conditions and neurodegenerative disorders. As sleep deprivation leads to impaired cognition, cognitive function may be further compromised in individuals with this *BDNF* polymorphism when exposed to a prolonged period of wakefulness. This *BDNF* polymorphism may therefore be a novel genetic marker of vulnerability to sleep deprivation, however, further research is needed. The following section reviews evidence for the role of the *BDNF* Val66Met polymorphism in vulnerability to sleep loss.

1.4.1. Brain derived neurotrophic factor (BDNF) Val66Met polymorphism

BDNF, a member of the neurotrophin family of growth factors, plays an important role in neuronal development and survival, context-dependent synaptic plasticity and long-term potentiation (Murer, Yan, & Raisman-Vozari, 2001). The *BDNF* gene encodes the pro-peptide form of BDNF (proBDNF), which is proteolytically cleaved to form the mature BDNF protein (Egan et al., 2003). This mature form of BDNF is expressed throughout the central nervous system and is particularly abundant in the hippocampus and prefrontal cortex (Murer et al., 2001; Pezawas et al., 2004). A

common single nucleotide polymorphism (SNP; rs6265) has been found in the *BDNF* gene, which produces an amino acid substitution, from valine (Val) to methionine (Met), at codon 66 [Val66Met; (Chen et al., 2004)]. This Met substitution leads to impairments in intracellular processing, trafficking and activity-dependent secretion of the pro-BDNF protein, such that the Met allele is thus associated with reduced mature BDNF expression (Chen et al., 2004; Egan et al., 2003). The *BDNF* Met allele is also associated with structural brain differences, such as reductions in cortical volume and thickness in hippocampal and prefrontal regions (Ho et al., 2006; Kim et al., 2013; Pezawas et al., 2004; Schofield et al., 2009; Szeszko et al., 2005); of note, Met allele carriers also show impaired cognitive functioning associated with these brain regions, including memory and executive function.

1.4.2. BDNF Val66Met polymorphism and cognitive function

In healthy adults and elderly populations, several studies have reported that *BDNF* Met allele carriers perform more poorly on cognitive tasks, especially in the domains of memory, learning and executive function (Egan et al., 2003; Ghisletta et al., 2014; Gong et al., 2009; Hariri et al., 2003; Kennedy et al., 2015; Miyajima et al., 2008; Raz, Rodrigue, Kennedy, & Land, 2009; Richter-Schmidinger et al., 2011; Schofield et al., 2009). Despite some studies showing a disadvantage of the Met allele, however, a number of studies have also reported a null effect or an advantage of the Met allele (Alfimova, Korovaitseva, Lezheiko, & Golimbet, 2012; Beste, Baune, Domschke, Falkenstein, & Konrad, 2010; Gajewski, Hengstler, Golka, Falkenstein, & Beste, 2011, 2012; Getzmann, Gajewski, Hengstler, Falkenstein, & Beste, 2013; Stuart, Summers, Valenzuela, & Vickers, 2014). While results in healthy populations are inconsistent, memory and executive function impairments associated with the Met allele have been reported more consistently in populations with psychiatric pathologies in which cognitive function is impaired, such as schizophrenia, bipolar disorder and Obsessive-Compulsive Disorder (Cao et al., 2016; Chung, Chung, Jung, Chang, & Hong, 2010; Da Rocha, Malloy-Diniz, Lage, & Correa, 2011; Egan et al., 2003; Lu et al., 2012; Rybakowski, Borkowska, Czerski, Skibińska, & Hauser, 2003; Rybakowski et al., 2006; Tükel et al., 2012). Furthermore, the *BDNF* Met allele has been associated with a

greater decline in cognition in individuals at risk of Alzheimer's disease (Boots et al., 2017; Lim et al., 2013; Lim et al., 2015) and with greater decline in hippocampal volume in individuals who already show symptoms of mild cognitive impairment (Lim et al., 2014). Taken together, these findings suggest that the Met allele may result in a certain vulnerability to memory impairment and executive dysfunction under conditions where functioning is already vulnerable, for example in some psychiatric conditions and in abnormal, pathological aging. Therefore, the Met allele may not be disadvantageous under normal, healthy conditions, but instead becomes detrimental when the brain regions involved in specific cognitive processes are compromised.

The inconsistencies in the literature examining the effects of the *BDNF* Val66Met polymorphism on cognitive function in healthy populations, may be a lack of insight into environmental or lifestyle factors that modulate the relationship between *BDNF* genotype and cognitive performance. For example, level of physical activity has been shown to interact with the Val66Met polymorphism to moderate the effects of genotype on cognitive performance in healthy individuals (Canivet et al., 2015; Erickson et al., 2013; Thibeau, McFall, Wiebe, Anstey, & Dixon, 2016). A mechanism underlying this may be the effect that physical activity has on the expression of BDNF, where physical activity promotes the secretion of the BDNF protein and the BDNF protein in turn improves brain plasticity (Cotman & Berchtold, 2002). In support of this, it was found in a population of healthy male adolescents that increased aerobic fitness was associated with greater prefrontal volume in carriers of the *BDNF* Val allele, but not the Met allele (Herting, Keenan, & Nagel, 2016), suggesting that the *BDNF* Val66Met polymorphism may modulate exercise induced neuroplasticity. As activity-dependent secretion of the BDNF protein is impaired in Met allele carriers, the exercise induced increase in BDNF may be reduced in Met allele carriers relative to Val/Val homozygotes, therefore attenuating the BDNF mediated benefits of physical activity on neuroplasticity and cognitive performance.

Obesity has also been shown to modulate the relationship between *BDNF* genotype and cognition, whereby obese *BDNF* Met allele carriers showed reduced cortical thickness in frontal

regions and impaired executive function compared to obese Val/Val homozygotes and healthy controls (Marqués-Iturria et al., 2014). Again, the mechanism involved may be the effects of obesity on the BDNF protein. Kaur et al. (2016) showed that circulating BDNF levels mediated the relationship between abdominal adiposity and executive function, such that individuals with greater adiposity had lower levels of BDNF and performed more poorly on executive function tasks. Similarly, BDNF expression is also altered by sleep loss, suggesting that inadequate sleep may be considered another compromised state that moderates the relationship between the *BDNF* Val66Met polymorphism and cognitive performance in healthy populations.

1.4.3. BDNF, sleep loss and cognition

Acute total sleep deprivation and short-term partial sleep restriction in rodents increases BDNF messenger ribonucleic acid (mRNA) and protein levels in the frontal cortex, basal forebrain and hippocampal brain regions (Cheng et al., 2015; Cirelli, Faraguna, & Tononi, 2006; Cirelli & Tononi, 2000; Fujihara, Sei, Morita, Ueta, & Morita, 2003; Wallingford, Deurveilher, Currie, Fawcett, & Semba, 2014; Zielinski et al., 2014). Longer periods of acute or partial sleep deprivation, however, eventually result in a decrease in cortical BDNF protein and mRNA similar to baseline levels, such that one day of acute or partial sleep loss results in greater cortical BDNF expression than three or more days of sleep loss (Cirelli et al., 2006; Wallingford et al., 2014; Zielinski et al., 2014). It has been suggested that the initial increase in cortical BDNF in response to acute or partial sleep deprivation is involved in a neuroprotective response to sleep loss, such that BDNF initially triggers molecular and cellular processes to compensate for sleep loss and is no longer required once these processes have begun (Wallingford et al., 2014). In humans, individuals with chronic sleep restriction due to insomnia show reduced levels of circulating BDNF (Giese et al., 2013; Giese, Unternährer, et al., 2014), whereas one night of total or partial sleep deprivation increases circulating BDNF in individuals with depression (Giese, Beck, et al., 2014; Gorgulu & Caliyurt, 2009). Thus, in humans and animals it appears that short-term acute sleep loss results in an increase in BDNF, while chronic sleep loss reduces BDNF.

In humans, changes in circulating BDNF have been associated with cognitive function. For example, several studies have described decreased concentrations of serum BDNF in individuals with Alzheimer's disease or mild cognitive impairment [for meta-analysis see (Qin et al., 2017)], which can be an antecedent to development of Alzheimer's disease (Petersen et al., 2001). Furthermore, the progression of cognitive decline is slower in individuals with Alzheimer's disease that have higher levels of serum BDNF (Laske et al., 2011) and post-mortem examination of BDNF gene expression in the prefrontal cortex showed that higher BDNF expression was associated with slower cognitive decline in individuals with Alzheimer's disease (Buchman et al., 2016). Therefore, while speculative, higher BDNF levels in the early stages of the disease may reflect a neuroprotective mechanism to slow the rate of cognitive decline and neurodegeneration. Similarly, the initial increase in BDNF in response to sleep loss may protect against cognitive dysfunction associated with inadequate sleep.

In support of a protective role for BDNF during acute sleep deprivation, Giacobbo et al. (2016) found no differences in Stroop task performance between controls and individuals who underwent a night of sleep deprivation; however, serum BDNF levels were significantly higher in the sleep deprived group and were also correlated to Stroop performance, such that lower serum BDNF was associated with poorer performance. The authors suggested that the increase in peripheral BDNF following sleep deprivation may be a compensatory mechanism that allows for maintenance of cognitive function during extended wakefulness. Therefore, given that the *BDNF* Met allele is associated with impaired activity-dependent secretion of the BDNF protein in the brain, the increase in BDNF during prolonged wakefulness may be attenuated in BDNF Met carriers, thus leading to poorer cognitive outcomes in response to sleep loss. BDNF genotype, however, was not investigated in the study conducted by Giacobbo et al. (2016).

Only one study has investigated cognitive performance during sleep deprivation in relation to the *BDNF* Val66Met polymorphism. Bachmann, Klein, et al. (2012) found that *BDNF* Met allele carriers performed more poorly on a working memory task during 40-h of prolonged wakefulness,

however, attention and vigilance was not affected. Although this suggests Met allele carriers appear to be more vulnerable to executive dysfunction during sleep deprivation, other aspects of executive function have yet to be investigated to confirm this.

1.5. Biochemical biomarkers of alertness state

Regulation of the sleep-wake cycle involves various biochemicals that act on areas of the brain to promote either sleep or wakefulness (Arias-Carrion, Huitron-Resendiz, Arankowsky-Sandoval, & Murillo-Rodriguez, 2011). Sleep propensity, or Process S, is thought to be signalled by the accumulation of sleep inducing substances in the brain (Porkka-Heiskanen & Kalinchuk, 2011). When concentrations of these substances reach threshold levels, sleep is induced. The idea that sleep was initiated due to the build-up of biochemicals in the brain was first given evidence when cerebrospinal fluid (CSF) from sleep deprived animals induced sleep in well rested animals following injection (Fencl, Koski, & Pappenheimer, 1971). Subsequently, the sleep promoting substance 'Factor S' was identified (Krueger, Pappenheimer, & Karnovsky, 1978; Pappenheimer, Koski, Fencl, Karnovsky, & Krueger, 1975) and more recently several 'sleep regulatory substances' have been found in both animals and humans.

To be considered a sleep regulatory substance (SRS) a substance must meet several criteria—the substance should 1) vary with sleep propensity, 2) enhance or reduce sleep when administered, 3) change sleep when its action is inhibited, 4) be altered in disease states associated with changes to sleep, and 5) act on known sleep regulatory systems (Clinton, Davis, Zielinski, Jewett, & Krueger, 2011; Rachalski, Freyburger, & Mongrain, 2014). While many endogenous substances exhibit some characteristics of an SRS, very few meet all the required criteria. Identified SRSs involved in the promotion of sleep include interleukin-1β, tumor necrosis factor-α, growth hormone stimulating hormone, prolactin, adenosine, nitric oxide and prostaglandins (Clinton et al., 2011; Gautier-Sauvigne et al., 2005; Porkka-Heiskanen & Kalinchuk, 2011; Sengupta, Roy, & Krueger, 2011; Urade & Hayaishi, 2010). While these substances appear to be good candidates as biochemical markers of alertness, their sleep regulatory function takes place within the brain which

makes them difficulty to measure. Furthermore, these substances are produced in response to stimuli other than sleep loss alone, thus lacking the specificity required for a biomarker of alertness. Nevertheless, it may be possible to investigate changes in SRSs by measuring other downstream molecules involved in the chemical cascades of the sleep homeostat.

Recent research has established that sleep and circadian disruption leads to measurable alterations in the levels of gene transcripts, proteins and metabolites in blood, urine and saliva. Therefore, changes in these biochemicals may thus serve as biomarkers of alertness state; however, given that the biological actions of many of the substances that change with sleep are not specific to sleep alone, it is likely that a panel of biomarkers will be required in order to reliably measure an individual's level of sleep propensity at any given time.

Two approaches can be used to identify biomarkers of alertness state—1) targeting specific biochemicals related to sleep regulatory pathways or related to genotypes or phenotypes associated with vulnerability to sleep loss, and 2) performing an untargeted screen of global changes associated with sleep loss to identify markers that are most sensitive to sleep loss and circadian phase. This thesis will explore both of these approaches, which are discussed below.

1.5.1. Targeted biomarker approaches

Identification of biomarkers using a targeted approach may involve targeting specific biochemicals that are known to play a role in the regulation of sleep and wake, as done previously in identifying SRSs. Another approach, however, is to target biochemicals that are related to genotypes or phenotypes that are associated with vulnerability to sleep loss. For example, several inflammatory markers have been targeted given the bi-directional relationship between sleep and the immune system, in which the immune system contributes to regulation of normal sleep, while sleep also responds to challenges to our immune system, for example from infection (Imeri & Opp, 2009). Using this targeted approach, cytokines and other immune markers have been shown to change in response to acute sleep deprivation (Frey, Fleshner, & Wright, 2007; Hui, Hua, Diandong, & Hong, 2007; Sauvet et al., 2010; Shearer et al., 2001), highlighting the utility of a targeted approach in

identifying biomarkers of sleep loss. Another phenotype that may be associated with vulnerability to performance impairment during sleep loss is menstrual phase (Wright & Badia, 1999) and the female sex hormones involved in the regulation of the menstrual cycle may therefore be useful markers of this vulnerability.

1.5.1.1 Reproductive hormones across the menstrual cycle

The female menstrual cycle is characterised by changes in the sex steroids estrogen, and progesterone, and the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). A typical menstrual cycle is on average approximately 28 days long in normally menstruating women (Fehring, Schneider, & Raviele, 2006) and consists of two main phases – the follicular phase and the luteal phase. The follicular phase lasts from menses onset until ovulation, and the luteal phase lasts between ovulation and menses onset.

At the beginning of the follicular phase, during menses, levels of estrogen, progesterone and LH are low (Landgren, Unden, & Diczfalusy, 1980; Sherman & Korenman, 1975), whereas FSH increases slightly to stimulate the development of follicles within the ovary (Couzinet, Lestrat, Brailly, Forest, & Schaison, 1988). As the dominant follicle develops, estrogen production increases and has a negative feedback action which reduces FSH production (Shaw et al., 2010). Throughout the follicular phase estrogen continues to rise and peaks approximately 1 day before ovulation (Landgren et al., 1980). The sustained elevation in estrogen now has a positive feedback effect which stimulates the LH surge (J. R. Young & Jaffe, 1976). The LH surge begins approximately 36-h before ovulation (Hoff, Quigley, & Yen, 1983) and is essential for the final development of the follicle, luteinisation of the follicle, which becomes the corpus luteum, and follicle rupture resulting in ovulation of the oocyte [egg; (K. A. Young, Chaffin, Molskness, & Stouffer, 2003)].

During the LH surge there is an abrupt decline in estrogen concentration, while concentrations of progesterone start to increase (Hoff et al., 1983), causing the mid-cycle surge in FSH (Liu & Yen, 1983). Following ovulation, FSH and LH levels decline and remain low for the remainder of the luteal phase (Landgren et al., 1980; Sherman & Korenman, 1975). Progesterone

levels, however, continue to rise throughout the luteal phase, and levels of estrogen also increase, although they remain lower than the preovulatory estrogen surge (Landgren et al., 1980; Sherman & Korenman, 1975). Toward the end of the luteal phase, if pregnancy does not occur, the source of progesterone and estrogen production, the corpus luteum, begins to degrade (Baerwald, Adams, & Pierson, 2005). The resulting withdrawal of progesterone and estrogen leads to shedding of the uterus lining through menstruation, thus starting the cycle anew.

In summary, throughout the menstrual cycle estrogen has periods of high concentration during both the mid- to late-follicular phase and throughout the luteal phase, whereas progesterone is low throughout the follicular phase and increases in concentration during the luteal phase. LH and FSH remain low throughout both phases of the menstrual cycle (FSH has a slight increase during menses), but peak during midcycle when ovulation occurs.

1.5.1.2. Cognitive performance across the menstrual cycle and associations with female reproductive hormones

Performance on many cognitive tasks has been shown to vary according to menstrual phase in the well-rested state. Women are thought to perform better on tasks such as verbal tasks, and tasks requiring perceptual speed and fine motor skills, during the luteal phase, when concentrations of progesterone and estrogen are both high, and better on tasks such as visuospatial tasks and visual memory, during the early follicular phase (menses) when estrogen and progesterone concentrations are low (Broverman et al., 1981; Hampson, 1990; Hausmann, Slabbekoorn, Van Goozen, Cohen-Kettenis, & Güntürkün, 2000; Maki, Rich, & Shayna Rosenbaum, 2002; Phillips & Sherwin, 1992; Schöning et al., 2007; Silverman & Phillips, 1993; Wuttke et al., 1975). Therefore, changes in reproductive hormones during the different phases of the menstrual cycle may underlie differences in cognition. In support of a role for the sex steroids in cognition, male-to-female sexual transition with hormone therapy has been shown to result in improvement on tasks performed better by women in the luteal phase when concentrations of female reproductive hormones are high (Miles, Green, Sanders, & Hines, 1998), whereas individuals undergoing female-to-male transitions show an increase in performance on tasks performed better by women during the early-follicular phase

when female reproductive hormones are low and a decrease in performance on tasks performed better during the luteal phase (Slabbekoorn, Van Goozen, Megens, Gooren, & Cohen-Kettenis, 1999; Van Goozen, Cohen-Kettenis, Gooren, Frijda, & Van de Poll, 1994).

Despite evidence to support differences in cognition across the menstrual cycle, several studies have shown inconclusive, or contradictory results (Epting & Overman, 1998; Gordon & Lee, 1993; Mordecai, Rubin, & Maki, 2008; Symonds, Gallagher, Thompson, & Young, 2004). The discrepancy in results may be due to the way in which menstrual phase was calculated. Self-report measures of menstrual phase using the date of last menses and typical menstrual cycle length have been used in several studies although post-hoc assays of hormone levels, which were used to validate menstrual phase in some studies, resulted in between 13 and 33% of participants being excluded due to incorrect classification based on self-report measures (Hausmann et al., 2000; Maki et al., 2002; Phillips & Sherwin, 1992). Furthermore, differences in findings between studies may arise from comparing different parts of the menstrual cycle (e.g. menses versus luteal, or late follicular versus luteal) which have different hormonal characteristics. Despite these methodological challenges, a recent review of the literature examining performance across the menstrual cycle could not find sufficient evidence to support the hypothesises that performance on sexually dimorphic tasks differs across the menstrual cycle in relation to the changes in the female reproductive hormones (Poromaa & Gingnell, 2014). Moreover, the differences in performance that have been reported in in the literature are typically small and this may explain the null findings in some studies. These small differences may not have clinically significant implications, in that these changes would not have a noticeable effect on everyday function, although they may become exacerbated in a compromised state, for example during sleep deprivation, as was described previously for the *BDNF* Val66Met polymorphism. Furthermore, the mechanisms underlying differences in performance between the menstrual phases may be different between the well-rested and sleep deprived states. As described below, differences in body temperature between the menstrual phases, which are most pronounced during the night, appear to result in improved night

time performance in the luteal phase, relative to performance observed in the follicular phase of the menstrual cycle.

1.5.1.3. Association between menstrual phase and performance during sleep deprivation

Two studies have investigated the effects of menstrual phase on performance during sleep deprivation in pre-menopausal women. Wright and Badia (1999), found that women in the follicular phase performed more poorly on a range of cognitive tasks following one night of sleep deprivation compared to women in the luteal phase. Similarly, Vidafar et al. (Under review) showed greater attentional lapses in women in the follicular phase during 30-h of wakefulness under CR conditions. The authors of both papers suggested that the differences in performance between the menstrual phases may be due to differences in core body temperature (CBT).

CBT displays a robust circadian rhythm with a minimum that typically occurs around 04:00 to 06:00 hours, and an average daily variation of approximately 1˚C (Duffy, Dijk, Klerman, & Czeisler, 1998; Kräuchi & Wirz-Justice, 1994). Kleitman, Titelbaum, and Feiveson (1938) described a relationship between body temperature and reaction time, suggesting that the diurnal variation in reaction time is dependent on the diurnal variation in temperature. Since these initial observations, it has been shown that performance on a wide range of cognitive tasks follows the 24-h rhythm in CBT (Dijk et al., 1992; Johnson et al., 1992; Monk et al., 1983; Wyatt et al., 1999). A more causal role of CBT in modulating cognitive performance was demonstrated by Wright, Hull, and Czeisler (2002), whereby performance testing that occurred across multiple cycles during an FD protocol were categorised as being associated with either the highest or lowest CBT value for each circadian phase or time since wake bin where testing occurred. This analysis showed that performance was better on tests performed in the same circadian phase or time since wake bin when CBT was higher, and for some tasks this effect was particularly pronounced during the biological night, when CBT reaches its minimum. Overall, an increase of approximately 0.17˚C was associated with an improvement in working memory, cognitive throughput, subjective alertness and the 10% fastest reaction times (Wright et al., 2002). While participants in this study included both women and men, menstrual cycle phase was not controlled.

During the menstrual cycle, daily average CBT is approximately 0.2-0.4˚C higher in the luteal phase compared to the follicular phase (Baker et al., 2001; Cagnacci, Arangino, Tuveri, Paoletti, & Volpe, 2002; Cagnacci, Soldani, Laughlin, & Yen, 1996; Cagnacci, Volpe, Paoletti, & Melis, 1997; Driver, Dijk, Werth, Biedermann, & Borbély, 1996; Kattapong, Fogg, & Eastman, 1995). Furthermore, in the luteal phase, the circadian rhythm in CBT is blunted, such that temperatures during the biological night are increased (Cagnacci et al., 2002; Cagnacci et al., 1996; Cagnacci et al., 1997; Coyne, Kesick, Doherty, Kolka, & Stephenson, 2000; Kattapong et al., 1995; Lee, 1988; Shechter, Varin, & Boivin, 2010).

Progesterone is thought to play a role in modulating the body temperature changes throughout the menstrual cycle as the increase in CBT during the luteal phase is concomitant with the natural increase in progesterone during this phase (Baker et al., 2001; de Mouzon, Testart, Lefevre, Pouly, & Frydman, 1984; Driver et al., 1996; Wright & Badia, 1999). Further evidence for a thermoregulatory role of progesterone, however, comes from studies which have monitored acute changes to temperature following administration of progesterone or synthetic progestins. These studies have shown that administration of progesterone in men (Rothchild & Barnes, 1952), in women following hysterectomy (Israel & Schneller, 1950) and in women with amenorrhea (Buxton & Atkinson, 1948) acutely increases body temperature. Moreover, it has also been shown that healthy pre-menopausal women taking progestin containing oral contraception (OC) have elevated temperatures relative to women in the natural follicular phase (Baker et al., 2001; Kattapong et al., 1995; Stachenfeld, Silva, & Keefe, 2000; Wright & Badia, 1999). These findings indicate a direct causal role of progesterone in the regulation of body temperature; however, the mechanisms underpinning the hyperthermic effects of progesterone are not well understood.

A study conducted in rabbits has shown that progesterone administration reduces the firing rate of cold-sensitive neurons and increases the firing rate of warm-sensitive neurons in the area of the brain involved in the homeostatic regulation of temperature: the preoptic area of the hypothalamus (Nakayama, Suzuki, & Ishizuka, 1975). Moreover, in humans, progesterone receptors have been shown to be located in the SCN (Kruijver & Swaab, 2002). Therefore,

progesterone may exert its effect on temperature by acting directly on the brain regions involved in thermoregulation. Another possible mechanism of action may be the effects of progesterone on other hormones involved in thermoregulation. For example, progesterone has been shown to attenuate the hypothermic effects of melatonin (Cagnacci et al., 1996), which may explain the attenuated nocturnal decline in body temperature during the luteal phase of the menstrual cycle (Kattapong et al., 1995; Lee, 1988; Shechter et al., 2010; Wright & Badia, 1999).

In addition to progesterone, estrogen is also thought to regulate temperature through its hypothermic actions, such that is lessens the hyperthermic effects of progesterone. For example, in both naturally cycling and hysterectomised women, the combination of estrogen and progesterone administration together results in an increase in body temperature which is less pronounced than the increase shown in response to administration of progesterone alone (Israel & Schneller, 1950). More recently, these findings have been replicated showing that the administration of OC containing progestin alone results in a greater increase in temperature than OC containing both estrogen and progestin in naturally cycling pre-menopausal women (Stachenfeld et al., 2000). Together these findings suggest that the hypothermic effects of estrogen can modify the hyperthermic actions of progesterone, such that both hormones play a thermoregulatory role throughout the menstrual cycle. In support of this, Cagnacci et al. (1997) showed that the progesterone/estradiol ratio, but not progesterone or estradiol (the main estrogen) alone, was associated with 24-h mean body temperature and the amplitude of the 24-h temperature rhythm in naturally cycling women who were studied in both the follicular and luteal phases of their menstrual cycle. The progesterone/estradiol ratio is positively associated with the 24-h mean in CBT and negatively associated with the amplitude of the circadian CBT rhythm, such that temperature increases, and the amplitude of the rhythm decreases when the ratio of progesterone to estradiol is higher (Cagnacci et al., 2002; Cagnacci et al., 1997), which is characteristic of the luteal phase.

While there is inconclusive evidence to support a role for female reproductive hormones modulating cognition in the well-rested state, given the thermoregulatory effects progesterone and

estradiol and the relationship between temperature and cognitive performance, progesterone and the progesterone/estradiol ratio may be useful biomarkers of performance impairment associated with sleep and circadian disruption in women of reproductive age. Despite this possibility, however, the relationship between performance, temperature and these reproductive hormones during prolonged wakefulness has not yet been investigated. If progesterone and/or the progesterone/estradiol ratio fluctuate with the daily rhythm in temperature and performance, they may be useful biomarkers of alertness mediated performance impairment associated with sleep loss and adverse circadian phase in premenopausal women. Furthermore, if these hormones are to be found to be effective alertness biomarkers it would lend support to the targeted approach for identifying future biomarkers of alertness state, particularly for specific groups where there is an identified biological determinant for vulnerability to alertness failure.

1.5.2. Untargeted biomarker approaches

In the past several years the need for a biomarker of alertness has been widely discussed (Czeisler, 2011; Mullington et al., 2016; Mullington et al., 2011; Quan et al., 2011). As reviewed above, several attempts have been made to target specific biochemicals, however, more recently untargeted omics techniques including transcriptomics, proteomics, and metabolomics have been used in the search for a biomarker of alertness. These omics techniques have been recommended in a recent workshop dedicated to identifying opportunities for developing novel sleep and circadian biomarkers (Mullington et al., 2016) as they allow for the simultaneous measurement of hundreds and thousands of potential biomarkers from a single sample which far exceeds the limited range and scope of candidates examined in targeted biomarker discovery studies. Transcriptomics studies have identified a number of gene transcripts that are altered by sleep deprivation (Arnardottir et al., 2014; Laing et al., 2017; Pellegrino et al., 2012), sleep restriction (Laing et al., 2017; Möller-Levet et al., 2013), and circadian misalignment (Archer et al., 2014; Laing et al., 2017), and more recently metabolomics approaches have been used to examine circadian and wake-dependent control of the human metabolome. This thesis will focus on this latter approach.

1.5.2.1. A brief introduction to metabolomics

Metabolomics is the analysis of small molecule (<1kDa) metabolic profiles in a biological system. The human metabolome consists of both endogenous metabolites synthesised within the organism, and exogenous metabolites, which are foreign substances derived from food, drugs or organism specific microbes. The metabolome is the final downstream product of the genome and is considered to be the closest to the organisms expressed phenotype (Dettmer, Aronov, & Hammock, 2007; Dunn, Broadhurst, Atherton, Goodacre, & Griffin, 2011). Thus, metabolomics, while being a relatively new technique, is a valuable tool for studying the influence of environmental, disease related and genetic disturbances on a phenotype.

Two techniques commonly used for metabolomics include nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) coupled with separation techniques such as gas chromatography (GC-MS) or liquid chromatography [LC-MS; (Dettmer et al., 2007)]. NMR is a useful tool for screening purposes as it has a high throughput and requires minimal sample preparation. A disadvantage of this technique, however, is that only medium to high abundance metabolites, those with concentrations in the micromolar range and upwards, will be detected. In contrast, mass spectrometry-based techniques can analyse metabolite concentrations well below the micromolar range with high selectivity and sensitivity and can aid in the identification of metabolites. Mass spectrometry-based methods, however, can be more time consuming and laborious with regard to sample preparation. Nevertheless, LC-MS in particular, is a powerful analytical tool that has been used most commonly in human metabolomics studies within the field of sleep and circadian science.

The use of different metabolomics platforms is necessary given the diverse range of molecules that comprise the metabolome. The varied chemical properties, sizes and charges of the molecules means that currently there is no one metabolomics platform than can capture the entire metabolome. Within the LC-MS methods, reverse phase (RP) chromatography with a C18 column has been most commonly used in the literature examining circadian and wake-dependent control of

the metabolome. RP chromatography captures a range of moderately polar and non-polar compounds, but highly polar compounds are poorly retained (Rojo, Barbas, & Rupérez, 2012). As an alternative to typical RP chromatography, hydrophilic interaction liquid chromatography (HILIC) is a separation technique that retains highly polar compounds well (Hemström & Irgum, 2006). To ensure coverage of the entire metabolome, these different separation techniques need to be employed.

Irrespective of the type of chromatography used, metabolomics experiments can be broadly classified as either targeted or untargeted (Dunn et al., 2011). As their names suggest, targeted metabolomics studies typically target a small number of known metabolites for measurement based on a hypothesis, whereas untargeted metabolomics studies measure a broad range of metabolites without a specific hypothesis regarding metabolites of interest. Given the large number of metabolites measured in untargeted studies and a lack of knowledge regarding the metabolites of interest, relative changes in metabolites (e.g. area under the peak) are measured rather than precise concentrations based on calibration curves derived from chemical standards (Dunn et al., 2011). Therefore, while a disadvantage of this technique is that no quantitative concentration data are obtained, it does mean that a large and broad range of metabolites can be detected. For the application of metabolomics in biomarker discovery programs, an untargeted approach would be most beneficial given the large numbers of metabolites measured. Once specific metabolites of interest have been putatively identified, a follow-up targeted study would allow for quantification of the candidate metabolites which may lead to development of cut-off concentrations that indicate an abnormal state.

A major limitation of metabolomics is the time consuming and expensive process of identifying metabolites from untargeted metabolomics experiments. Being a relatively new field, better techniques to identify unknown compounds are still being developed (Dunn et al., 2013). Raw metabolomic data are displayed as a series of mass spectra acquired at a given detection time (retention time), thus, further analysis is required in order to identify specific metabolites from the

features detected. Experimental data including accurate mass, retention time and tandem MS (MS/MS) data are often used to identify metabolites along with comparison to chemical standards and metabolite database searches (e.g. Human Metabolome Database). Despite this drawback, metabolomics is a promising technique for identification of biomarkers of alertness state. For example, physiological and environmental changes in an organism can influence the metabolome immediately and can be measured within seconds to minutes, whereas changes in the proteome and transcriptome typically occur on a longer time scale from minutes to hours (Dunn et al., 2012). Therefore, while the metabolome is extremely diverse and complex, it can provide a real-time indication of the state of an organism, such that a metabolomics approach may be useful in detecting a biomarker of alertness.

Given that the vast majority of published studies investigating circadian and wake-dependent modulation of the metabolome have investigated the plasma metabolome, this review will focus on studies that have collected and analysed blood samples; although metabolomic profiles in urine (Giskeødegård, Davies, Revell, Keun, & Skene, 2015), saliva (Dallmann, Viola, Tarokh, Cajochen, & Brown, 2012) and breath (Martinez-Lozano Sinues et al., 2014) have also been examined previously.

1.5.2.2. Circadian control of the human plasma metabolome

Understanding how the circadian system regulates the metabolome has several important applications. Firstly, characterisation of the circadian control of the metabolome is integral given the use of metabolomics as a technique for identifying biomarkers of many physiological and pathological states (Mikami, Aoki, & Kimura, 2012; Serkova, Standiford, & Stringer, 2011). For example, understanding the circadian variation in metabolites used in a diagnostic test would be important as the time that the test is given may significantly alter its interpretation. Secondly, understanding how the circadian system regulates the metabolome may help to elucidate the mechanisms underlying the association between sleep and circadian disruption and metabolic disease (Depner, Stothard, & Wright, 2014). Lastly, knowledge of circadian regulation of the

metabolome is also important for the development of biomarkers of alertness. Since many established behavioural and physiological markers of alertness are modulated by both the circadian system and the sleep homeostat, it is likely that a biomarker of alertness will display similar properties. Furthermore, development of a biomarker that reflects only the homoeostatic system would still require knowledge of whether that marker also displayed a circadian rhythm. If an identified marker of the sleep homeostat was also under control of the circadian system, its interpretation would change depending on the timing that the sample was collected. Therefore, while a biomarker of sleep propensity does not necessarily need to show a circadian rhythm, knowing whether it is under circadian control is crucial.

Investigations of circadian control of the plasma metabolome involves the collection of multiple blood samples across 24 to 40-h in healthy non-medicated participants under highly controlled laboratory conditions. In a group of 8-middle aged healthy male adults, LC-MS was used to analyse plasma metabolites from blood samples taken every 3 to 6-h during a normal 24-h sleep/wake cycle (Ang et al., 2012). Using an untargeted metabolomics approach approximately 200 metabolite features (19% of all features detected) were found to exhibit a 24-h rhythm. The identities of 24 oscillating metabolites were determined and these belonged to a variety of chemical classes including acetylcarnitines, lysophospholipids, corticosteroids and amino acids. In a similar study, plasma metabolite rhythms were examined in 12 healthy young males under two conditions, 1) during a normal 24-h sleep/wake schedule, and 2) during 24-h of sleep deprivation (Davies et al., 2014). Using LC-MS, the authors observed 109 metabolites that exhibited a significant circadian rhythm on day 1 under normal sleep/wake conditions. On day 2, during sleep deprivation, 78 metabolites remained rhythmic, while 31 metabolites lost rhythmicity. In addition, 15 metabolites that were not rhythmic on day 1 exhibited rhythmicity on day 2. While both of these studies were conducted in highly controlled laboratory environments they did not adhere to traditional constant routine (CR) procedures which eliminate or control factors that may mask endogenous circadian rhythms, specifically with respect to lighting conditions, and sleep in the first study (Ang et al., 2012) and lighting conditions, sleep, movement and meals in the second study (Davies et al., 2014).

The loss and generation of rhythms during sleep deprivation compared to the sleep condition in the Davies et al. (2014) study suggest that some of the metabolite rhythms were evoked or masked by sleep. While informative, this does suggest it is important to study circadian control of the metabolome under CR conditions that would best allow for the determination of endogenously generated circadian rhythms.

To date, only three studies have investigated circadian rhythmicity of the human metabolome under CR conditions. In the first of these studies, GC-MS and LC-MS were used to analyse plasma metabolites collected during a 40-h CR in a group of 10 middle age male participants (Dallmann et al., 2012). Blood samples were taken every hour and were then pooled across participants in 4-h bins. From these pooled samples, it was found that ~15% metabolites exhibited circadian variation, with a high proportion (75%) of these rhythmic metabolites being lipids, in particular fatty acids. A major limitation of this study lies in the pooling of blood samples across participants: pooling samples at the same clock time does not account for individual differences in circadian phase, where even in young healthy individuals the range in DLMO can be as large as ~5 hours (Sletten, Vincenzi, Redman, Lockley, & Rajaratnam, 2010; Wright, Gronfier, Duffy, & Czeisler, 2005).

Also utilising the CR protocol (36-h duration), Kasukawa et al. (2012) examined circadian rhythms in plasma metabolites in a group of 6 healthy young males. LC-MS was used to analyse plasma samples collected every 2-h in an untargeted metabolomics approach where approximately 300 (~7%) circadian oscillating metabolites were detected. A large proportion of the metabolites whose identities were determined belonged to a steroid hormone metabolism pathway. A number of lipids also displayed significant circadian rhythmicity; however, they had weak amplitude and/or high variability among individuals. This variability in lipids was investigated in a lipidomics study which examined the circadian regulation of 263 targeted lipids using both group- and individual-level analyses (Chua et al., 2013)

In a group of 20 healthy young males, LC-MS was used to examine the circadian modulation of the human plasma lipidome in blood samples collected every 4-h during a 40-h CR (Chua et al., 2013). Using a targeted lipidomics based approach, approximately 13% of the lipids analysed showed circadian variation at the group level, whereas approximately 18% of lipid metabolites showed circadian variation when analysed at the individual level. At the individual level, there were large differences observed between participants in the timing of lipid rhythms with peak concentration levels occurring up to 12-h apart in some participants. There were also substantial differences in the set of lipid species identified as being rhythmic across participants with a median agreement of 20% between participants. Additionally, several lipid species that were not rhythmic in the group-level analysis showed circadian rhythmicity at the individual level. Despite these large inter-individual differences, Chua et al. (2013) were able to cluster participants into different phenotypic groups based on the peak time (acrophase) of rhythmic lipids, where participants were classified into one of three groups— morning peak, evening peak or no phase predominance. Further cluster analyses showed that participants could also be grouped based on the strength of rhythmicity for different lipid species. This study was the first to highlight the importance of individual level analysis for the assessment of circadian modulation of the metabolome.

Despite clear evidence that certain metabolites in the human metabolome are under circadian control, there is a need for further investigation and characterisation of individual differences in circadian modulation of the metabolome, particularly among other classes of metabolites [e.g. non-lipid, polar metabolites as the one study which examined individual differences focused exclusively on lipids (Chua et al., 2013)]. Furthermore, and likely due to the differences in methodology employed across the studies, few metabolites have consistently been shown to be rhythmic between studies, such that further research using the gold-standard CR protocol is needed to confirm rhythmicity of previously identified metabolites. Characterisation of circadian modulation of highly polar metabolites, such as organic and amino acids, sugars, and nucleotides, is also required as to date only moderately polar metabolites and lipids have been

examined. Polar metabolites represent an important study target as many are being identified as biomarkers and drug targets of various disease states (Urayama et al., 2010; Wang et al., 2011; Ibanez et al., 2012; Munger et al., 2008; Vastag et al., 2011). Moreover, they are also involved in many important biological pathways including synthesis of large macromolecules, the urea cycle and essential energy pathways including glycolysis and the Krebs cycle.

1.5.2.3. Wake-dependent control of the human metabolome

To date, five studies have investigated the effects of sleep loss on the expression of metabolites in the plasma metabolome. The first of these studies used LC-MS in an untargeted metabolomics approach to investigate the metabolic profile of 10-middle aged male subjects during a 40-h CR [rhythms analysis reviewed above; (Dallmann et al., 2012)]. Initially this study aimed to determine the circadian rhythmicity of metabolites found in human plasma and saliva, however, the authors noticed that 34 (of 281; ~12%) plasma metabolites displayed a monotonic increase or decrease across the 40-h of sleep deprivation. More recently, linear changes in individual participant lipid profiles in 20 young, healthy males during a 40-h CR was examined (Chua et al., 2015). Of the 5,260 individual lipid profiles analysed, 9.3% showed a linear decrease and 17.8% showed a linear increase with prolonged wakefulness. As with the rhythmic lipid profiles previously published by this group (Chua et al., 2013), there was a significant amount of individual variation in linear changes among participants. Despite this variation, however, 25 lipids were identified that showed a consistent pattern of change in at least half of the participants. Sixteen of these 25 individually-stable lipids, were also found to be rhythmic and displayed a pattern of change similar to the time course observed in behavioural and physiological alertness as measured by neurobehavioural performance and EEG activity. Therefore, these lipids may be regulated by both the circadian and homeostatic systems and could be considered as candidate biomarkers of alertness.

While CR protocols are the gold standard for assessing endogenous, self-sustained circadian rhythms and the impact of acute sleep deprivation, the highly controlled nature of the protocol is not a realistic way in which sleep deprivation might occur in a real-life scenario. Three studies have

investigated the effects of sleep deprivation or chronic sleep restriction in less highly controlled conditions to determine the effects that a more realistic model of sleep loss has on the plasma metabolome. As described earlier, Davies et al. (2014) compared the metabolic profile of 12 healthy young males under low sleep pressure, during a normal 24-h sleep/wake cycle, and under high sleep pressure, during 24-h of sleep deprivation. Davies et al. (2014) found that 41 metabolites increased in concentration during sleep deprivation compared with normal sleep conditions. Majority (66%) of the metabolites that increased during prolonged wakefulness did so during the normal hours of sleep (00:00-06:00h), and 34% increased significantly during waking hours (14:00-22:00h). The reason for the increase in metabolites during the afternoon and evening hours on day two compared to day one of the study is unclear as samples collected at this time point were taken during a typical waking day, before sleep deprivation had occurred. The authors suggested that the increase may be due to inactivity and lack of exercise during the protocol. The increase in metabolites observed during a night without sleep compared to during sleep, however, gives an indication of the acute effects of sleep deprivation on plasma metabolites. Changes in these metabolites may reflect an increase in the homeostatic drive for sleep and as such may be useful biomarkers of alertness.

In real-world settings individuals are more likely to suffer from repeated nights of inadequate sleep (i.e. chronic sleep restriction) rather than acute sleep deprivation. Therefore, it is important to examine the effects of not only total sleep deprivation, but also sleep restriction on metabolite expression. This type of investigation has been conducted in a recent study of 11 young adults [~50% female; (Bell et al., 2013)], where participants completed two experimental sessions of eight nights of either 5.5-h or 8.5-h time-in-bed. Morning fasting blood samples were taken at the end of each condition and analysed using LC-MS and GC-MS. From the 362 metabolites identified using an untargeted approach, 13 exhibited a significant increase, 3 showed a significant decrease, and 12 and 5 displayed trends toward increasing and decreasing, respectively. A more recent study has investigated the effects of sleep restriction on the metabolome in 10 healthy adults aged between 20 and 55 years of age, where sleep was restricted to 4-h in bed for 5 nights. Using an

untargeted approach, 37 metabolites were identified as having changed following sleep restriction. Together, these two studies (Bell, 2013; Weljie, 2015) provide evidence to suggest that expression of metabolites are sensitive not only to acute sleep deprivation, but also sleep restriction.

Sleep restriction studies to date have relied on obtaining two samples at different timepoints but collected at the same clock time on different study days (i.e. before and after sleep restriction). This analysis approach, despite comparing samples at the same clock time, does not account for individual differences in circadian phase, where even under highly controlled conditions, there is wide inter-individual variability in circadian phase (Sletten et al., 2010; Wright et al., 2005). Therefore, changes in the concentration of a metabolite may be indicative, not of a steady increase or decrease over time, but instead of a change in the amplitude of a rhythm, or a shift in the timing of a rhythm across study days. For example, the metabolites tryptophan and phenylalanine have been shown to increase when sleep was restricted to 4-h per night for 5 nights (Weljie et al., 2015), yet were also shown to be rhythmic in previous research (Kasukawa et al., 2012; Ang et al., 2012). It is possible therefore that the change in concentration of some metabolites that was attributed to sleep restriction may actually reflect a change in concentration due to a shift in circadian phase (induced by the study protocol) rather than a change due to accumulating sleep debt.

The current research examining changes in the human metabolome following sleep loss suggest that changes in metabolite concentrations may be useful biomarkers for identifying an individual's sleep propensity at any given time. The extent to which systemic changes in metabolite concentration are actually representative of the biological response to sleep loss is unclear, however. For example, changes to these metabolites might represent a build-up in some metabolites due to inactivity, as described by Davies et al. (2014), or a build-up in metabolites in response to frequent meals given in CR protocols, as suggested by Chua et al. (2013). Consideration of these metabolites in the context of their metabolic pathway will aid in interpretation of the underlying processes contributing to changes observed during sleep loss. Furthermore, while sleep restriction and sleep deprivation studies conducted under less controlled conditions offer some insight into the

effects of sleep loss under real-life conditions, more research is needed using the gold-standard CR protocol. This approach will help to determine the extent of circadian control of the metabolome, which is integral in interpreting changes in some metabolites as discussed above, and to further understand inter-individual differences in the metabolic response to sleep deprivation, which is currently relatively unknown. Less controlled studies are needed in future, however, particularly for the validation of any identified biomarkers.

To determine the best metabolic candidates for inclusion in a panel of biomarkers for sleep pressure, replication of previous findings is required before any one metabolite or group of metabolites can be said to reliably change with sleep propensity. Despite the need for replication of previous findings, the research to date has shown that global metabolic profiling appears to be a useful technique for identifying metabolites that change in response to sleep loss and/or circadian phase. Given the rapid and dynamic changes in the metabolome in response to changes in our environment and physiology, this technique appears to be a promising method for identifying biomarkers of alertness state.

1.6. Conclusion and current research directions

Inadequate sleep, extended duration work shifts and non-standard work hours are becoming more common in today's 24-h society. The need for adequate alertness management strategies is therefore crucial to maintain productivity, health and safety. In order to appropriately apply alertness management strategies those who are at risk need to be identified. Identification of genotypes and other biological factors (e.g. sex, menstrual phase) associated with enhanced vulnerability to sleep loss will allow for the targeted application of countermeasures to reduce the impact of alertness failure on safety and productivity. Although these markers of vulnerability may help to prevent alertness mediated errors, accidents and injuries in those individuals who are most susceptible, detection of alertness state and prediction of future, within shift, alertness failure would be of additional benefit particularly in high risk environments. Therefore, while identification of biomarkers of vulnerability to alertness failure is important, identification of biomarkers of

alertness state will further contribute to the effective management of alertness in the workplace and on the roads.

Current research has examined both biomarkers of vulnerability to alertness failure and associated cognitive impairment and biomarkers of alertness state, using both targeted and untargeted approaches. To date, these approaches have not yet yielded a definitive biomarker of alertness, however, further investigation and validation of previous findings will help to narrow the field of biochemicals that may serve as biomarkers of alertness and expand the genetic and phenotypic candidates that contribute to vulnerability to sleep loss.

1.7. Thesis aims

The broad aim of this thesis is to examine a genetic marker of vulnerability cognitive impairment during sleep loss and, using a targeted and untargeted approach, identify endocrine and metabolic biomarkers of alertness state. The *BDNF* Val66Met polymorphism (**Chapter 3**) and menstrual phase (**Chapter 4**) will be examined as biomarkers of vulnerability to alertness failure and the progesterone/estradiol ratio (**Chapter 4**) and plasma metabolites (**Chapter 5**) will be examined as biomarkers of alertness state. More specifically, the aims of this thesis are to:

1) investigate the effects of the *BDNF* Val66Met polymorphism on a novel aspect of executive function, response inhibition, during 30-h of prolonged wakefulness (**Chapter 3**),

2) investigate female reproductive hormones associated with menstrual phase as novel biomarkers of performance impairment during 50-h of prolonged wakefulness and determine the effects of a light exposure intervention on both performance and candidate endocrine biomarkers (**Chapter 4**), and

3) characterise circadian and wake-dependent modulation of the human plasma polar metabolome during 40-h of prolonged wakefulness (**Chapter 5**).

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Chapter 2

Methodology

In **Chapter 2** the materials and methods, and rationale for the methodologies used in the three studies included in this thesis are discussed. Below is a brief description of the relevant aspects of the experimental designs of the three studies featured in the experimental **Chapters 3-5**:

Chapter 3: Participants included 30 healthy adults who underwent a 30-h CR. Eighteen participants had the BDNF Val/Val genotype and 12 were Val/Met heterozygotes. Participants completed the Stroop task every 2-h during the CR.

Chapter 4: Participants included 16 healthy pre-menopausal women who underwent a 49-h 40-min CR, followed by a 6.5-h light exposure centred in middle of the recovery day following the CR. Participants completed the Karolinska Sleepiness Scale, and the auditory Psychomotor Vigilance Task during the CR and light exposure, and the addition and digit symbol substitution tasks during the CR only. Blood samples were also collected at 0.5 to 2-h intervals for the purpose of analysing plasma melatonin, progesterone, estradiol, FSH, LH and sex hormone binding globulin (SHBG) concentrations.

Chapter 5: Participants included 13 healthy, young adults (9 males, 4 females) who underwent a 40-h CR. Four-hourly plasma samples collected during the CR were analysed using HILIC LC/MS to target polar metabolites.

Raster plots for each of the study protocols are shown in Figure 1. While the entire laboratory protocols are presented, this thesis includes data from only some aspects of these protocols, mainly the CRs. Studies 1 and 2 (**Chapters 3** and **4**) were conducted at the Center for Clinical Investigations at the Brigham and Women's Hospital, and Study 3 (**Chapter 5**) was conducted at the Monash University Sleep and Circadian Medicine Laboratory.

Further detailed descriptions of the methodologies for each of the studies can be found in the relevant experimental chapters. Given that the methodologies used in each of the studies were similar and designed based on similar principles, **Chapter 2** will provide justification for the experimental designs and methodologies that are common to the three experimental chapters. This

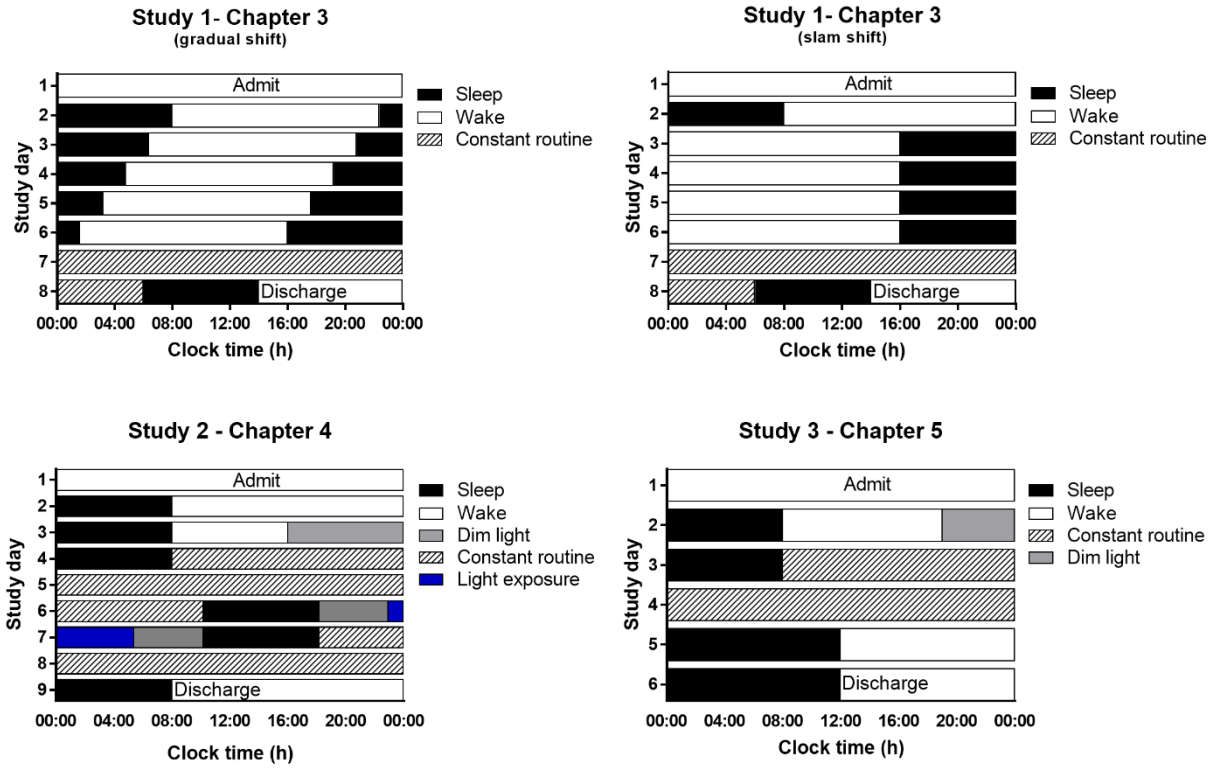

Figure 1. Study protocols for the three studies contributing to experimental chapters 3, 4 and 5. White bars represent scheduled wake episodes, black bars represent schedule sleep opportunities, grey bars represent dim light not under CR conditions, and bars with a diagonal pattern represent CRs. In Study 1 (Chapter 3), participants completed an 8-day laboratory protocol where prior to beginning a 30-h CR on day 7, sleep was advanced either gradually by 1.6-h per day over 5 days (*top left*), or by a 'slam shift' where the sleep episode is abruptly advanced by 8-h (*top right*). In Study 2 (Chapter 4), participants completed a 9-day laboratory study (*bottom left*). The protocol consisted of 3 baselines days, a 49-h 40-min CR (50-h 10-min wake episode) followed by an 8-h recovery sleep opportunity, a 16-h light exposure day (blue bar represents a 6.5-h light exposure occurring 4.75-h post wake on day 6) followed by an 8-h recovery sleep opportunity, and finally a second 29-h 50-min CR (30-h 10-min wake episode). Finally, in Study 3 (Chapter 5), participants completed a 6-day laboratory protocol, which consisted of 2 baseline days, a 40-h CR and 2 recovery days with up to 12-h sleep opportunities, followed by discharge (*bottom right*).

chapter will also expand on aspects of the methodologies where information is not provided in manuscripts featured within the experimental chapters.

2.1. Exclusion criteria and participant screening

Participants in the three studies described in **Chapter 3, 4 and 5**, underwent extensive screening to determine eligibility for each research study. Strict exclusion criteria ensured that participants did not have any sleep or circadian disruption prior to participating in the studies and were physically and psychologically capable of coping with the study requirements. Furthermore, these exclusion criteria were designed to generate an homogenous sample, such that the biomarkers could be investigated with minimal exogenous factors affecting the outcomes.

The exclusion criteria for each study are presented in Table 1. Details of the questionnaires and tests administered as part of the screening process are detailed below.

2.1.1. Sleep disorder questionnaires

2.1.1.1. Pittsburgh Sleep Quality Index (PSQI).

The PSQI is a 19-item self-report questionnaire designed to measure sleep quality in the past month (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989). The PSQI assesses seven components related to sleep including, subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleepiness medications and daytime dysfunction. Each component is given a score from 0 – "no difficulty", to 3 – "severe difficulty", and a global score is calculated by summing the scores from each of the seven components. A PSQI score greater than 5, which indicates poor sleep quality, was considered exclusionary for all studies in **Chapters 3-5.**

2.1.1.2. Epworth Sleepiness Scale (ESS)

The ESS is an 8-item self-report questionnaire that assesses levels of daytime sleepiness (Johns, 1991). The chance of dozing off or falling asleep in eight common situations, for example "sitting and reading", are rated from 0 – "would never doze" to 3 – "high chance of dozing". The scores for each item are summed to produce a composite score, with scores greater than 10 indicating excessive daytime sleepiness (Johns, 1991). The ESS was used in the screening process for all studies in **Chapters 3-5**, to exclude individuals with self-reported excessive daytime sleepiness (ESS> 10).

2.1.1.3. Fatigue Severity Scale (FSS)

The FSS is a 9-item self-report questionnaire that assesses the presence of fatigue and its impact on daily functioning (Krupp, Larocca, Muir Nash, & Steinberg, 1989). Each of the 9 items are rated from 1 – "strongly disagree" to 7– "strongly agree", with the scores from each item being summed to yield and overall score. Scores greater than 35, which suggests that an individual may be suffering from fatigue, were considered exclusionary for the study in **Chapter 5**.

2.1.1.4. Multivariable Apnea Predictor Questionnaire (MAPQ)

The MAPQ is a 3-item self-report questionnaire assessing obstructive sleep apnea (Maislin et al., 1995). The questionnaire requires the frequency of symptoms, including "snorting or gasping", "loud snoring", and "breathing stops, choke, or struggling for breath" to be rated on a 5-point scale from "Never" to "Always – 5-7/week". MAPQ scores greater than 0.5 were considered exclusionary for the study in **Chapter 5**. As detailed below in section 2.3., polysomnography (PSG) was also performed on the first laboratory night for the study in **Chapter** 5 to further confirm the absence of sleep apnea.

A multiple logistic regression equation including the average of the responses from the three questions (Index 1), age, BMI and gender is used to calculate the MAP index as follows:

MAP index = e^x / (1 + e^x) where,

x = -8.160 + 1.299*Index 1 + 0.163*BMI – 0.028*Index 1*BMI + 0.032*Age + 1.278*Male

Note. Male = 1 if male or 0 if female (Maislin et al., 1995).

2.1.1.5. Insomnia Severity Index (ISI)

The ISI is a 7-item self-report questionnaire that assesses symptoms of the sleep disorder insomnia (Bastien, Vallières, & Morin, 2001). The items on the ISI assess the subjective experience and consequences of insomnia symptoms in addition to the level of distress associated with these symptoms. Each item is rated from 0 – "none/ satisfied", to 4 – "very/ dissatisfied", and a global score is calculated by summing the scores for each item. Individuals with scores greater than 7, which indicates at least possible insomnia, were exclusionary for the study in **Chapter 5**.

2.1.1.6. Restless legs syndrome (RLS)

The presence of RLS was assessed with a brief questionnaire. The questionnaire included the following items, 1) "Do you have unpleasant sensations (such as creepy-crawly, electric shocks, pain, burning, or tightness) in your legs combined with an urge or need to move your legs?", 2) "Do these feelings occur mainly or only at rest and do they improve with movement?", 3) "Are these feelings worse in the evening or night than in the morning?", and 4) "If yes to all of the above, how often do these feelings occur?". The first three items require a yes or no response and the final item asks for the frequency of symptoms to be reported on a 7-point scale from 1 – "Less than one time a year" to 7 – "6-7 times per week". A positive test (i.e. indicating yes to the first question) was considered exclusionary for the study in **Chapter 5**. As detailed below in section 2.3., PSG was also performed on the first laboratory night for the study in **Chapter 5** to further confirm the absence of RLS.

2.1.1.7. The Sleep Disorders Questionnaire (SDQ)

The presence of sleep disorders was determine using the SDQ for the studies conducted in **Chapters 3** and **4**. The SDQ is a 175-item self-report questionnaire that measures typical sleep habits and sleep disturbances experienced in the past month (Douglass et al., 1994). Four categories of sleep disorders, including sleep apnea, narcolepsy, periodic limb movements and sleep disorders associated with psychiatric pathology (e.g. insomnia, nightmares, etc.), are evaluated. Each item is rated from 0 – "never/strongly disagree" to 5 – "always/ strongly agree" and the sores for each scale are produced by summing the ratings for each item within a scale. Scores greater than 35 (31 for women), 29 (30 for women), 18 (20 for women) and 20 were considered exclusionary for the sleep apnea, narcolepsy, psychiatric sleep disorders and periodic leg movement scales, respectively.

2.1.2. Psychiatric pathology questionnaires

2.1.2.1. Patient Health Questionnaire-9 (PHQ-9)

The PHQ-9 is a 9-item self-report questionnaire that assesses depressive symptomology over the past 2 weeks (Kroenke, Spitzer, & Williams, 2001). Each item represents one of the Diagnostic and

Statistical Manual of Mental Disorders-IV (DSM-IV) criteria for depressive disorders. The 9 items are rated from 0 – "not at all", to 3 – "nearly every day" and the score for each item is summed to obtain an overall score. Scores greater than 4, which represent at least mild symptomatology, were considered exclusionary for the study in **Chapter 5**.

2.1.2.2. Depression Anxiety Stress Scales 21 (DASS-21)

The DASS-21 is a 21-item self-report questionnaire that assesses negative emotional symptoms related to depression, anxiety and stress (Lovibond & Lovibond, 1995). The Depression scale measures symptoms associated with low mood (dysphoria), lack of interest and motivation, and other negative mood symptoms such as hopelessness and self-depreciation. The Anxiety scale assesses physiological aspects of anxiety such as the autonomic nervous system arousal, and also subjective experiences of anxiety such as panic and fear. Finally, the Stress scale assesses feelings of tension, irritability and agitation. Each of the 21 items are rated from 0 – "did not apply to me at all— never", to 3 – "applied to me very much, or most of the time— almost always". Scores for the Depression, Anxiety and Stress scales are calculated by summing the 7 items relevant to each scale, a multiplying the total score by two. Scores greater than 4, 3 and 7 for the Depression, Anxiety and Stress scales, respectively, were considered exclusionary for the study in **Chapter 5**.

2.1.2.3. Generalised Anxiety Disorder-7 (GAD-7)

The GAD-7 is a 7-item self-report questionnaire that assesses symptoms of generalised anxiety disorder in the past two weeks (Spitzer, Kroenke, Williams, & Löwe, 2006). Each item is rated from 0 – "not at all", to 3 – "nearly every day" and the score for each item is summed to obtain an overall score. Scores greater than 4, which represents mild anxiety, were considered exclusionary for the study in **Chapter 5**.

2.1.2.4. Beck Depression Inventory (BDI)

The BDI is a 21-item self-report questionnaire that assesses depressive symptomatology (Beck, Ward, Mendelson, Mock, & Erbaugh, 1961). For each item individuals must select one of four statements that most relates to them, where the first statement, given a score of 0, represents no

symptomatology (e.g. "I do not feel sad) and the last statement, given a score of 3, represents the greatest symptomatology (e.g. "I am so sad and unhappy that I can't stand it"). An overall score is then calculated by summing the scores for each item. A BDI score greater than 10, which indicates at least mild mood disturbance, was considered exclusionary for the studies in **Chapters 3** and **4**.

2.1.2.5. State Anxiety Scale (S-Anxiety)

The State Anxiety Scale, which is one of the subscales of the State-Trait Anxiety Inventory, is a 20-item self-report questionnaire that assesses current (state) levels of anxiety (Spielberger, 1983). Each of the 20 items are rated from 1 – "not at all" to 4 – "very much so". The S-Anxiety subscale includes both anxiety absent (e.g. "I am calm") and anxiety present (e.g. "I am tense") items, where anxiety absent items are reverse scored. Scores greater than 40 were considered exclusionary for the studies in **Chapters 3** and **4**.

2.1.2.6. Symptom Checklist 90-Revised (SCL-90-R)

The Symptom Checklist 90-R is a 90-item self-report questionnaire that assesses psychological symptoms and distress (Derogatis, 1994). The SCL-90-R assesses 9 symptom domains including somatisation, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation and psychoticism. Respondents are required to rate how bothered they are by different symptoms in the past week from 0 – "not at all", to 4 – "extremely". Distress levels on the depression, anxiety and paranoid ideation scales, characterised by scores greater than 1.25, on the hostility and psychoticism scales, characterised by scores greater than 1, and on the phobic anxiety scale, characterised by a score greater than 0.75, were considered exclusionary for the studies in **Chapters 3** and **4**.

2.1.2.7. Minnesota Multiphasic Personality Inventory-2(MMPI-2)

The MMPI-2 consists of 567 true/false questions that assess personality and psychopathology (Butcher et al., 2001). The MMPI-2 has 10 clinical subscales including, hypochondriasis, depression, hysteria, psychopathic deviate, masculinity/femininity, paranoia, psychasthenia (obsessive-compulsive), schizophrenia, hypomania and social introversion. In addition, there are

three validity scales including, the lie (L) scale which assesses dishonest answers, the F scale which assesses unusual or random answers, and the K scale which assesses under-reporting or psychopathology in individuals who otherwise have scores within the normal range. Raw MMPI-2 scores for each of the clinical subscales and validity scales are converted to T-scores based on normative data. A T-score of greater than 69 on the depression scale, and greater than 74 on the psychopathic deviance, schizophrenia and hypomania scale were considered exclusionary for the studies in **Chapters 3** and **4**. Scores greater than 79 on the L, F and K validity scales were also considered exclusionary.

2.1.3. Medical and psychiatric screening.

2.1.3.1. Psychiatric examination

Participants in all three studies underwent psychiatric screening via an interview with a clinical psychologist or psychiatrist. For the studies in **Chapters 3** and **4**, this included the administration of the MMPI-2, SCL-90-R, BDI, the State Anxiety Scale, and a structured clinical interview using the Structured Clinical Interview for DSM-III-R (SCID-R; note this data was collected before the release of DSM-V). For the study in **Chapter 5**, individuals underwent a structured clinical interview using the Structured Clinical Interview for DSM-V (SCID-5). Individuals with evidence of psychopathology based on this testing and review of their psychiatric history were excluded from participation. Furthermore, individuals with a family history of an Axis I disorder, including major depressive illness, bipolar disorder, schizophrenia, agoraphobia, or panic disorders were excluded. This thorough psychiatric screening was conducted to ensure that participants would be able to tolerate the conditions of the studies, including confinement, temporal isolation, and sleep deprivation. Moreover, these interviews were used to further ensure that participants understood the requirements of the study and that non-compliance with the protocol would not be an issue.

2.1.3.2. Medical examination

A medical examination by a physician was conducted in all three studies to ensure that all participants were free from any acute, chronic or debilitating medical conditions. The medical

examination included an analysis of medical history and a physical examination. As part of the medical examination for all three studies, routine pathology tests were also conducted to assess full blood count, thyroid stimulating hormone, erythrocyte sedimentation rate, C-reactive protein, urea, electrolyte and creatinine, and liver function. Furthermore, ECG recordings were collected during screening for all three studies to detect any cardiac abnormalities. This medical screening was conducted to ensure that participants were medically fit to tolerate the study requirements and that they had no underlying pathology that may alter the study outcomes.

2.1.3.3. Ophthalmic examination

For the studies in **Chapters 3** and **4**, an eye examination was conducted by an ophthalmologist to rule out the presence of disease or impairment of the visual system. Ensuring the absence of any visual abnormalities was important as the presence of eye disease or impairment may have affected the results of computerised testing and light exposure interventions conducted in these studies. While an ophthalmic examination was not conducted for the study in **Chapter 5**, any impairment or disease of the visual system was assessed via self-report and during the medical history examination with the physician.

2.1.3.4. Ishihara test

The Ishihara Test for Colour-Blindness (Ishihara, 2000) was used to assess whether participants were colour blind. Study lighting interventions and some cognitive tests (e.g. Stroop test) require normal colour vision, such that individuals with colour blindness were excluded in all three studies.

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Table 1. Exclusion criteria for the three thesis studies presented in **Chapters 3-5**.

Criteria	Chapter 3 BDNF	Chapter 4 Menstrual phase and sex hormones	Chapter 5 Metabolomics
Age	<18 or >50 years	<18 or >55 years	<20 or >65 years
Sex	-	Males	-
BMI	<18 or >30 kg/m^2	<18 or >30 kg/m^2	<18 or >30 kg/m^2
Bed times	Bedtime <10pm or >1am	Bedtime <10pm or >1am	Bedtime <10pm or >1am
Sleep duration	<7 or >9 hours/night	<7 or >9 hours/night	<7 or >9 hours/night
Shift work	In the past three years	In the past three years	In the past three years
Transmeridian travel	>2 time zones in the past three months	>2 time zones in the past three months	>2 time zones in the past three months
Medications	Any use (assessed via self-report, blood and urine toxicology)	Any use (assessed via self-report, blood and urine toxicology)	Any use (assessed via self-report and urine toxicology)
Medical	Any acute, chronic or debilitating medical conditions; current symptoms of active illness (e.g. hypertension, fever, leucocytosis—assessed via medical examination and blood tests)	Any acute, chronic or debilitating medical conditions; current symptoms of active illness (e.g. hypertension, fever, leucocytosis—assessed via medical examination and blood tests)	Any acute, chronic or debilitating medical conditions; current symptoms of active illness (e.g. hypertension, fever, leucocytosis—assessed via medical examination and blood tests)
Visual system	Any non-correctable impairment (assessed via ophthalmologist) and colour blindness (assessed via Ishihara Test)	Any non-correctable impairment (assessed via ophthalmologist) and colour blindness (assessed via Ishihara Test)	Any non-correctable impairment (assessed via self-report) and colour blindness (assessed via Ishihara Test)
Psychiatric	Current or previous diagnosis of any psychiatric disorder (assessed by psychologist/psychiatrist), or first degree relative with an Axis 1 disorder (assessed via self-reported)	Current or previous diagnosis of any psychiatric disorder (assessed by psychologist/psychiatrist), or first degree relative with an Axis 1 disorder (assessed via self-reported)	Current or previous diagnosis of any psychiatric disorder (assessed by psychologist), or first degree relative with an Axis 1 disorder (assessed via self-reported)
Sleep disorders	Diagnosis of any sleep disorder (assessed via self-report)	Diagnosis of any sleep disorder (assessed via self-report)	Diagnosis of any sleep disorder (assessed via self-report and confirmed during laboratory study via PSG)
Females only	Current pregnancy (assessed via urine test); Current breastfeeding; History of gynaecological pathology; Women in menopause or unable to become pregnant (e.g. from hysterectomy); Menstrual cycle length <26 or >35 days; Irregular menstrual cycle	Current pregnancy (assessed via urine test); Current breastfeeding; History of gynaecological pathology; Women in menopause or unable to become pregnant (e.g. from hysterectomy); Menstrual cycle length <26 or >35 days; Irregular menstrual cycle	Current pregnancy (assessed via urine test); Current breastfeeding; History of gynaecological pathology; Women in menopause or unable to become pregnant (e.g. from hysterectomy); Menstrual cycle length <26 or >35 days; Irregular menstrual cycle
Cigarette smoking	Current cigarette smoking (assessed via self-report)	Current cigarette smoking (assessed via self-report)	Current cigarette smoking (assessed via self-report)
Drugs of abuse	History of abuse or dependence; use during study (assessed via blood and urine toxicology)	History of abuse or dependence; use during study (assessed via blood and urine toxicology)	History of abuse or dependence; use during study (assessed via urine toxicology)

Table continues on the next page.

Criteria	Chapter 3 BDNF	Chapter 4 Menstrual phase and sex hormones	Chapter 5 Metabolomics
Alcohol	History of abuse or dependence; use during study (assessed via self-report)	History of abuse or dependence; use during study (assessed via self-report)	History of abuse or dependence; use during study (assessed via self-report)
Caffeine use	>300mg/day; use during study (assessed via urine toxicology)	>300mg/day; use during study (assessed via urine toxicology)	>300mg/day; use during study (assessed via self-report)
PSQI	>5	>5	>5
ESS	>10	>10	>10
DASS-21	-	-	Depression >4; Anxiety >3; Stress >7
MAPQ	-	-	>0.5
ISI	-	-	>7
RLS	Positive test (assessed via SDQ questionnaire)	Positive test (assessed via SDQ questionnaire)	Positive test (assessed via RLS questionnaire and PSG)
PHQ-9	-	-	>4
GAD-7	-	-	>4
FSS	-	-	>35
SDQ	Sleep apnea scale > 35 (31 for women) Narcolepsy scale > 29 (30 for women) Psychiatric sleep disorders > 18 (20 for women) Periodic leg movement > 21 for men and women	Sleep apnea scale > 35 (31 for women) Narcolepsy scale > 29 (30 for women) Psychiatric sleep disorders > 18 (20 for women) Periodic leg movement > 21 for men and women	-
BDI	>10	>10	-
S-Anxiety	>40	>40	-
SCL-90-R	Distress level on depression scale >1.25; Hostility scale >1; Phobic anxiety >0.75; Paranoid ideation >1.25; Psychoticism >1; Anxiety >1.25	Distress level on depression scale >1.25; Hostility scale >1; Phobic anxiety >0.75; Paranoid ideation >1.25; Psychoticism >1; Anxiety >1.25	-
MMPI-2	Depression scale T> 69; Psychopathic deviance, schizophrenia, hypomania scale T> 74; L, F and K validity scales & other clinical scales T> 79	Depression scale T> 69; Psychopathic deviance, schizophrenia, hypomania scale T> 74; L, F and K validity scales & other clinical scales T> 79	-

2.2. Pre-study requirements

In all three studies participants were required to maintain a regular 8:16-h sleep/wake schedule for at least two weeks prior to entering the laboratory. The schedule was self-selected by the participant and based on their habitual sleep and wake times reported during screening. Adherence to the participants' sleep/wake schedule was assessed using time-stamped call-ins at bed and wake times, and for at least one week prior to the laboratory admission, actigraphy and daily sleep diaries. Deviations of greater than 15 minutes either side of bed and wake times on one occasion on the week before admission resulted in exclusion or rescheduling of the participant, such that they had to

undergo additional pre-laboratory sleep/wake monitoring that did not deviate from their prescribed sleep and wake time prior to entering the laboratory. The strict sleep/wake schedule was used to ensure that participants maintained a consistent schedule of light exposure and sleep in order to minimise the effects of light on phase shifting the circadian clock (Boivin, Duffy, Kronauer, & Czeisler, 1996; Zeitzer, Dijk, Kronauer, Brown, & Czeisler, 2000) and ensuring participants did not enter the laboratory with a sleep debt that might affect study outcomes (Spiegel, Leproult, & Van Cauter, 1999; Van Dongen, Maislin, Mullington, & Dinges, 2003). During the pre-laboratory monitoring, participants were also asked to abstain from use of prescription and non-prescription medications, supplements, caffeine, recreational drugs and alcohol, and this was confirmed with urine toxicology upon admittance to the laboratory. These restrictions were in place to ensure that participants were not taking anything that could affect sleep or circadian rhythms.

2.3. Study protocols

Prior to starting study interventions (e.g. light exposure) or testing, participants in all three studies underwent one adaptation night to habituate them to the laboratory environment. During admit on the adaptation night participants were oriented to the laboratory and underwent initial practice testing to familiarise them with the tasks that would be completed throughout the study and to reduce potential learning effects on the cognitive tasks. For the study in **Chapter 5**, participants also underwent additional screening on the adaptation night to rule out the presence of sleep disorders. PSG was assessed by a trained technician following the sleep episode to rule out the presence of sleep apnea (Apnea-Hypopnea Index >5 exclusionary), restless legs syndrome, and periodic limb movements. The PSG set up included the following:

1. Nasal cannula and thermistor were used to assess air flow.
2. Pulse oximeter was used to determine oxygen saturation.
3. Thoracic and abdominal respiratory belts were used to measure respiratory effort.
4. EEG was used to determine arousals from sleep.
5. Electromyography recorded from the legs and chin were used to assess muscle movements.

Throughout all three of the studies, participants were monitored constantly by staff in individual, time-cue free suites where they did not have access to windows, clocks, live television or radio, and newspapers. All staff were trained not to reveal the time of day when interacting with participants. Participants were studied in a time-cue free environment as knowledge of time of day has been suggested to be weak Zeitgeber capable of circadian entrainment (Czeisler, Richardson, Zimmerman, Moore-Ede, & Weitzman, 1981), and furthermore, knowledge of time of day may influence cognitive performance if a participant believes that he or she would typically be feeling less alert or perform more poorly at that time of day.

All of the study events (e.g. testing, sample collection, etc.) that occurred in each of the three protocols were timed relative to each participant's habitual wake time during the pre-study sleep monitoring period, which was also maintained when participants were in the laboratory. Timing the study relative to habitual wake time was to ensure that study events occurred at equivalent times since wake for each participant.

2.3.1. Constant routine

In each of the three studies, participants underwent a CR protocol. CRs are designed to keep constant or distribute evenly across the study protocol any environmental cue, for example, light, posture, sleep, temperature, and meal timing, that may mask or influence circadian rhythms (Duffy & Dijk, 2002; Minors & Waterhouse, 2009). CR protocols therefore allow for the assessment of endogenously generated circadian rhythms, and given that participants remain awake throughout the protocol, also allow for the assessment of the influence of extended wakefulness on study outcomes. The duration of the protocol typically lasts for more than 24-h in order to measure at least one circadian cycle. During the CR protocols, participants remained awake in a constant semi-recumbent posture (head of bed at 45˚) in dim light conditions of <3 lux. Room temperature was also kept constant (~22˚C) and participants receive their daily fluid and caloric intake in hourly iso-caloric snacks. The meal composition in each study was as follows: 1) 150mEq Na^+/ 100 mEq K^+ (±20%), 1.5 x basal energy expenditure, 2500mL fluids/24 h (**Chapter 3)**, 2) 150mEq Na^+/ 100

mEq K^+ (±20%), 1.5 x basal energy expenditure, 2000-2500mL fluids/24 h (**Chapter 4)**, and 3) 1804 ± 99 cal/ day, 1.1 x basal energy expenditure, 2400mL fluids/ 24 h (**Chapter 5**). Participants were monitored closely by technicians at all times during these protocols to ensure that they did not fall asleep. EEG was also recorded constantly to confirm that participants remained awake throughout the protocol and during times (e.g. toilet breaks) where participants were not directly monitored.

2.4. Lighting conditions

Given the phase resetting (Boivin et al., 1996; Lockley, Brainard, & Czeisler, 2003; Wright, Hughes, Kronauer, Dijk, & Czeisler, 2001; Zeitzer et al., 2000) and acute alerting effects of light (Lockley et al., 2006; Phipps-Nelson, Redman, Dijk, & Rajaratnam, 2003; Rahman et al., 2014), lighting conditions were highly controlled throughout the studies. During baseline days, if no light intervention was used, participants remained in normal room lighting of 100-200 lux and lights were turned off (0 lux) during sleep episodes. During CR and other aspects of the protocol that required dim light, for example DLMO assessments, lights were lowered to <3 lux. Routine light measurements taken from both the horizontal and vertical plane were conducted throughout the studies to confirm lighting conditions. Further details of study specific lighting conditions are given in the relevant experimental **Chapters 3-5**.

2.5. Blood sample collection and storage

Blood samples were collected via an indwelling cannula located in the antecubital vein or forearm. Blood samples were aliquoted into blood tubes with an ethylenediaminetetraacetate (EDTA) additive and then centrifuged to separate the plasma. Following separation, the plasma fraction was aliquoted into plastic storage tubes and placed into long term storage at -20˚C (melatonin and reproductive hormone samples) or -80˚C (metabolomics samples), depending on the type of sample.

The collection, processing and storage of metabolomics samples (analysed in **Chapter 5**) was of particular importance given the effects that preanalytical variability can have on LC-MS based metabolomics analyses. The temperature of samples during processing, the presence of haemolysis, the blood tube additive and storage temperature can all affect measurement of metabolites in plasma [for review see (Yin, Lehmann, & Xu, 2015)]. Attempts to reduce the effects of these variables on metabolomics samples analysed in **Chapter 5** are detailed below.

2.5.1 Haemolysis

Haemolysis, the most common preanalytical error in clinical settings (Gimenez-Marin, Rivas-Ruiz, Perez-Hidalgo, & Molina-Mendoza, 2014), is caused by the rupture of red blood cells causing their contents, including metabolites, to leak into the plasma fraction of the sample. In study three (**Chapter 5**), haemolysis of samples was caused in large part by difficult or slow bleeds and overly vigorous mixing of the blood tubes. To reduce the extent of haemolysis, participants were kept well hydrated, cannulas were placed in the forearm rather than in the antecubital vein (whenever possible) to avoid kinking, and standard operating procedures, distributed to nursing staff, highlighted the importance of mixing in the blood tube's EDTA additive gently. Samples with moderate to severe haemolysis, judged based on the colour of the sample (dark orange to red), were excluded from the metabolomics analysis (Kamlage et al., 2014; Yin et al., 2013). Haemolysed blood samples were also excluded from analysis of hormonal assays (melatonin and reproductive hormones) used in **Chapters 3** and **4**.

2.5.2. Blood tube additive

Blood tube additives have been shown to affect the metabolomic profile of plasma samples (Gonzalez-Covarrubias, Dane, Hankemeier, & Vreeken, 2012; Yin et al., 2013). As such, the same blood tube additive, K^2EDTA, was used consistently throughout the study in **Chapter 5**.

2.5.3. Pre-processing storage and temperature

Following collection of whole-blood, samples were placed immediately into the centrifuge at 4˚C, or were stored in a refrigerator (4˚C) when the centrifuge was occasionally already in use. All

samples were processed within 30 minutes of collection and in the exact same manner (except when briefly stored in a refrigerator before centrifugation) to ensure that storage temperature would not affect the samples. Storing samples at this temperature for a short period of time stops enzymatic activity and metabolic activity of cells within the sample (Kamlage et al., 2014; Yin et al., 2013).

2.5.4. Post-centrifugation storage and temperature

Following centrifugation blood samples were kept in the centrifuge at 4˚C until being aliquoted into cryo-tubes. Aliquots sizes were kept small (500µL) to avoid multiple freeze-thaw cycles during later analysis. At this temperature, plasma samples have been shown to stay relatively stable for up to 16-h (Kamlage et al., 2014). Samples collected for metabolomics analysis remained at 4˚C for a maximum of 30 to 60-min before being aliquoted at room temperature in a laminar flow cabinet. After being aliquoted, the plasma samples were transferred immediately onto dry ice and stored for up to 12-h before being transferred to long term storage at -80˚C. When stored on dry ice, the samples were frozen within 1 to 2-min and, given the stability of samples for short periods of time even when stored at -20˚C, this should have been adequate to stop further changes occurring within the plasma samples (Pinto et al., 2014).

2.5.5. Long term freezer storage

As recommended for these types of samples (Vaught, 2006), plasma for metabolomics analysis was stored at -80˚C. The metabolic profile of samples stored at -20˚C for one month shows small changes compared to samples stored at -80˚C, with these small changes occurring at -20˚C being attributed to the activity of proteins (Pinto et al., 2014). Metabolomics analysis of samples stored at colder temperatures (-70˚C to -80˚C) for up to 30 months show only small changes (Mitchell, Yasui, Li, Fitzpatrick, & Lampe, 2005; Pinto et al., 2014). At the time of analysis, samples had been stored at -80˚C for up to approximately 12 months.

2.6. Metabolomics quality control measures

Several quality control measures were used for the metabolomics analysis in **Chapter 5**. Each of these measures are described below:

2.6.1. Plasma quality control (PQC)

PQCs were created from pooled plasma, which included 20µL of plasma from each sample prior to metabolite extraction. The pooled mixture was then aliquoted into 12 20µL PQCs. PQCs were extracted every tenth sample per batch of 24, such that each extraction batch included 22 samples and 2 PQCs. PQCs were then inserted into the run every tenth sample, starting after the fifth sample (Figure 2). Metabolite extraction were conducted in batches of 24 due to equipment restrictions on the number of samples that could be prepared at once. The PQCs were used to monitor any batch effects, where one batch may have been prepared differently to another.

1 S	2 S	3 S	4 S	5 S	6 PQC	7 S	8 S	9 S	10 S	11 S	12 PBQC
13 S	14 S	15 S	16 S	17 S	18 PQC	19 S	20 S	21 S	22 S	23 S	24 PBQC
25 S	26 S	27 S	28 S	29 S	30 PQC	31 S	32 S	33 S	34 S	35 S	36 PBQC
37 S	38 S	39 S	40 S	41 S	42 PQC	43 S	44 S	45 S	46 S	47 S	48 PBQC
49 S	50 S	51 S	52 S	53 S	54 PQC	55 S	56 S	57 S	58 S	59 S	60 PBQC
61 S	62 S	63 S	64 S	65 S	66 PQC	67 S	68 S	69 S	70 S	71 S	72 PBQC
73 S	74 S	75 S	76 S	77 S	78 PQC	79 S	80 S	81 S	82 S	83 S	84 PBQC
85 S	86 S	87 S	88 S	89 S	90 PQC	91 S	92 S	93 S	94 S	95 S	96 PBQC

Figure 2. Example of the plate layout of samples (S), PQCs and PBQCs. PQCs and PBQCs were inserted every tenth sample in the run, alternating after every fifth sample.

2.6.2. Pooled biological quality control (PBQC)

Following extraction of the samples, 10µL from each sample was pooled to create a PBQC mixture. From this mixture, 12 PBQCs were created and these were inserted into the run every

tenth sample, starting after the eleventh sample (see Figure 2). These PBQCs were used to monitor instrument performance (e.g. retention time drift) throughout the ~2 day run time.

2.6.3. Spiked internal standards

The extraction solvent used to extract metabolites was spiked with 2µM ^{13}C-sorbitol, 2 µM $^{13}C^{15}N$-AMP, and 2 µM $^{13}C^{15}N$-UMP as internal standards. These internal standards were used to monitor sample processing (i.e. to ensure the same amount of extraction solvent was pipetted into each sample) and recovery of metabolites from the mass spectrometer.

2.6.4. Sample randomisation

Each of the samples were randomised by participant and timepoint before being loaded onto the plates. Sample randomisation was used to ensure that any analytical variability would not affect only samples from any one participant (i.e. if participant order was not randomised), or any one timepoint (i.e. if time was not randomised).

2.7. Marker of the biological clock

Dim light melatonin onset (DLMO) is the most commonly used phase marker of the melatonin rhythm. Calculation of DLMO can be done in several different ways including the 1) threshold method, 2) two standard deviation method, and 3) percentage method (Benloucif et al., 2008). In the current thesis, both a threshold and percentage method were used and these are discussed below.

2.7.1. Threshold method

The threshold method uses a set threshold of melatonin concentration that once reached is used as the time of melatonin onset. The time at which melatonin levels reach the set threshold is determined by linear interpolation between adjacent samples. A threshold of 10pg/mL (Brown, Choe, Shanahan, & Czeisler, 1997; Klerman, Gershengorn, Duffy, & Kronauer, 2002; Voultsios, Kennaway, & Dawson, 1997) is typically reported in the literature for plasma melatonin. Lower thresholds (e.g. 5pg/mL), however, are less influenced by the amplitude of the melatonin rhythm,

whereas the use of higher thresholds can lead to estimations of later DLMO times for individuals who secrete low levels of melatonin (Lewy, Cutler, & Sack, 1999). Despite the 5pg/mL cut-off being less influenced by amplitude, the higher 10pg/mL threshold is more comparable to salivary DLMO assessments, which typically use a cut-off of 3-4pg/mL, as the levels of melatonin in plasma are approximately three times greater than in saliva (Voultsios et al., 1997). As plasma melatonin alone was used to estimate DLMO, a threshold of 5pg/mL was used to calculate DLMO for study three (**Chapter 5**), such that interindividual differences in the amplitude of the melatonin rhythm would have less impact on the estimation of DLMO.

2.7.2. Percentage method

The other method used for DLMO calculation in studies one and two (**Chapters 3** and **4**) was the percentage of maximum peak. Here DLMO was defined as the time at which plasma melatonin levels rose to 25% of the nightly fitted peak. The nightly fitted peak was calculated by fitting a 3-harmonic waveform to the plasma melatonin data collected during CR (Brown et al., 1997). The 3-harmonic fit assumes that the slope of the melatonin rhythm can be fit using a fundamental and 2-harmonic sinusoidal curve, for example 24 (fundamental), 12 and 8-h harmonics (St Hilaire, Gronfier, Zeitzer, & Klerman, 2007). The amplitude of the fitted waveform was used to determine the 25% crossover threshold. After calculating 25% of the amplitude, linear interpolation was used between adjacent samples to determine the minute when plasma levels rose to 25% of the nightly fitted peak as described for the threshold method above. Th percentage method normalizes differences in the amplitude of the melatonin curve between individuals and thus can be used to compare DLMO between groups (Benloucif et al., 2008). This method is the standard practice for calculating DLMO in the laboratory in which the data for studies one and two (**Chapters 3** and **4**) were collected. In certain situations, for example where there is a large number of missing samples near DLMO, or in very low secretors [e.g. in some older individuals (Hughes, Sack, & Lewy, 1998; Zeitzer et al., 1999)], this method may be more useful than threshold based methods for calculating DLMO. A comparison of different methods for assessing DLMO, however, showed that in young healthy individuals there is no one method that is better than the others, and for all methods the

standard deviation of the DLMO estimate was low (Klerman et al., 2002). Therefore, while the method used to calculate DLMO differed in study three (**Chapter 5**) compared to studies one and two (**Chapters 3** and **4**), the method was kept consistent within individual studies and DLMO times were not compared between studies.

2.8. Alertness and performance testing

The following tests were used to assess performance and alertness in the three studies that comprise the experimental chapters of this thesis. Not all tests were used in each study, and the study that each test is relevant to is listed in the description for that test.

2.8.1. The Stroop task

The Stroop task has been shown to be sensitive to sleep deprivation and circadian phase, such that reaction times are longer and more errors are made as time awake increases and during the biological night, particularly on trials that require the inhibition of a prepotent response (Burke, Scheer, Ronda, Czeisler, & Wright, 2015; Cain, Silva, Chang, Ronda, & Duffy, 2011; Sagaspe et al., 2006). The version of the Stroop task used in study one (**Chapter 3)** included three trial types: congruent, incongruent and neutral. In incongruent trials, the font colour and word colour were different (e.g. "green" written in red ink), such that to complete these trials correctly, the prepotent response of reading the word must be inhibited to correctly name the colour of the ink. Neutral trails were presented as a row of four coloured X's (e.g. "XXXX" written in green ink) and the purpose of these trails was to provide a measure of the time taken to name a colour in the absence of interfering (incongruent trials) or facilitating (congruent trials) words. Finally, in the congruent trials, the font colour and the word colour were the same (e.g. "green" written in green ink). Not all versions of the Stroop task include the congruent trial; however, inclusion of this trial type is thought to reduce the use of a reading suppression strategy in which individuals avoid reading the word, for example by not focusing their gaze (Cain et al., 2011). For all trial types, the word or Xs were presented on a computer monitor against a black background and participants were asked to respond by naming the colour of the text (red, green, blue or yellow) by typing the first letter of the

colour on the keyboard (R, G, B or Y). The main outcomes of the Stroop task included reaction time and error rate for each trial type. An additional measure of inhibition was calculated as the reaction time of neutral trials subtracted from the reaction time of incongruent trials. This calculation yields a score of the cost of inhibition, or the additional time needed to respond when inhibiting a prepotent response. The Stroop task was used as a measure of response inhibition in the study in **Chapter 3**.

2.8.2. Psychomotor Vigilance Task

The PVT is one of the most commonly used tasks to assess attention and vigilance in sleep and circadian studies [for review see Lim and Dinges (2008)]. The PVT is sensitive to acute sleep deprivation and chronic sleep restriction (Banks, Van Dongen, Maislin, & Dinges, 2010; Basner & Dinges, 2011; Dinges et al., 1997; Doran, Van Dongen, & Dinges, 2001; Van Dongen et al., 2003), and to changes in performance associated with circadian phase (Burke et al., 2015; Graw, Kräuchi, Knoblauch, Wirz-Justice, & Cajochen, 2004; Shekleton et al., 2013; Wright, Hull, & Czeisler, 2002; Wyatt, Ritz-De Cecco, Czeisler, & Dijk, 1999). The task requires participants to respond to a visual (e.g. counter counting up in milliseconds) or auditory (e.g. a tone played) stimulus presented at random intervals (1 to 10 seconds) as quickly as possible by pressing a button on a hand-held response box. While the visual PVT is more commonly used, the auditory version (aPVT) has also been shown to be sensitive to sleep loss and circadian phase (Jung, Ronda, Czeisler, & Wright, 2011) and is useful during light exposure protocols as it does not require the use of a computer screen which would provide a light source other than the experimental light manipulation. The outcome measures of the PVT used in the current thesis are the mean reaction time of the overall 10-minute task, and lapses of attention which are response times greater than 500ms. Due to the light exposure portion of the protocol, the aPVT was used as a measure of vigilance and sustained attention in the study in **Chapter 4**.

2.8.3. Addition task

The addition task requires participants to add together as many 2-digit numbers as possible within a 2-min timeframe. The test outcomes include how many additions are attempted, the number of correct and the number of incorrect additions. The addition task has been shown to be sensitive to both time awake and circadian phase (Burke et al., 2015; Frey, Badia, & Wright, 2004). The number of correct additions on the addition task was used as a measure of cognitive throughput in the study in **Chapter 4**.

2.8.4. Digit Symbol Substitution Task (DSST)

The DSST is a measure of working memory and processing speed. At the top of the computer screen the digits 0 to 9 are paired with symbols (e.g. 1 – triangle, 2 – circle, etc.). Each trial during the 1.5-min task requires participants to respond as quickly as possible by entering the corresponding number of a symbol that is displayed in the centre of the screen. The number-symbol pairs remain the same throughout the task, while the symbol displayed in the centre of the screen changes for each trial. The number of correct responses on the DSST, the outcome measure used in this thesis, is sensitive to time awake and circadian phase (Burke et al., 2015; Groeger et al., 2008; Shekleton et al., 2013). The DSST was used as a measure of measure of working memory and processing speed in the study in **Chapter 4**.

2.8.5. Karolinska Sleepiness Scale

The Karolinska Sleepiness Scale (KSS) is a self-report measure of subjective sleepiness (Åkerstedt & Gillberg, 1990). Participants rate their level of sleepiness on a 9-point scale from 1 – "Extremely alert" to 9 – "Extremely sleepy, fighting sleep". KSS scores are highly correlated with both behavioural and physiological measures of sleepiness (Gillberg, Kecklund, & Akerstedt, 1994; Kaida et al., 2006). The KSS was used as a measure of subjective sleepiness/alertness in the study in **Chapter 4**. The revised version of the scale, which has labels for each of the 9 steps on the scale (Baulk, Reyner, & Horne, 2001) was used for the study in **Chapter 5**, whereas the original scale,

which has labels on every second step, was used in **Chapters 3** and **4**. While KSS scores were collected in all three studies, KSS scores were only used in analyses for **Chapter 4**.

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Chapter 3

Impaired cognitive flexibility during sleep deprivation among carriers of the Brain Derived Neurotrophic Factor (*BDNF*) Val66Met allele

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Preface to Chapter 3

As reviewed in **Chapter 1**, several genes have been identified as genetic markers of vulnerability to alertness mediated performance impairment resulting from acute sleep loss. A common polymorphism in the Brain Derived Neurotrophic Factor (*BDNF*) gene has previously been associated with working memory impairment (Bachmann et al., 2012) and in **Chapter 3** the effects of this polymorphism on another executive function, response inhibition, was investigated.

While measures of executive function are typically not used to measure alertness, it could be argued that higher order cognitive functions are likely subservient to the level of cortical arousal, such that a basic level of alertness is required not only to respond to a simple reaction time task, but also to engage with more complex cognitive tasks, such as response inhibition. During acute sleep deprivation, brain regions showing deactivation include the thalamus and prefrontal cortex (Thomas et al., 2000). Both of these regions subserve alertness and attention (Sadaghiani et al., 2010; Sturm & Willmes, 2001), and the prefrontal cortex is additionally involved in higher order cognition, including response inhibition (Garavan, Ross, & Stein, 1999; Menon, Adleman, White, Glover, & Reiss, 2001; Ridderinkhof, Van Den Wildenberg, Segalowitz, & Carter, 2004). Furthermore, inhibition on the Stroop task specifically has been shown to predominantly activate the anterior cingulate cortex (Bench et al., 1993; Pardo, Pardo, Janer, & Raichle, 1990; Peterson et al., 1999), another brain region involved in alertness (Sadaghiani et al., 2010; Sturm & Willmes, 2001). Therefore, given the overlap in brain regions controlling both alertness and higher order cognitive functions such as response inhibition, performance impairment on the Stroop task, particularly during sleep loss, may be due to a failure of inhibitory control or momentary lapses of underlying alertness.

During prolonged wakefulness, reaction time and error rates on the Stroop task show a similar pattern of impairment to other measures of neurobehavioural performance that are commonly used to measure alertness (Bratzke, Steinborn, Rolke, & Ulrich, 2012; Cain, Silva, Chang, Ronda, & Duffy, 2011), suggesting that the Stroop task is sensitive to changes in alertness.

The executive component of the task, however, as measured by the difference score between the reaction time of neutral and incongruent trials, appears to be more strongly regulated by the circadian system, rather than the homeostatic system (Burke, Scheer, Ronda, Czeisler, & Wright, 2015). As the circadian system plays a central role in alertness regulation, vulnerability on the inhibition aspect of the task may indicate vulnerability to circadian mediated alertness failure and cognitive impairment. Therefore, in **Chapter 3**, response inhibition, measured by the Stroop task, was used to determine whether the *BDNF* Val66Met polymorphism is a marker of vulnerability to performance impairment, which may, in part, be due to an underlying alertness failure. It was expected that carriers of the vulnerable Met allele would perform more poorly, particularly on incongruent trials, compared to individuals with the homozygous Val/Val genotype.

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Short communication

Impaired cognitive flexibility during sleep deprivation among carriers of the Brain Derived Neurotrophic Factor (*BDNF*) Val66Met allele

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ABSTRACT

Accumulating evidence points to a genetic contribution to explain inter-individual vulnerability to sleep deprivation. A functional polymorphism in the *BDNF* gene, which causes a valine (Val) to methionine (Met) amino acid substitution at Codon 66, has been associated with cognitive impairment, particularly in populations with impaired frontal functioning. We hypothesised that sleep deprivation, which affects frontal function, may lead to cognitive dysfunction in Met allele carriers. To examine this, we investigated, in different *BDNF* genotypes, the effects of sleep deprivation on cognitive flexibility, as measured by response inhibition using the Stroop Color Naming Task. Thirty healthy, adults of European ancestry, including 12 heterozygous Met allele carriers and 18 Val/Val homozygotes, underwent 30-h of extended wakefulness under constant routine conditions. A computerised Stroop task was administered every 2 h. Error rate and reaction times increased with time awake for all individuals. Participants with the Val/Met genotype made more errors on incongruent trials after 20 h awake. While Val/Met participants also took significantly longer to respond when inhibiting a prepotent response irrespective of time awake, this was particularly evident during the biological night. Our study shows that carriers of the *BDNF* Met allele are more vulnerable to the impact of prolonged wakefulness and the biological night on a critical component of executive function, as measured by response inhibition on the Stroop task.

At the group level, sleep deprivation has a detrimental impact on cognitive function (See [1,2] for reviews). Some individuals, however, are more vulnerable than others to the adverse effects of sleep deprivation on performance [3]. Importantly, this vulnerability appears to be trait-like and likely has a genetic basis[4]. A number of candidate genes, such as *PER3*, *COMT* and *ADORA2A*, have been identified as contributing to individual variability in cognitive function following sleep deprivation [5–7]. More recently, a polymorphism in the Brain Derived Neurotrophic Factor (*BDNF*) gene has shown promise as a genetic marker of vulnerability to sleep deprivation [8]. While this gene influences cognitive function in well-rested, healthy adults, the effects of this polymorphism on vulnerability to sleep deprivation is comparatively less well-studied.

BDNF, a member of the neurotrophin family of growth factors, plays an important role in neuronal development and survival, context-dependent synaptic plasticity and long-term potentiation [9]. *BDNF* is expressed throughout the central nervous system, and is particularly abundant in the prefrontal cortex [9,10]. A common single nucleotide polymorphism has been identified in the *BDNF* gene which produces an amino acid substitution from valine (Val) to methionine (Met) at codon 66 (Val66Met) [11]. This Met substitution leads to impairments in intracellular processing, trafficking and activity dependent secretion of the *BDNF* protein [12], and is associated with reductions in cortical volume and thickness in prefrontal regions [10,13]. The *BDNF* Val66Met polymorphism has been linked to individual differences in cognitive performance, such that *BDNF* Met allele carriers perform more poorly on cognitive tasks, especially those relating to memory, learning and executive function [11,13–15], when well-rested. This finding is not consistent however, with other studies reporting no effects [16–18]. As Met allele impairments are more consistently found where frontal functioning is compromised, such as in schizophrenia, bipolar disorder and Obsessive Compulsive Disorder [11,19–21], we hypothesise that the Met allele may result in enhanced vulnerability to sleep deprivation where frontal function is also impaired [1,2].

To our knowledge, only one study has investigated the relationship between sleep deprivation, performance and *BDNF* genotype in

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humans. Although they reported no effect of the polymorphism on the psychomotor vigilance task, they did report that response accuracy on a working memory task was significantly poorer in Met allele carriers compared to Val/Val homozygotes during sleep deprivation [8]. To determine whether this effect may be evident across other cognitive domains, our study examined whether individuals with the *BDNF* Val/Met genotype, compared to Val/Val homozygotes, were more vulnerable to sleep deprivation on a task of response inhibition.

Thirty participants of self-reported European ancestry completed an 8-day inpatient study. Twelve participants carried the Val/Met genotype (4 women (33.3%); 23.17 ± 4.22 years) and 18 carried the Val/Val genotype (8 women (44.4%); 22.89 ± 5.18 years). All participants were free from medical, psychiatric and sleep disorders. Individuals with color blindness, and those who had engaged in night and/or shift work in the past 3 years, or who travelled across more than one time zone in the previous 3 months were excluded. Participants maintained a consistent, self-selected 8:16 h sleep/wake schedule for three weeks prior to the study, confirmed by time-stamped calls at sleep/wake times and actigraphy (Actiwatch-L, Minimitter, Inc, Bend, OR). The use of any medications, supplements, recreational drugs, nicotine, caffeine, and/or alcohol were prohibited for the duration of the study, and was confirmed by urine and blood toxicology prior to study admission. The protocol was approved by the Partners Human Research Committee at the Brigham and Women's Hospital, and participants provided full written informed consent.

Participants were continuously monitored for 8 consecutive days in a time free environment (no windows, clocks, radio, live TV, newspapers, and technicians trained to not reveal the time). Prior to commencing a 30-h constant routine (CR), participants underwent a phase advance protocol involving the 8-h advance of the sleep-wake cycle over 5 days – reported elsewhere [22,23]. During the CR, participants remained awake in dim light conditions (3 lx), in a semi-recumbent posture (head of bed at 45°), and received their equivalent daily fluid and caloric intake in hourly snacks. Participants completed a computerised version of the Stroop Color Naming Task every two hours, starting three hours post wake. The task included three trial types, where the font color and word color were the same (congruent) or different (incongruent), or simply consisted of a row of 4 colored "X"s (neutral). For all trial types, participants were asked to respond by naming the color of the text by typing the first letter of the color on a keyboard (See [24] for more details).

To determine circadian phase, Dim Light Melatonin Onset (DLMO) was defined as the time at which plasma melatonin levels rose to 25% of the nightly fitted peak. This was determined using the 3-harmonic method (See [25] for more details). DNA was extracted from whole blood and genotyped for the rs6265 (coding DNA variant Val66Met) variant of the *BDNF* gene using TagMan SNP genotype assays (Applied Biosystems, Assay ID: C1159275810). PCR amplification and allele specific discrimination was performed using LightCycler 480 (Roche Applied Science, USA). The reaction consisted of 10 ng DNA, 6.25 µl of 2X master mix (Applied Biosystems, catalog number: 4371357), 0.65 µl of 20X Taqman SNP genotyping assay in a PCR reaction volume of 12.5 µl. Annealing was performed at 60 °C. The LightCycler 480 software was used to detect specific SNP alleles using the end-point detection method. Genotyping was performed in duplicate to ensure reproducibility and a call rate > 95% was obtained.

Polysomnography (PSG) was recorded using a Vitaport-3 recording system (TEMEC Instruments, B.V. Kerkrade, The Netherlands). EEG (C3, C4 referenced to contralateral mastoids), EOG (upper and lower outer-canthi) and submental EMG was obtained. Electrode impedances were < 10 kΩ, and EEG signals were filtered (high-pass EEG filter 0.23 Hz; low-pass EEG filter 70.1 Hz; 24 dB/octave, sampling rate 256 Hz) and sleep staged in 30-s epochs according to the Rechtschaffen and Kales scoring system. Total sleep time (TST); sleep efficiency (% TST of time in bed) and wake after sleep onset (WASO) was calculated.

Mean reaction time (RT) and the error rate (percent incorrect responses) for congruent, incongruent and neutral trials were calculated for each Stroop session. A difference score (RT of incongruent trials - RT of neutral trials) was also calculated within each session as an index of inhibition. The inhibition score, or the cost of inhibition, reflects the additional time needed to inhibit the prepotent response for incongruent trials. Test sessions were averaged over three time periods in order optimise power for the analysis: Tertile 1: 0–10-h, Tertile 2: 11–20-h and, Tertile 3: 21–30-h. To examine the effect of sleep deprivation, PROC MIXED analyses (SAS 9.4) were performed with tertile and genotype modelled as fixed factors and participant and circadian phase modelled as random factors. Degrees of freedom were calculated using the Kenward-Rogers method and the covariance type with the lowest Schwarz Bayesian Criterion (BIC) was chosen. To control for familywise error, post hoc pairwise comparisons were conducted using a false discovery rate (FDR) comparison, reported as p_{adj}. As a secondary aim, we examined the impact of circadian time. Independent samples *t*-tests (SPSS Statistics 20) compared performance during the 'biological day' (average of three tests occurring at least one hour before DLMO) versus 'biological night' (average of three tests occurring at least one hour after DLMO). Only participants who had data for at least three sessions before and after DLMO were included (Val/Met: n = 11 (~92%); Val/Val: n = 16 (~89%)).

Data were successfully obtained from 30 participants, except for phase where we could not estimate DLMO for n = 1 (Val/Met) and sleep where there was not a polysomnography recording for n = 2 (Val/Met). Participant demographic information is shown in Table 1. The genotype frequencies observed in the current sample did not differ from the expected Hardy-Weinberg equilibrium ($\chi^2_1 = 2.2$, $p > 0.10$). There were no participants with the Met/Met genotype; however, this was expected given the low frequency of the genotype in populations of European ancestry (< 5%) [11]. To ensure the different phase advance protocols did not affect Stroop outcomes during CR, we conducted a linear mixed model to determine whether the different light or phase shift conditions during the phase advance affected any of the Stroop outcomes. There was no effect of light condition or phase advance protocol on any Stroop outcome (see Table S1 in supplemental material). Means and standard deviations for all Stroop outcomes by genotype, and the results of the linear mixed model are reported in Table 2.

Overall, response times were slower and the error rate of responses was greater as time awake progressed (See Table 2; all trial types $p < 0.0001$). Post hoc comparison revealed that reaction times were slowest and more errors were made in the third tertile of sleep deprivation (20–30 h awake), compared to both the first (0–10 h awake; congruent and incongruent: $p < 0.0001$) and second tertiles (10–20 h awake; all trial types: $p < 0.0001$). There was no difference between the first and second tertiles for either reaction time (all trial types: $p > 0.3$) or error rate (all trial types: $p > 0.7$). Post-hoc paired *t*-test statistics for the main effect of time are shown in supplemental Table S2.

Table 1
Participant demographic information by genotype.

Demographic	Val/Val (M ± SD)	Val/Met (M ± SD)	t (df)	p
n	18	12	–	–
Age	22.89 ± 5.18	23.17 ± 4.22	0.16 (28)	0.878
Sex males n(%)	10 (55.56%)	8 (66.67%)	–	0.709[a]
Wake time	08.05 ± 0.86	8.11 ± 0.80	0.20 (28)	0.847
DLMO	18.81 ± 1.92	20.03 ± 3.18	1.28 (27)	0.212
DLMO shift (h)	2.95 ± 1.43	2.23 ± 2.06	1.11 (27)	0.277
TST (mins)	388.22 ± 68.92	408.70 ± 67.35	0.76 (26)	0.455
SE (%)	80.77 ± 14.46	85.05 ± 13.97	0.76 (26)	0.455
WASO (mins)	65.08 ± 73.79	56.91 ± 66.62	0.29 (26)	0.774

Dim light melatonin onset (DLMO); total sleep time (TST); sleep efficiency (SE); wake after sleep onset (WASO). Wake time refers to wake time before the 8-h phase advance and DLMO refers to DLMO assessed during the CR post phase shift.

[a] Fisher's exact test p-value (2-sided).

Table 2
Performance on the Stroop task for genotype (Val/Met vs. Val/Val) and time awake (1st, 2nd and 3rd tertile) and the genotype*time awake interaction.

Stroop Outcome	Stroop Trial Type	Time Tertile	Val/Met (M ± SD)	Val/Val (M ± SD)	Genotype F (df)	Time Awake F (df)	Genotype*Time Awake F (df)
Reaction Time (ms)	Congruent	T1	816.22 ± 165.67	780.76 ± 106.15	0.45 (1, 27.1)	30.94*** (2, 53.9)	0.32 (2, 53.9)
		T2	802.30 ± 175.34	765.33 ± 94.56			
		T3	912.57 ± 186.82	896.33 ± 133.66			
	Neutral	T1	829.83 ± 149.58	825.17 ± 123.89	0.26 (1, 27.1)	38.38*** (2, 53.9)	0.71 (2, 53.9)
		T2	833.14 ± 169.23	801.43 ± 102.32			
		T3	951.93 ± 173.68	954.08 ± 134.56			
	Incongruent	T1	923.37 ± 206.32	873.57 ± 132.44	0.45 (1, 27.1)	30.94*** (2, 53.9)	0.32 (2, 53.9)
		T2	926.39 ± 229.87	866.65 ± 114.17			
		T3	1055.23 ± 217.14	1009.36 ± 134.98			
Error rate	Congruent	T1	2.55 ± 2.27	2.35 ± 1.64	3.01 (1, 28)	20.35*** (2, 54.5)	1.28 (2, 54.5)
		T2	3.26 ± 2.76	1.89 ± 1.03			
		T3	7.11 ± 4.22	4.90 ± 3.69			
	Neutral	T1	4.05 ± 2.52	2.87 ± 2.20	5.25* (1, 27.8)	22.56*** (2, 54.3)	1.30 (2, 54.3)
		T2	4.24 ± 2.86	3.03 ± 1.85			
		T3	8.99 ± 4.48	5.62 ± 3.89			
	Incongruent	T1	4.43 ± 2.55	4.13 ± 2.00	2.10 (1, 27.3)	24.65*** (2, 53.9)	3.65** (2, 53.9)
		T2	4.90 ± 3.18	4.24 ± 2.40			
		T3	10.15 ± 5.05	6.45 ± 4.41			
Inhibition	n/a	T1	93.54 ± 69.85	48.19 ± 27.87	4.13*	0.47	0.48
		T2	93.78 ± 68.33	65.89 ± 37.05	(1, 27.1)	(2, 53.7)	(2, 53.7)
		T3	103.31 ± 84.13	55.43 ± 69.03	0.003	0.03	0.02

Values for the F-statistic and degrees of freedom are shown for the main effects of time and genotype, and the time*genotype interaction. Significance is denoted by *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. p-values for comparison between timepoints and for post-hoc tests for the significant interaction are reported in the text.

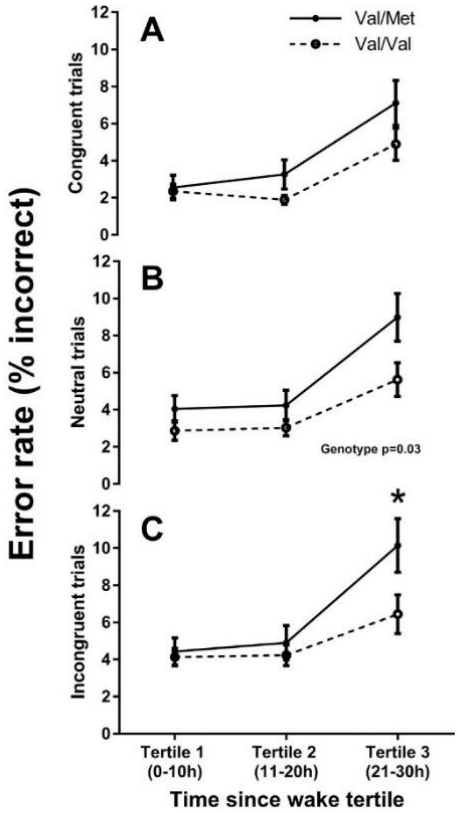

Fig. 1. Mean and standard error of the number of errors committed for each genotype. Each trial type is plotted separately: (**A**) Congruent (**B**) Neutral, and (**C**) Incongruent. *$p < 0.05$. Untransformed data are plotted.

No significant differences were found between *BDNF* genotypes for reaction time on any trial type, or the error rate for both congruent and incongruent trials (Table 2). For neutral trials, however, individuals with the Val/Met genotype made more errors compared to those homozygous for the Val/Val genotype (Val/Met 5.76 ± 4.03 ms; Val/Val 3.84 ± 3.02; p = 0.03). As seen in Fig. 1, a significant genotype*time interaction was found for the error rate on incongruent trials (p = 0.032). Post hoc comparison showed that Val/Mets made 3.54% [95% CI 0.9590, 6.1267] more errors, relative to Val/Val homozygotes, in Tertile 3 ($t_{55.3}$ = 2.75, p = 0.008, p_{adj} = 0.017). Other interactions were not significant as described in Table 2.

As seen in Fig. 2A, Val/Met heterozygotes took significantly longer to respond, relative to Val/Val homozygotes, when required to inhibit a prepotent response, such that the Val/Met homozygotes had a higher cost of inhibition (Val/Met 96.88 ± 72.43 ms; Val/Val 56.50 ± 47.66 ms; p = 0.047). There was no effect of time awake or a significant genotype*time interaction on cost of inhibition, however. As the main effect of genotype appeared to be most apparent at 0–10 h awake and 21–30 h awake (See Fig. 2A), which, due to the protocol design, corresponded with night time hours, we conducted a follow-up analysis comparing the genotypes during the biological day and the biological night. As seen in Fig. 2B, participants with the Val/Met genotype took significantly longer to respond when inhibiting the prepotent response than those with the Val/Val genotype during the biological night (Val/Met 112.46 ± 76.09 ms; Val/Val 43.54 ± 73.27 ms; t_{25} = 2.37, p = 0.026, d = 0.92), but not during the biological day (Val/Met 71.53 ± 59.25 ms; Val/Val 63.26 ± 44.35 ms; t_{25} = *0.42,* p > 0.68, d = 0.16) – see Fig. 2B.

Our data provide evidence that individuals who carry the Met allele of the *BDNF* genotype exhibit more impairment of cognitive flexibility following sleep deprivation, specifically in their ability to inhibit a prepotent response, as indicated by an increased error rate on the incongruent trials of the Stroop task following 20 h awake. The cost of inhibition, or the extra time required to inhibit the response, was also higher in those carrying the Met allele, but this was irrespective of sleep loss. This difference in cost of inhibition between the two groups appeared greatest at 0–10 h awake and 21–30 h which corresponded with the biological night in our study. Follow up analyses confirmed that the cost of inhibition was higher during the biological night for those with the Met allele, compared to those homozygous for the Val/Val *BDNF* genotype. Our results also support previous findings showing that Met allele carriers perform worse on a range of cognitive tasks even under well rested conditions [11,13,14], such that participants with the Val/Met genotype in the current study made more errors overall for neutral trials, irrespective of time awake.

The impact of *BDNF* genotype on vulnerability to sleep deprivation

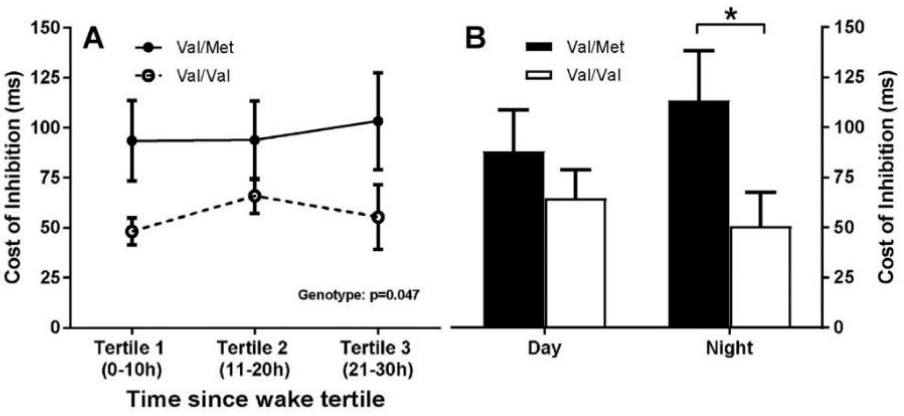

Fig. 2. Mean and standard error of inhibition for each genotype. (**A**) Inhibition score for each tertile (**B**) Inhibition scores for the biological day and the biological night. *$p < 0.05$. Untransformed data are plotted.

may be due to the effect of sleep loss on *BDNF* expression and subsequent cortical function. For instance, expression of *BDNF* is increased in the frontal cortex, basal forebrain and hippocampus following sleep loss in rats [26–28], which is associated with improved cognitive performance [26]. These findings suggest that *BDNF* may act to enhance cortical functioning in the areas where it is upregulated during sleep loss. As the *BDNF* Met allele is associated with impaired activity-dependent secretion of the *BDNF* protein, we speculate that any sleep deprivation-induced increase in cortical *BDNF* may be attenuated in Met allele carriers leading to poorer cognitive outcomes. In support of this interpretation, imaging studies in humans show reduced activation in the prefrontal cortex and hippocampus of *BDNF* Met carriers [10,11,13,29], which was attributed to impaired secretion of *BDNF* protein in the Met allele carriers affecting synaptic events underlying performance [29]. The Stroop task relies on activation of frontal brain regions [30], thus, the structural and functional brain differences [10,13] between the *BDNF* genotypes may underlie the variation in performance by affecting the way in which the brain responds to the task during sleep loss. These structural and functional abnormalities in Val/Met individuals may affect the allocation of frontal resources and alter the brain's compensatory response to sleep loss, leading to impaired cognitive performance relative to Val/Val homozygotes. As this interpretation is speculative, future work comparing frontal activation and compensatory responses between those with and without the *BDNF* Met allele during sleep deprivation would provide crucial insight.

While our study outcomes are novel and provide further evidence for a genetic marker of vulnerability to sleep loss, our study has a number of limitations. Firstly, the results of this study should be interpreted with caution due to the small sample size, and our results should be replicated under similar conditions. Despite this, our sample size is similar to previous studies investigating the effects of a polymorphism on performance during sleep deprivation [5–8] and our findings replicate previous research showing impaired frontal functioning in *BDNF* met allele carriers in well-rested individuals [13,14,19,20] and during sleep deprivation [8]. Secondly, individuals were exposed to varying light exposure and phase shifting protocols prior to the CR as this was part of a larger study. To address whether these conditions had an impact on our outcomes, we conducted a linear mixed model (See supplementary material Table S1) which showed that there was no effect of light and phase shift condition on Stroop outcomes. There was also no difference in the average phase shift between genotypes in response to the phase advance protocol. Finally, as the main protocol induced a phase advance prior to the CR, resulting in (some) participants sleeping at a non-optimal circadian time, we again examined sleep outcomes prior to the CR as this may have exacerbated performance deficits. As we found no difference in total sleep time, wake after sleep onset, and sleep efficiency between genotypes, we argue that differences in prior sleep between the groups does not explain our observed differences in performance during sleep deprivation (CR).

In summary, our study shows that the *BDNF* Met allele carriers are more vulnerable to the effect of sleep deprivation and the biological night on a measure of inhibition. *BDNF* Met allele carriers made more inhibitory errors on the Stroop task during sleep deprivation and the cost of inhibition, the additional time taken to respond when inhibiting a response, was greater regardless of time awake, and particularly so during the biological night. These data indicate that *BDNF* genotype merits further inquiry as a potentially important genetic determinant for individual vulnerability to sleep loss and performance impairments at adverse circadian times. Our data both support and extend the findings presented by [8] in that the *BDNF* Val66Met polymorphism is associated with vulnerability to sleep deprivation, and that this vulnerability goes beyond working memory to other cognitive domains, such as cognitive flexibility, which is a critical component of executive function. Taken together, these results have important implications for individuals working extended duration shifts, particularly at night.

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Author contributions

All authors have contributed to this manuscript. C.A.C., A-M.C., S.C., C.A. contributed to the study concept and design. A-M.C., S.C. and C.A. contributed to the collection of data. R.S. conducted the genetic analysis. L.G., S.C. and C.A. contributed to analysis and interpretation of the data and drafting of the manuscript. All authors were involved in editing and reviewing the manuscript. The manuscript has been approved by all authors.

Disclosure statement

Drs. Cain, Chang and Saxena report no financial conflicts of interest in relation to this work. Ms. Grant has received scholarship funding from the Cooperative Research Centre for Alertness, Safety and Productivity. Dr Czeisler has received consulting fees from or served as a paid member of scientific advisory boards for: Bose Corporation; Boston Celtics; Columbia River Bar Pilots; Institute of Digital Media and Child Development; Klarman Family Foundation; Quest Diagnostics, Inc.; Vanda Pharmaceuticals and V-Watch/PPRS. He has also received education/research support from Cephalon Inc., Mary Ann & Stanley

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2017.09.025.

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Chapter 3 Additional information supplement

3.1. Supplemental material to Grant et al., 2018

The following material was published as supplemental material to Grant et al., 2018, *Behavioural Brain Research.*

3.1.1. Effect of phase advance protocol on Stroop outcomes

To determine whether there was an effect of the phase advance protocol conditions for light or type of phase shift on Stroop outcomes we performed a linear mixed model with time, genotype (Val/Val and Val/Met) and time or condition modelled as fixed factors, and subject as a random factor. There were three different light types (white, green, combined) and three different phase shift conditions (slam, slam with nap, and gradual). The results of the linear mixed model analyses are shown in Table S1. None of the tests reached statistical significance indicating that the light and phase shifting conditions did not affect Stroop outcomes during the CR.

Table S1. Results of linear mixed model analyses to determine the effects of lighting condition and phase shift condition on Stroop outcomes measured during CR.

Stroop outcome	Trial type	Light F (df)	Genotype* Light F (df)	Time* Genotype* Light F (df)	Condition F(df)	Genotype* Condition F (df)	Time* Genotype* Condition F (df)
Reaction time (ms)	Congruent	1.21 (2, 24)	1.02 (2, 24)	0.62 (10, 37.8)	0.01 (1, 23.9)	1.33 (2, 23.9)	0.59 (10, 37.7)
	Neutral	1.48 (2, 24)	2.46 (2, 24)	0.81 (10, 37.8)	1.24 (2, 39.9)	1.36 (2, 23.9)	0.47 (10, 37.8)
	Incongruent	1.32 (2, 24.1)	1.98 (2, 24.1)	0.49 (10, 37.5)	1.42 (2, 24.3)	2.12 (2, 24.3)	0.33 (10, 37.8)
Error rate	Congruent	1.16 (2, 22.2)	0.53 (2, 22.2)	0.98 (10, 38.2)	1.67 (2, 22.1)	0.45 (2, 22.1)	0.86 (10, 38.4)
	Neutral	0.13 (2, 22.6)	0.19 (2, 22.6)	0.65 (10, 38.6)	1.24 (2, 22.6)	0.03 (2, 22.6)	0.61 (10, 38.9)
	Incongruent	1.43 (2, 23.3)	0.26 (2, 23.3)	1.21 (10, 38.4)	1.38 (2, 23.6)	0.60 (2, 23.6)	1.62 (10, 38.4)
	Inhibition	0.51 (2, 23.6)	0.23 (2, 23.6)	0.92 (10, 39)	0.28 (2, 23.4)	1.58 (2, 23.4)	1.51 (10, 46)

The F-statistic and degrees of freedom, are shown for the main effect of light and condition, the interaction between genotype and light or condition, and the interaction between genotype and light or condition with time awake.

3.1.2. Post-hoc t-test comparisons for the main effect of time

Statistics of the post-hoc tests for the main effect on time are shown in Table S2.

Table S2.

Post-hoc paired t-test comparisons of time tertiles for the main effect of time.

Stroop outcome	Stroop trial type	Comparison	t	df	P	Mean difference	Lower CI	Upper CI
Reaction time (ms)	Congruent	1,2	1.03	53.9	0.3058	17.55	-16.49	51.59
		1,3	-6.24	53.9	<.0001	-105.87	-139.91	-71.83
		2,3	-7.27	53.9	<.0001	-123.42	-157.46	-89.39
	Neutral	1,2	0.73	53.9	0.4705	12.68	-22.30	47.66
		1,3	-7.20	53.9	<.0001	-125.56	-160.53	-90.58
		2,3	-7.92	53.9	<.0001	-138.24	-173.21	-103.26
	Incongruent	1,2	0.09	54.0	0.9282	1.85	-39.06	42.75
		1,3	-6.64	54.0	<.0001	-135.50	-176.40	-94.59
		2,3	-6.73	54.0	<.0001	-137.34	-178.25	-96.44
Error rate	Congruent	1,2	-0.12	54.5	0.9051	-0.08	-1.35	1.20
		1,3	-5.58	54.5	<.0001	-3.55	-4.83	-2.28
		2,3	-5.46	54.5	<.0001	-3.48	-4.75	-2.20
	Neutral	1,2	-0.32	54.3	0.7475	-0.22	-1.57	1.13
		1,3	-5.97	54.3	<.0001	-4.02	-5.36	-2.67
		2,3	-5.65	54.3	<.0001	-3.80	-5.15	-2.45
	Incongruent	1,2	-0.45	53.9	0.6523	-0.30	-1.60	1.01
		1,3	-6.29	53.9	<.0001	-4.11	-5.42	-2.80
		2,3	-5.84	53.9	<.0001	-3.81	-5.12	-5.12

The mean difference and confidence intervals represent the within-group means adjusted for other effects in the model (LS Means).

3.1.3. Individual participant data

Given the relatively small sample size for a genetic study the individual participant data is shown in Figures S1 and S2 to demonstrate the absence of outliers influencing the data. Standardized z-scores also confirmed the absence of outliers as none of the individual participant scores for any Stroop variable fell outside ±3 standard deviations of the mean.

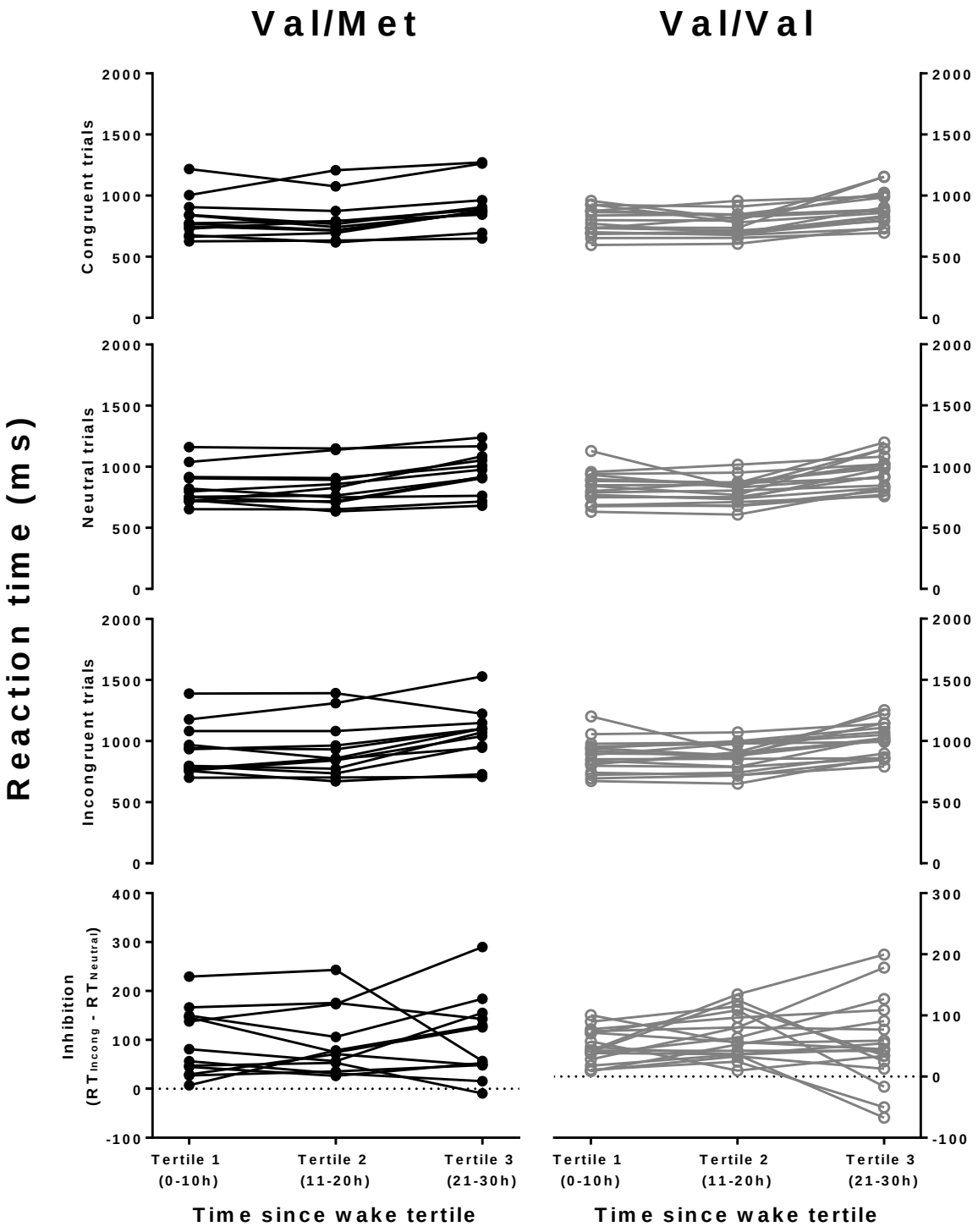

Figure S1. Individual participant data showing reaction times for all Stroop trial types, including the measure of inhibition (calculated as Inhibition= $RT_{\text{incongruent trials}} - RT_{\text{neutral trials}}$). Participants with the Val/Met genotype are shown in black on the left, and participants with the Val/Val genotype are shown in grey on the right.

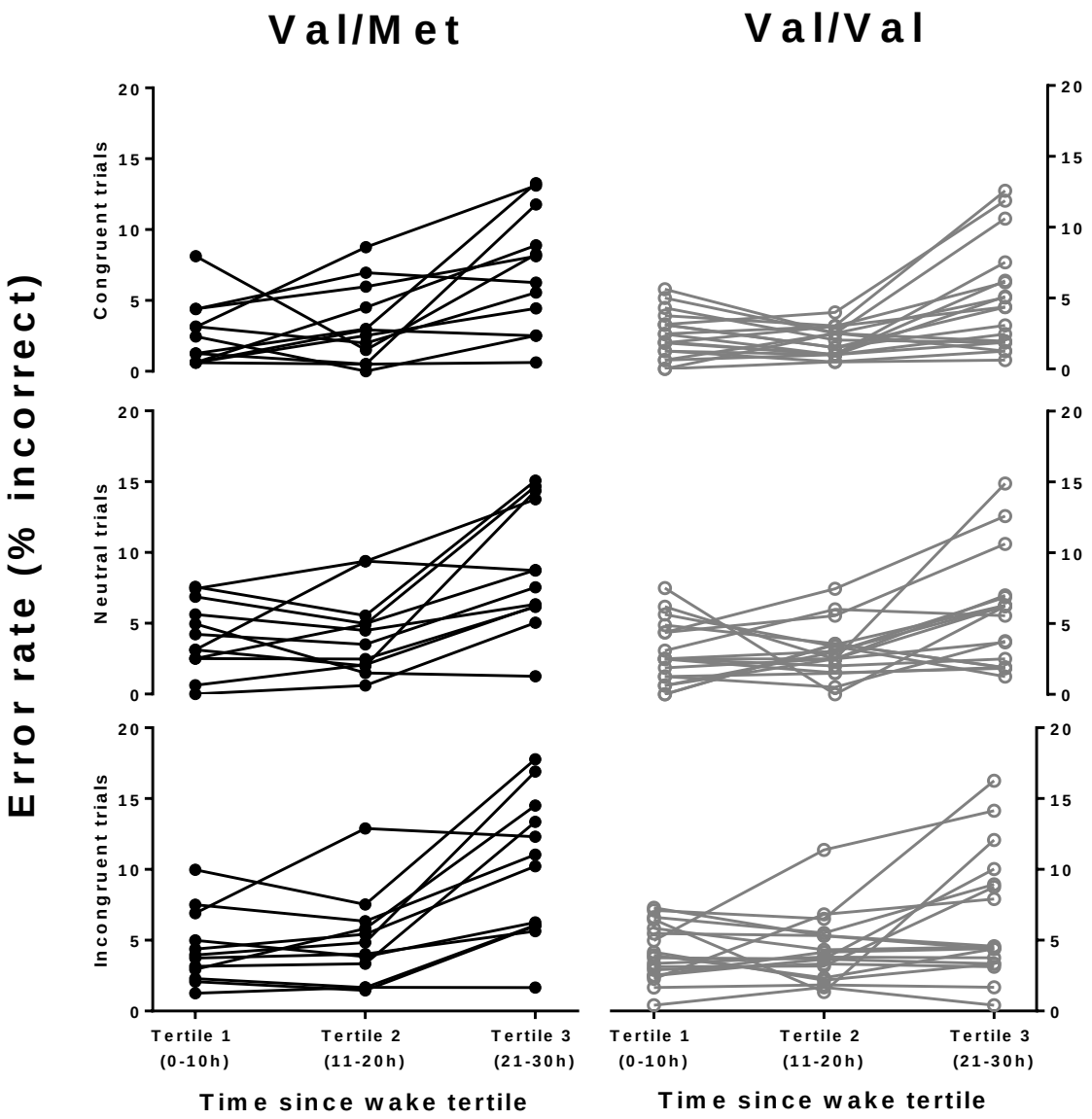

Figure S2. Individual participant data showing the error rate for all Stroop trial types. Participants with the Val/Met genotype are shown in black on the left, and participants with the Val/Val genotype are shown in grey on the right.

3.2. Combined effects of *BDNF* Val66Met polymorphism and menstrual phase on Stroop performance

Self-report data of the date of last menses and menstrual cycle was used to estimate menstrual phase. Of the n= 12 women (Val/Met: n=4; Val/Val: n=8) in the sample, the data needed to determine menstrual phase was available for n=8 women. Data was missing for n= 1 woman with the Val/Met genotype, and n= 3 women with the Val/Val genotype. Of the three women with the Val/Met genotype, n= 1 was in the follicular phase and n= 2 were in the luteal phase, and of the five women with the Val/Val genotype, n= 3 were in the follicular phase, and n= 2 were in the luteal phase. As seen in Figure S3, the one woman with both vulnerability factors (Val/Met genotype and follicular phase) appeared to perform most poorly, particularly on the reaction time measures. Due to the limited sample size, statistical analyses were not conducted. Furthermore, the effects of menstrual phase on the sleep episode prior to the CR were also examined. Sleep data was available for n=7 participants (n=4 follicular and n=3 luteal). There were no differences between the menstrual phases for total sleep time, sleep efficiency, wake after sleep onset, and the percentage of time or minutes spent in REM sleep or the NREM stages 1, 2, 3 and 4. Given the limited sample size these results should be interpreted with caution, however.

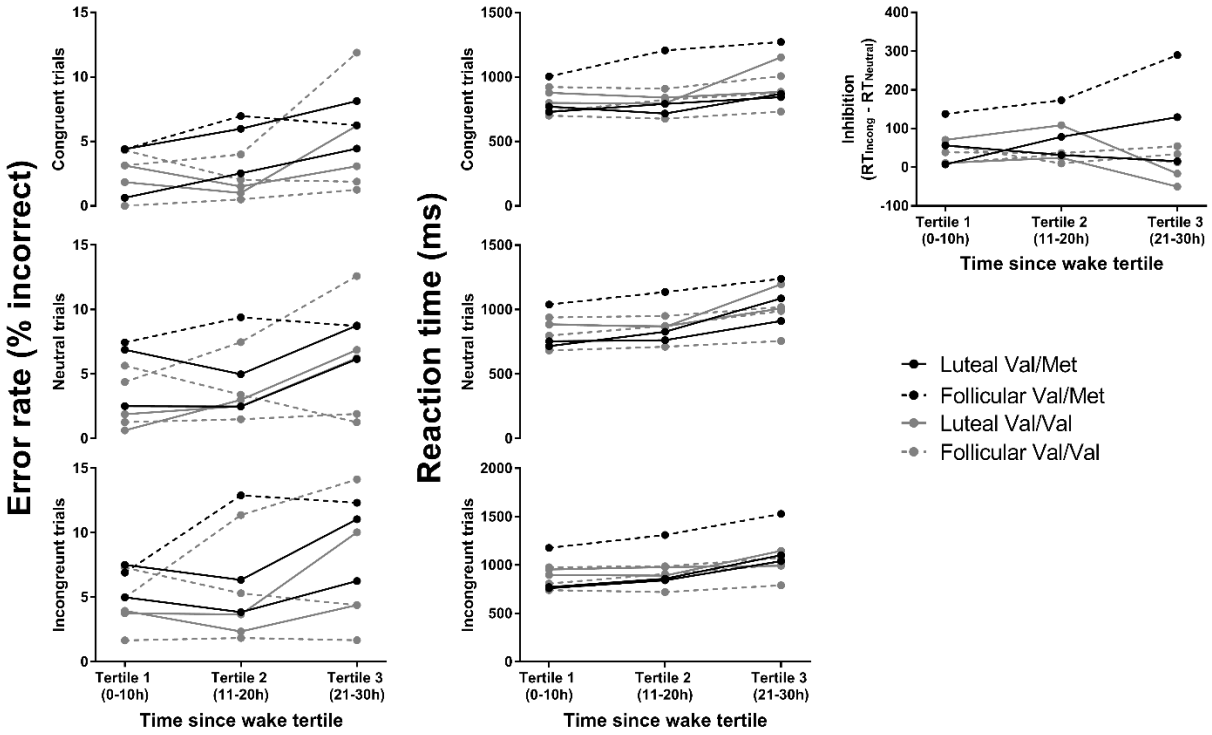

Figure S3. Individual participant data by menstrual phase showing error rate and reaction time for all Stroop trial types, including the measure of inhibition (calculated as Inhibition= $RT_{\text{incongruent trials}} - RT_{\text{neutral trials}}$). Participants with the Val/Met and Val/Val genotype are shown in black and grey, respectively. The follicular phase of the menstrual cycle is denoted by the dashed line, and the luteal phase is denoted by the solid line.

Chapter 3 Concluding remarks

In this chapter the *BDNF* Val66Met polymorphism was identified as a marker of vulnerability to cognitive imapirment during sleep loss. While *BDNF* genotype has previously been identified as a potential marker of vulnerability to working memory impairment during sleep deprivation (Bachmann et al., 2012), the current study expands on those findings by showing that carriers of the *BDNF* Val66Met polymorphism also show impaired cognitive flexibility during sleep deprivation, as measured by response inhibition on the Stroop task. As described above, we found that individuals with the Val/Met genotype made more errors on Stroop trials requiring them to inhibit a prepotent response following 20-h of wakefulness, and also took longer to respond when inhibiting a prepotent response, compared to Val/Val homozygotes, particularly during the biological night. Our data show that the *BDNF* Val66Met polymorphism is associated with vulnerability to sleep deprivation and that this vulnerability goes beyond working memory to other cognitive domains, specifically response inhibition. The findings of this study suggest that carriers of the *BDNF* Val66Met polymorphism may be more vulnerable to performance failure and this has important implications for individuals who work extended duration shifts, particularly during night time hours.

Knowledge of which genes may be contributing to enhanced vulnerability to alertness failure will allow for the identification of individuals most in need of effective sleepiness countermeasures, such as prophylactic naps, caffeine, and appropriately timed light exposure, which are well validated countermeasures that can enhance alertness (Cajochen et al., 2005; Horne & Reyner, 1996; Penetar et al., 1993; Phipps-Nelson, Redman, Dijk, & Rajaratnam, 2003; Schweitzer, Randazzo, Stone, Erman, & Walsh, 2006; Viola, James, Schlangen, & Dijk, 2008; Wyatt, Cajochen, Ritz-De Cecco, Czeisler, & Dijk, 2004). The ability to understand the underlying cause of vulnerability, in this case carrying the *BDNF* Val66Met polymorphism, may allow us to better understand which countermeasures will be most effective for different individuals. For example, the *BDNF* Val66Met polymorphism has also been shown to affect sleep intensity, such

that Val/Met heterozygotes have decreased SWA during a sleep episode and accumulate SWA more slowly during wake compared to Val/Val homozygotes during both normal sleep and in recovery sleep following prolonged wakefulness (Bachmann et al., 2012). Therefore, the efficacy of interventions such as slow wave sleep enhancement, which has been shown to be protective against performance deficits associated with sleep restriction (Walsh et al., 2006), may be modulated by the *BDNF* Val66Met polymorphism. While this has not been investigated to date, future research should explore the efficacy of different sleepiness countermeasures in carriers of the *BDNF* Val66Met polymorphism and other functional polymorphisms. Future research may also investigate the effects of combinations of genotypes, or genotypes and phenotypes known to result in enhanced vulnerability to alertness failure. For example, in the current chapter, the combination of carrying the *BDNF* Val66Met polymorphism and being in the vulnerable follicular phase of the menstrual cycle appeared to have an additive effect, such that the participant with both of these vulnerability factors appeared to have the poorest performance, particularly on reaction time measures. Although these results warrant further investigation, they must be interpreted with caution given the extremely small sample size.

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Chapter 4

Differences in neurobehavioural performance due to menstrual phase: The role of temperature, progesterone and the progesterone/estradiol ratio

This chapter constitutes a manuscript prepared for submission to the *Journal of Clinical Endocrinology & Metabolism* as:

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Preface to Chapter 4

In **Chapter 3**, the *BDNF* Val66Met polymorphism was shown to be a genetic marker of vulnerability to response inhibition during sleep deprivation, such that individuals with heterozygous Val/Met genotype made more errors on incongruent Stroop trials following 20-h of wakefulness and took longer to respond when inhibiting a prepotent response, especially during the biological night. Continuing from **Chapter 3**, in **Chapter 4** an endocrine rather than genetic marker is investigated. Differences in performance between menstrual phases have previously been reported (Vidafar et al., under review; Wright & Badia, 1999) and this chapter seeks to extend these findings by examining the potential endocrine mechanisms underlying these behavioural differences and also examining the effectiveness of a photic countermeasure in reducing vulnerability to sleep loss in pre-menopausal women.

Although two previous studies have shown that women in the follicular phase of the menstrual cycle appear more vulnerable to performance impairment associated with sleep loss (Vidafar et al., under review; Wright & Badia, 1999), the hormones that characterise the different phases of the menstrual cycle have not been investigated as potential biomarkers capable of predicting performance impairment in pre-menopausal women. Due to the relationship between temperature and performance (Wright, Hull, & Czeisler, 2002), differences in temperature between the menstrual phases (Baker, Waner, et al., 2001; Cagnacci, Volpe, Paoletti, & Melis, 1997; Kattapong, Fogg, & Eastman, 1995), and evidence to support a role for progesterone in modulating temperature throughout the menstrual cycle (S. L. Israel & Schneller, 1950; Stachenfeld, Silva, & Keefe, 2000), this investigation focused predominantly on progesterone and the progesterone/estradiol ratio as candidate biomarkers, although other female reproductive hormones were also investigated. Furthermore, given the well documented effects of light on both temperature and performance, in a novel aspect, this study also investigated whether the alerting effects of light were equally effective during both menstrual phases. Therefore, the aims of the current study were to 1) investigate female reproductive hormones as novel biomarkers of

performance impairment in naturally cycling women; and 3) determine the effects of a light exposure intervention on both performance and candidate endocrine biomarkers.

Chapter 4 includes an article prepared for submission to *Journal of Clinical Endocrinology & Metabolism.*

Chapter 4 Article prepared for submission

Differences in neurobehavioural performance due to menstrual phase:

The role of temperature, progesterone and the progesterone/estradiol ratio

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Abstract

The 24-h rhythm in core body temperature (CBT) is associated with changes in performance, whereby performance deteriorates in parallel to the nocturnal decline in CBT. Pre-menopausal women in the luteal phase have a higher CBT, particularly at night, and exhibit less cognitive impairment during sleep loss than women in the follicular phase of the menstrual cycle. As an association between progesterone and changes in CBT during the menstrual cycle has previously been reported, we aimed to investigate progesterone and the progesterone/estradiol ratio as biomarkers of performance impairment in naturally cycling women. Additionally, we compared the acute alerting effects of night time light exposure on performance between the menstrual phases. We studied 16 healthy young women (8 follicular; 8 luteal) during 50-h of continuous wakefulness under constant conditions and during a 6.5-h monochromatic light exposure (420-555nm) at night during the following wake episode. Cognitive performance, subjective sleepiness, CBT, melatonin and reproductive hormones were assessed throughout the protocol. Women in the follicular phase were more vulnerable to performance impairment, especially during the night. Exposure to light sufficient to suppress melatonin, however, completely recovered these night time performance impairments. Furthermore, we found that temperature, but not progesterone or the progesterone/estradiol ratio, was significantly associated with performance. Our results reiterate the importance of measuring menstrual phase when examining the effects of sleep loss on performance in women and may have implications for female shift workers.

4.1. Introduction

Daily fluctuations in alertness and performance are associated with the 24-h rhythm in core body temperature (CBT), which displays a daily variation of approximately 1˚C (Dijk, Duffy, & Czeisler, 1992; Kräuchi & Wirz-Justice, 1994). In normally menstruating women, however, the circadian rhythm in CBT is also affected by the phase of the menstrual cycle. As compared to the follicular phase, the amplitude of the 24-h CBT rhythm is decreased during the luteal phase during the night (Cagnacci, Arangino, Tuveri, Paoletti, & Volpe, 2002; Cagnacci, Soldani, Laughlin, & Yen, 1996; Cagnacci et al., 1997; Kattapong et al., 1995; Lee, 1988; Shechter, Varin, & Boivin, 2010), consequently leading to ~0.4˚C higher average daily temperature (Baker, Waner, et al., 2001; Cagnacci et al., 2002; Cagnacci et al., 1996; Cagnacci et al., 1997; Driver, Dijk, Werth, Biedermann, & Borbély, 1996). Given previous reports of improved working memory, cognitive throughput, subjective alertness and reaction times being associated with an increase of 0.17˚C in nocturnal CBT (Wright et al., 2002), the ~0.4˚C increase in CBT associated with the luteal phase of the menstrual cycle would be expected to increase alertness and improve performance relative to the follicular phase. Two studies investigating the effects of menstrual phase on performance during sleep loss have confirmed this relationship and shown that women in the luteal phase of the menstrual cycle perform better than women in the follicular phase, particularly during the night (Vidafar et al., under review; Wright & Badia, 1999), but neither of these studies examined the role of the reproductive hormones in mediating these differences in cognitive performance between menstrual phases.

The changes that occur in CBT during the different phases of the menstrual cycle are thought to be driven primarily by progesterone, which has an hyperthermic effect (S. L. Israel & Schneller, 1950; Stachenfeld et al., 2000). Circulating progesterone concentrations are low throughout the follicular phase but increase during the luteal phase, which occurs concomitantly with the elevation in CBT during this phase of the cycle (Baker, Waner, et al., 2001; Kattapong et al., 1995). While the mechanisms underlying the hyperthermic effects of progesterone are not well

understood, there is some evidence to suggest that progesterone directly affects brain regions involved in thermoregulation. For example, progesterone administration in rabbits reduces the firing rate of warm-sensitive neurons and increases the firing rate of cold-sensitive neurons in the hypothalamic preoptic area (Nakayama, Suzuki, & Ishizuka, 1975), which is the primary brain region involved in the homoeostatic regulation of CBT. Additionally, progesterone receptors are also found within the suprachiasmatic nuclei (SCN; (Kruijver & Swaab, 2002)), the hypothalamic region responsible for generating circadian rhythms, including the 24-h CBT rhythm (Eastman, Mistlberger, & Rechtschaffen, 1984). Moreover, estradiol through its hypothermic actions, modifies the hyperthermic effects of progesterone (Stachenfeld et al., 2000). Accordingly, the progesterone/estradiol ratio, rather than progesterone or estradiol alone, has been shown to be most associated with CBT in naturally cycling women (Cagnacci et al., 2002; Cagnacci et al., 1997).

The associations between reproductive hormones and temperature, and the modulatory effects of temperature on performance, suggest that these reproductive hormones may be useful biomarkers of performance impairment across the menstrual cycle. The relationship between changes in performance, temperature and reproductive hormones (i.e. progesterone and the progesterone/estradiol ratio) during sleep deprivation, particularly during the biological night, has not been investigated in detail, however. Furthermore, despite research suggesting that women in the follicular phase show altered circadian rhythmicity of CBT and neurobehavioural performance impairments, a countermeasure such as light exposure, which is well documented to both increase temperature (Cajochen et al., 2005; Wright, Myers, Plenzler, Drake, & Badia, 2000) and improve performance (Cajochen et al., 2005; Lockley et al., 2006; Rahman et al., 2014), has not been investigated in this group specifically. Therefore, in the current study we aimed to determine the relationship between progesterone, the progesterone/estradiol ratio, CBT and cognitive performance underlying the differences in neurobehavioural performance observed between menstrual phases during sleep loss. Additionally, we characterised the menstrual phase dependent effects of light exposure on cognitive performance and changes in progesterone, the progesterone/estradiol ratio and CBT during exposure to light. We hypothesised that higher CBT,

plasma progesterone and progesterone/estradiol ratio would be associated with better cognitive performance during sleep deprivation. We also expected that greater levels of plasma progesterone and a higher progesterone/estradiol ratio would be associated with higher CBT. Furthermore, we anticipated that light exposure would improve performance in both the follicular and luteal phases of the menstrual cycle and, given that light exposure is known to increase temperature, we also expected that progesterone and the progesterone/estradiol ratio would increase in response to light exposure.

4.2. Methods

4.2.1. Participants

Participants were 16 healthy women aged 19-29 years (mean age 22.94 ± 2.57 years), with 8 studied in the follicular phase and 8 in the luteal phase of their menstrual cycle. Criteria for menstrual phase determination is below. All participants underwent comprehensive physical, psychiatric and medical screening including an ophthalmologic examination and Ishihara color blindness test. All participants self-reported a regular menstrual cycle lasting 26 to 35 days and were not using oral contraception for at least three months prior to the start of the study. To determine menstrual phase, daily average progesterone levels were calculated for each participant and women with progesterone concentrations >3ng/mL were considered to have ovulated (R. Israel, Mishell Jr, Stone, Thorneycroft, & Moyer, 1972; Stricker et al., 2006) and were assigned to the luteal phase. Given the length of the protocol (9-days), some women changed menstrual phase during the study. Table 1 shows progesterone concentrations and allocation of menstrual phase for each participant on the different study days. As the effect of light exposure on progesterone is not well understood, the calculation of progesterone concentrations for study days 6 and 7, when scheduled light exposure occurred, excluded the timepoints collected during and after light exposure.

For three weeks prior to the laboratory study, participants maintained a self-selected 8:16 sleep/wake schedule that was confirmed with time-stamped call ins at bed and wake times for three

weeks, and with actigraphy (Actiwatch-L, Minimitter Inc., Bend, OR) and sleep diaries for at least 7-days prior to entering the laboratory. Participants were asked to refrain from using any prescription and non-prescription medications, supplements, recreational drugs, caffeine, alcohol or nicotine. Compliance was confirmed with urine toxicology during screening and upon admission to the laboratory. The study was approved by the Partners Human Research Committee and written informed consent was given by participants before commencing the study.

Table 1. Daily average plasma progesterone concentrations and menstrual phase allocation

Pt code	CR day 1		CR day 2		LE day	
	P4 ng/mL M(SD)	Phase	P4 ng/mL M(SD)	Phase	P4 ng/mL M(SD)	Phase
22K7V	2.04 (0.19)	F	2.04 (0.30)	F	2.20 (0.41)	F
25N6V6T2	2.64 (0.59)	F	2.60 (0.42)	F	2.66 (0.90)	F
2614V	2.39 (0.45)	F	2.30 (0.40)	F	2.57 (0.53)	F
2692V	2.06 (0.42)	F	2.25 (0.48)	F	2.51 (061)	F
26F2V	2.20 (0.59)	F	1.98 (0.32)	F	2.48 (0.68)	F
26G3V	2.28 (0.43)	F	2.19 (0.29)	F	2.36 (0.37)	F
26G6V	1.94 (0.43)	F	1.98 (0.32)	F	2.51 (0.49)	F
26H6V	2.04 (0.19)	F	2.56 (0.93)	F	3.26 (0.78)	L
2251V	3.89 (0.73)	L	5.75 (0.68)	L	8.28 (2.24)	L
21B8V	8.73 (2.00)	L	12.74 (2.78)	L	21.17 (0.67)	L
22A1V	21.70 (5.08)	L	12.92 (4.83)	L	8.22 (1.38)	L
22K3V	4.78 (1.32)	L	3.29 (0.57)	L	2.68 (0.65)	F
25Q2V	29.38 (3.71)	L	28.04 (5.31)	L	17.97 (4.90)	L
2622V	7.95 (3.59)	L	3.46 (0.63)	L	3.16 (0.75)	L
26P1V	8.73 (2.77)	L	3.88 (1.24)	L	3.29 (1.22)	L
26R1V	10.86 (1.24)	L	9.17 (2.53)	L	10.60 (1.68)	L
n follicular		8		8		8
n luteal		8		8		8

Note. The daily mean and standard deviation of progesterone is shown for each participant on the different study days: constant routine (CR) and light exposure (LE). Averages for the LE day include only samples collected before light exposure. Allocation to the luteal phase was based on a cut-off of progesterone>3ng/mL. The total number of participants (n) in each menstrual phase, follicular (F) and luteal (L), on different study days is presented in the bottom two rows.

4.2.2. Study Protocol

Participants were studied individually for 9-days in an environment free of time-cues (no access to windows, clocks, live TV, radio, internet and continually supervised by staff trained not to reveal the time). The study schedule consisted of 1) 3 baseline nights with 8:16 sleep/wake schedule, 2) a 49-h 40-min constant routine (CR) followed by an 8-h sleep opportunity, 3) a 6.5-h night time light exposure starting 4.75-h post wake, followed by an 8-h sleep opportunity, and finally 4) a second 29-h 40-min CR followed by an 8-h sleep opportunity and then discharge (Figure 1). During the CRs participants remained awake in a semi-recumbent posture in dim light (<3 lux) and were fed hourly-isocaloric snacks [150mEq Na+/100mEq K+ (± 20%); 1.5 x basal energy expenditure; 2000mL fluids/ 24h day]. The current analyses are limited to data collected from the first CR and until the end of the 6.5-h light exposure.

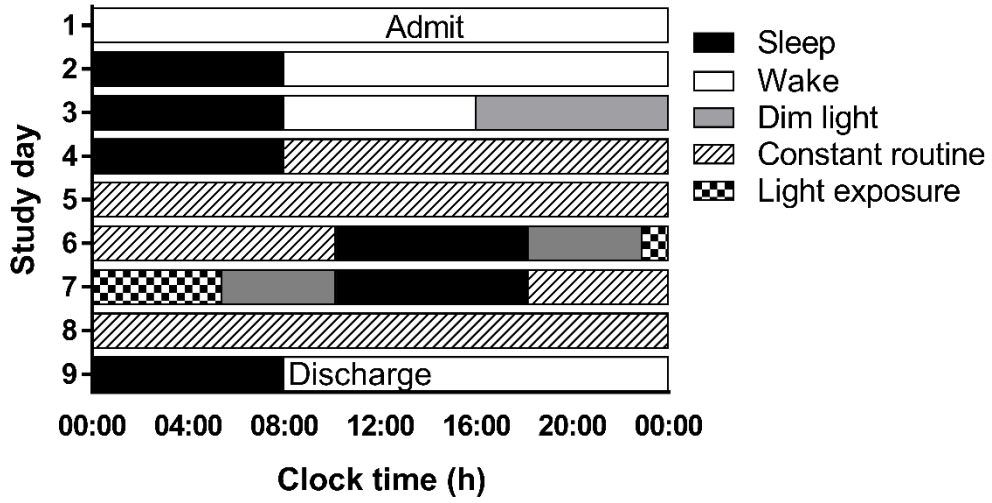

Figure 1. Participants completed a 9-day laboratory protocol. White bars represent wake episodes in ~190 lux, black bars represent scheduled sleep episodes with lights off (0 lux), grey bars represent wake episodes in dim light (<3 lux) not under CR conditions, and bars with a diagonal pattern represent the CRs in dim light (<3 lux). The blue bar represents the 6.5 h light exposure occurring 4.75 h post wake on day 6. Note: wake episodes for the CRs include an additional 30-min not under CR conditions, during which time participants prepared for bed.

4.2.3. Lighting

Study lighting conditions have been described in detail previously (Gooley et al., 2010; Lockley et al., 2006; Rahman et al., 2014). During baseline days, maximum ambient light (ceiling mounted 4100K fluorescent lamps F96T12/41U/HO/EW, 95W; F32T8/ADV841/A, 32W; F25T8/TL841, 25W; Philips Lighting, The Netherlands) during scheduled wake episodes was ~190 lux when measured vertically and ~88 lux when measured horizontally at a height of 187cm and 137cm, respectively. Midway through day 3, maximum ambient light was reduced to <3 lux when measured vertically and ~0.6 lux when measured horizontally. This level of light was maintained for the remainder of the study except during scheduled sleep episodes that occurred in darkness, and during the light exposure on day 6 and 7 when ambient lighting was switched off.

The 6.5-h monochromatic light exposure began on day 6 of the protocol (Figure 1) starting 4.75-h after wake (or 9.25-h prior to respective wake time during each participants' baseline days). Light wavelength and irradiance were selected as part of a different study examining the role of light on melatonin suppression and circadian phase resetting (Gooley et al., 2010). Participants were randomly assigned to exposure to 420-nm (follicular n= 3; luteal n= 1), 460-nm (follicular n= 3; luteal n= 2), 507-nm (follicular n= 0; luteal n= 2), or 555-nm (follicular n= 2; luteal n= 3) light. Participants wore black-out goggles for the 15-minutes prior to the light exposure following administration of a pupil dilator (ophthalmologic preparation of 0.5% cyclopentolate hydrochloride, Cyclohel, Alcon, TX). During the light exposure, participants remained seated under continuous supervision by study staff and were asked to maintain a fixed gaze for 90-min in the Ganzfeld dome followed by a 10-min free gaze. This procedure was repeated throughout the light exposure.

4.2.4. Performance, hormone, and temperature assessments

Participants completed alertness and performance assessments every hour starting 2.5-h post wake during the first CR, and every hour starting 1-h post wake on the light exposure day. Subjective sleepiness was assessed using the Karolinska Sleepiness Scale [KSS; (Åkerstedt & Gillberg, 1990)], a 9-point scale from 1 ("very alert") to 9 ("very sleepy, fighting sleep"). KSS scores were collected by pressing the appropriate number on a keyboard when prompted. During the

monochromatic light exposure, participants completed the KSS by responding verbally after the identical instructions and options presented during the CR were read to them. Objective performance was measured using the 10-minute auditory psychomotor vigilance task (aPVT) where a tone was presented at random intervals between 1-9 seconds and participants were asked to respond by pressing a button as quickly as possible after hearing the sound. Mean reaction time and lapses of attention (>500ms) were calculated for each 10-min aPVT session.

Plasma was collected from an indwelling IV cannula inserted into a forearm vein and kept patent with a heparinised saline infusion (5 UI heparin/mL 0.45% NaCl infused at 40-42mL/h). Blood samples were transferred to EDTA tubes and kept on ice before centrifugation. The plasma fraction was transferred into plastic tubes and stored at -20˚C. During the first CR, blood samples were collected every 30-60 minutes until the beginning of the light exposure where samples were collected every 20 minutes. Two-hourly samples from CR were assayed and all available samples on the light exposure day were assayed. Samples collected during the CR and light exposure were binned in 2-h and 1-h bins, respectively, prior to analysis. Plasma melatonin was assayed using radioimmunoassay (ALPCO Diagnositcs, Salem NH). Plasma intraassay and interassay coefficients of variation were <9% and <11% respectively, at 1.94 and 16.59pg/ml. Plasma reproductive hormones were assayed at the Specialty Assay Research Core (Brigham and Women's Hospital, Boston, MA). The progesterone/estradiol ratio was calculated as [progesterone ng/mL *1000) / estradiol pg/mL]. Given that data for other reproductive hormones involved in the menstrual cycle [estradiol, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and sex-hormone binding globulin (SHBG)] were available, these hormones were also included in the analyses to provide validation of the menstrual phase classification as also as potential markers of performance.

Core body temperature was measured every minute via a rectal thermistor (Yellow Springs Instruments Inc. Yellow Springs, OH) throughout the study protocol. CBT data were averaged in 1-h bins prior to analysis. Prior to binning, CBT data were cleaned to remove missing data, and data points where the sensor had been removed, unplugged, or failed.

4.2.5. Data analysis

The first 5-h of performance, hormone and temperature data were excluded from the analysis of CR data to remove masking effects from the prior sleep episode and changes in posture (Brown & Czeisler, 1992). For analyses of the light exposure data, only time points collected between lights on (4.75-h post wake) and lights off (11.25-h post wake) were analysed. The figures, however, include data prior to and post light exposure to illustrate temporal changes in hormone levels before, during and after the light exposure. All KSS data for one participant (2622V) were missing, and for another participant (26F2V) LH was below the assay's limit of detection. FSH was excluded for one participant (22A1V), due to abnormally high levels without abnormal values for any of the other hormones.

Linear mixed model analyses were performed to compare performance, hormones and temperature between 1) menstrual phases during the CR, and 2) menstrual phases and suppression non-suppression groups during the light exposure. For analyses of the light exposure data, women who showed greater than 30% melatonin suppression were assigned to the suppression group (Hull, Czeisler, & Lockley, in press). Time and group were modelled as fixed effects, participant was modelled as random effect, and time and participant were modelled as repeated effects. An autoregressive covariance type was chosen for all analyses, and degrees of freedom were calculated using the Kenward-Rogers method. To determine the relationship between temperature, hormones and performance during the CR (6 to 48-h), regression analyses were conducted on these variables using the unadjusted data averaged across the entire CR and also the area under the curve (AUC) calculated via the trapezoid method. As some participants had missing data at the start or end of the time series which altered the AUC calculation, linear interpolation was used to calculate scores where participants had missing data for the first or last timepoint. For these regression analyses, data were first analysed with a linear regression model. Given, however, that previous research has shown a non-linear quadratic ($y= ax^2 + bx + c$) model best describes the relationship between the progesterone/estradiol ratio and CBT (Cagnacci et al., 2002; Cagnacci et al., 1997), we also fit this model to the data. All statistical analyses were conducted in SAS 9.4. (Cary, NC, USA).

4.3. Results

4.3.1. Effect of menstrual phase on performance, hormones and temperature

Subjective sleepiness (KSS scores: $F_{42, 497}$= 5.01, p< 0.0001) and neurobehavioural performance impairment (aPVT lapses: $F_{42, 490}$= 7.28, p< 0.0001; reaction time: $F_{42, 486}$= 4.8, p< 0.0001) increased with increasing time awake (Figure 2). Women in the follicular phase had significantly slower reaction times ($F_{1, 13.9}$= 10.19, p< 0.01) and more lapses ($F_{1, 13.9}$= 6.52, p< 0.03) than women in the luteal phase. Additionally, a significant time by menstrual phase interaction for both reaction time ($F_{42, 486}$= 2.47, p< 0.0001) and lapses ($F_{42, 490}$= 1.77, p< 0.003) showed that differences in performance between the groups were most pronounced at night following the CBT minimum (Figure 2).

As expected, menstrual phase significantly affected overall levels of progesterone ($F_{1, 14}$= 8.62, p< 0.02), progesterone/estradiol ratio ($F_{1, 14}$= 10.84, p< 0.01), luteinizing hormone ($F_{1, 13}$= 5.54, p< 0.04), follicular-stimulating hormone ($F_{1, 13.1}$= 19.27, p< 0.001), and CBT ($F_{1, 14.8}$= 10.41, p< 0.01). Progesterone, progesterone/estradiol ratio and CBT were higher, and FSH and LH were lower in the luteal phase of the menstrual cycle (Figure 2). In contrast, melatonin, estradiol and SHBG levels were not different between menstrual phases. Moreover, there was a significant interaction effect of time and menstrual phase on CBT ($F_{42, 533}$= 1.72, p< 0.003), showing greatest differences in CBT between the menstrual phases occurring during the night time circadian nadir of CBT.

4.3.2. Relationship between temperature, performance and progesterone

To determine the relationship between temperature, performance and hormones, we conducted linear regression analyses between each of these variables using both mean and AUC of the ~50-h CR time series. Mean CBT was inversely correlated with lapses (r_{14}= -0.52, p=0.04; SI Figure 1). Progesterone (r_{14}= 0.57, p= 0.02) and the progesterone/estradiol ratio (r_{14}= 0.62, p= 0.01)

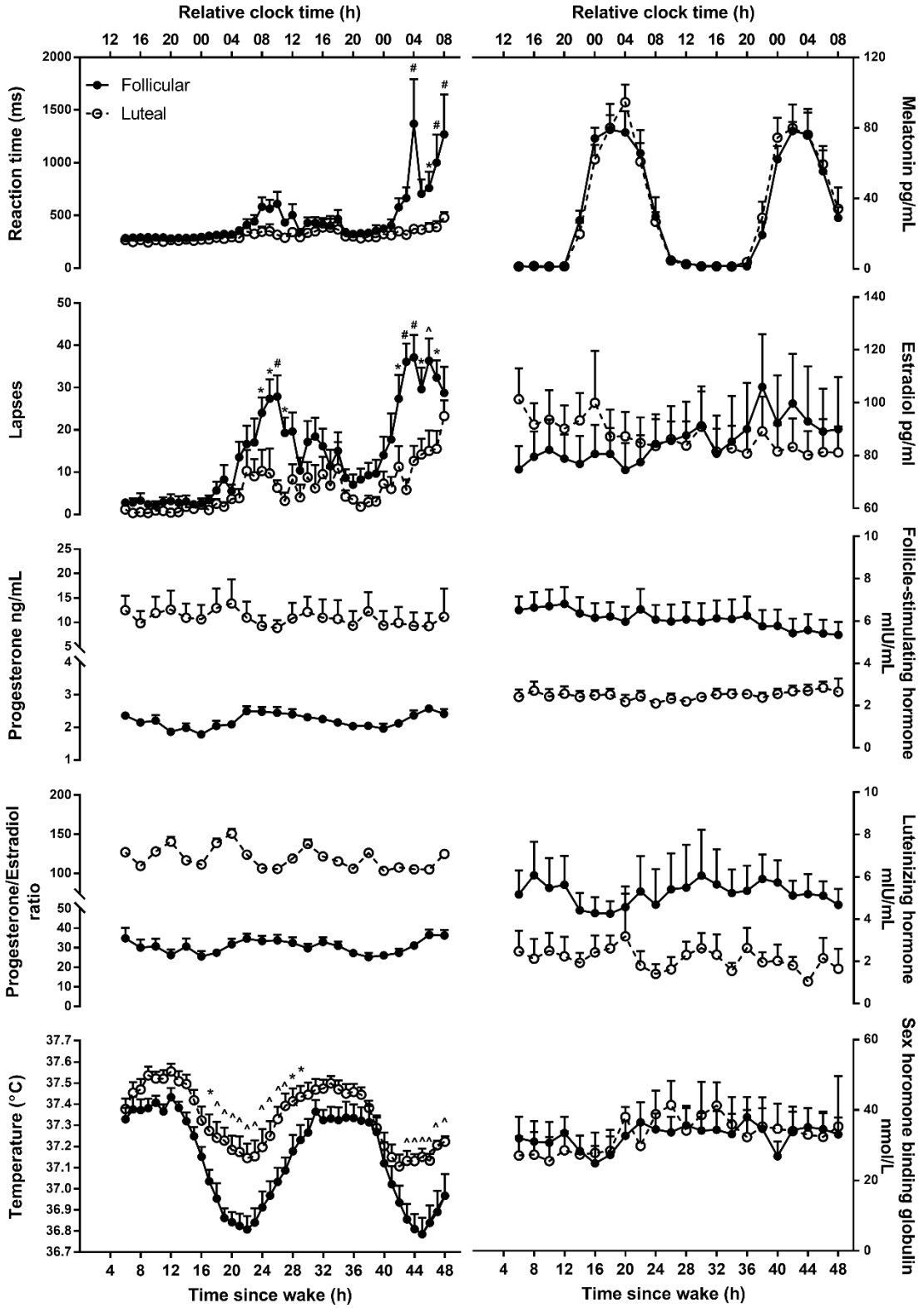

Figure 2. The mean ± SEM of performance on the aPVT (reaction time and lapses), hormones (progesterone, progesterone/estradiol ratio, melatonin, estradiol, FSH, LH and SHBG), and CBT are shown for women in the follicular (solid circles, solid line) and luteal (open circle, broken line) phases of the menstrual cycle during the CR. Significance for FDR corrected post-hoc t-tests is denoted by *($p<0.05$), ^ ($p<0.01$), and # ($p< 0.001$). Raw data are plotted.

were significantly correlated to CBT, but neither progesterone nor the progesterone/estradiol ratio predicted lapses (Figure 3). Regression analyses using AUC estimates of the same variables showed similar associations (SI Figures 1 and 3). CBT, mean reaction time or lapses did not show a linear association with any of the other reproductive hormones, however (SI Figures 2-3).

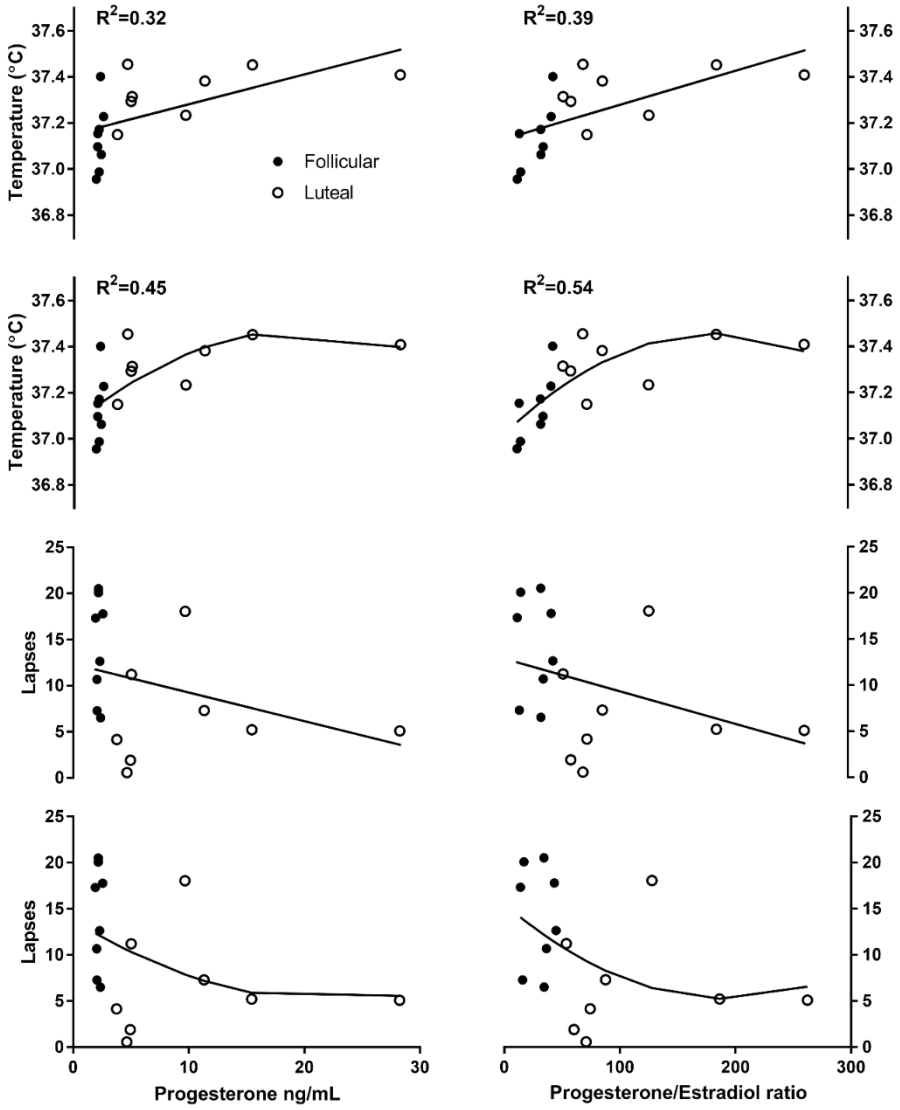

Figure 3. Linear and non-linear quadratic regression analyses of the association of progesterone (left) and the progesterone/estradiol ratio (right) with CBT (top four panels) and lapses (bottom four panels) for women in the follicular (closed circles) and luteal (open circles) phase of the menstrual cycle. R^2 values are shown for significant associations only.

A non-linear polynomial function has been reported previously to best model the relationship between CBT and the progesterone/estradiol ratio (Cagnacci et al., 2002; Cagnacci et al., 1997), however. Therefore, we further explored the association between CBT and reproductive hormones using a non-linear quadratic regression model. Both progesterone (unadjusted data: r_{13}= 0.67, p= 0.02; AUC data: r_{13}= 0.68, p= 0.02) and the progesterone/estradiol ratio (unadjusted data: r_{13}= 0.74, p= 0.006; AUC data: r_{13}= 0.73, p= 0.007), were significantly correlated with CBT (Figure 3). Similar to the linear model, however, neither progesterone nor the progesterone/estradiol ratio were significantly associated with lapses (unadjusted data: Figure 3; AUC data: SI Figure 4) or reaction time (SI Figure 4).

4.3.3. Effect of light exposure on performance, hormones and temperature

To examine the effects of light exposure on performance, hormones and temperature, we conducted a linear mixed model with four groups: 1) luteal, >30% melatonin suppression (n=4); 2) follicular, >30% melatonin suppression (n=4); 3) luteal, no suppression (<30%; n=4); and 4) follicular, no suppression (<30%; n=4). There was a significant effect of group for lapses ($F_{3,12.1}$= 5.38 p< 0.02), reaction time ($F_{3,12.2}$= 5.76, p< 0.02), CBT ($F_{3,12.3}$= 3.58, p< 0.05), and melatonin ($F_{3,12}$= 3.72, p< 0.05). Post-hoc analysis showed significantly less lapses ($t_{12.1}$= 3.23, p< 0.01) and faster reaction times ($t_{12.1}$= 3.39, p< 0.01) during the follicular phase with concomitant melatonin suppression than during the follicular phase without melatonin suppression (Figure 4). In contrast, melatonin suppression status did not affect lapses or reaction time during the luteal phase (Figure 4). Consistent with the results from the CR condition that had no light-exposure induced melatonin suppression, women demonstrated worse neurobehavioural performance during the follicular phase, compared to the luteal phase, as assessed by reaction time ($t_{12.2}$= 2.89, p< 0.02) and lapses ($t_{12.2}$= 2.73, p< 0.02), when there was no concomitant melatonin suppression induced by the experimental light exposure. This menstrual phase dependent difference in neurobehavioural performance was no longer significant when melatonin levels were suppressed by light exposure assessed by reaction time ($t_{12.1}$= 0.34, p= 0.740) or lapses ($t_{12.1}$= 0.42, p= 0.682).

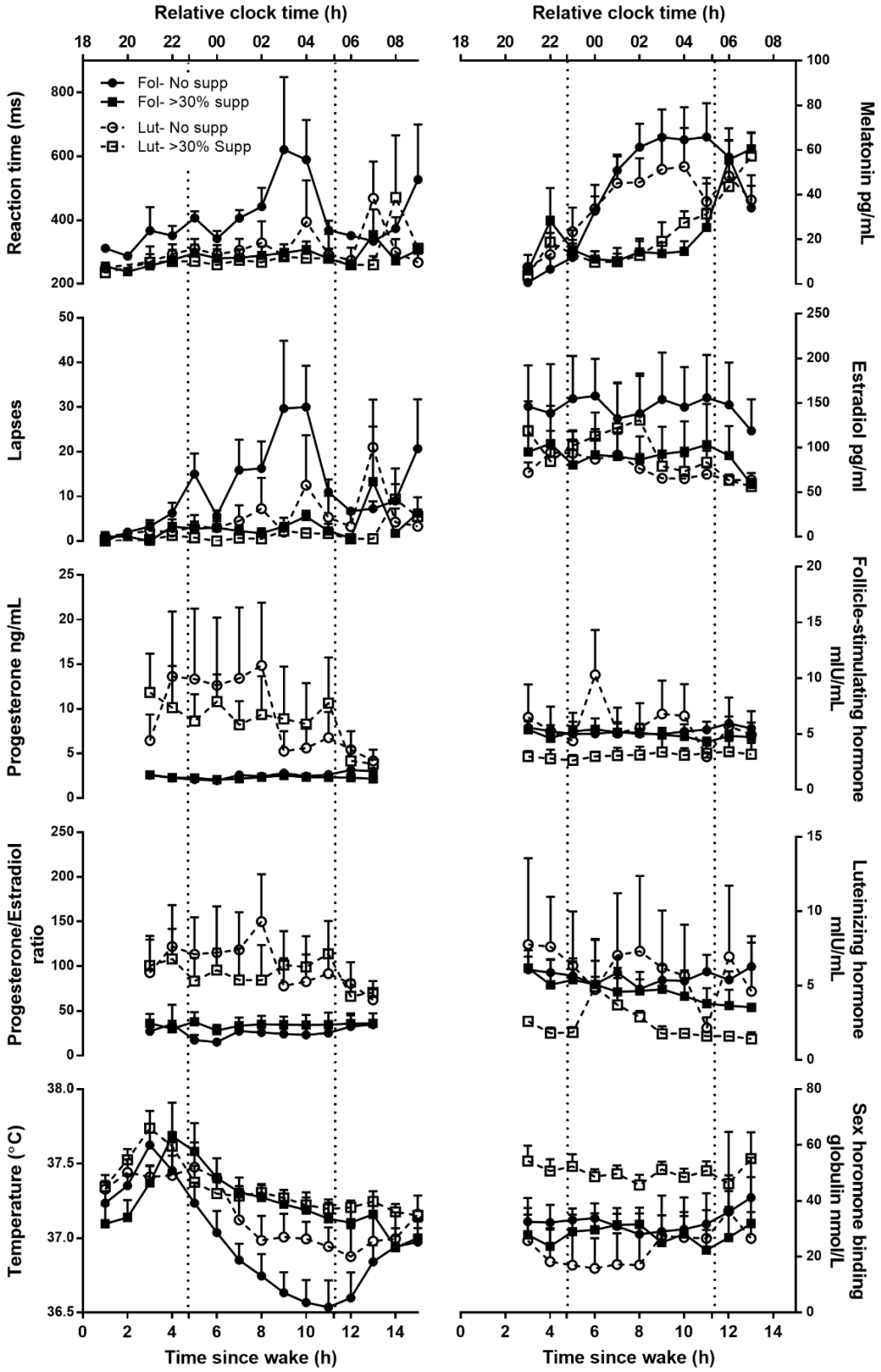

Figure 4. The mean ± SEM of performance on the aPVT (reaction time and lapses), hormones (progesterone, progesterone/estradiol ratio, melatonin, estradiol, FSH, LH and SHBG), and CBT for women in the follicular (closed symbols) and luteal phases (open symbols) who showed suppression (>30% melatonin suppression; squares) versus no suppression (<30% suppression; circles) during the 6.5-h light exposure. The dotted lines mark the start and the end of the light exposure. Raw data are plotted.

Melatonin levels were the same between the follicular and luteal phases within each of the two melatonin suppression categories (>30% suppression: $t_{12.1}$= 0.04, p= 0.971; <30% suppression: t_{12}= 0.52, p= 0.611). Consistent with the CR condition, temperature appeared to be higher in the luteal phase compared to the follicular phase when comparing the non-suppression groups, but this difference did not reach statistical significance ($t_{12.3}$= 1.86, p= 0.087). When light exposure induced melatonin suppression, however, temperature in the follicular phase was similar to that in the luteal phase ($t_{12.3}$= 0.09, p= 0.930; Figure 4). Analysing the data within each menstrual phase showed that during the follicular phase, the suppression group has significantly higher CBT than the non-suppression group ($t_{12.3}$= 2.88, p< 0.02), but in the luteal phase, CBT was not significantly different between the suppression and non-suppression group ($t_{12.3}$= -0.94, p= 0.366). None of the four groups differed significantly in any of the reproductive hormones (Figure 4).

4.4. Discussion

Our data show, for the first time, that the observed increase in vulnerability to performance impairment during the night in women in the follicular phase of the menstrual cycle can be recovered with sufficient exposure to light. Our analysis of the relationship between performance, temperature and reproductive hormones, and the effects of light on these variables suggest the differences in temperature is the main correlate associated with the differences in performance between the menstrual phases. Therefore, in the absence of photic countermeasures, interventions that manipulate temperature may be effective countermeasures to reduce vulnerability in women in the follicular phase.

Analysis of performance during the constant routine showed that women in the follicular phase of the menstrual cycle, as confirmed using endocrine markers, displayed impaired neurobehavioural performance during prolonged wakefulness, as was shown in a larger group of women from the same series of experiments (Vidafar et al., under review). The previous report relied on self-reported menstrual history to determine menstrual phase, however. Additionally, the association between performance and reproductive hormones was not assessed. With objective

confirmation of menstrual phase, we show here that women in the follicular phase were both slower to respond and had more lapses of attention particularly in the early morning hours following the CBT minimum. While a previous study has shown greater impairment in the follicular phase during the night on tasks measuring cognitive throughput there were no significant differences in reaction time between the menstrual phases (Wright & Badia, 1999). In the current study, the differences in reaction time between the menstrual phases appeared only following significant sleep loss (post-hoc comparisons significant only after 44-h awake), and these findings are consistent with those of Wright and Badia (1999) who found no difference in reaction time following a single night of sleep deprivation (~24-h awake). Our findings are also consistent with those of Vidafar et al. (under review), who showed differences between women on the PVT during 30-h of prolonged wakefulness under CR conditions, but in a larger cohort. We expand their findings by showing that the differences in PVT performance remain stable during a second night of sleep deprivation, such that women in the luteal phase remain more resilient even when prolonged wakefulness is extended past 30 hours. Taken together, these studies show that women are more vulnerable to the effects of sleep loss during their follicular phase than in their luteal phase, and underscore the importance of monitoring and reporting menstrual phase when examining performance in women during sleep deprivation.

Another novel finding in our study was the menstrual phase dependent efficacy of nocturnal light exposure as a countermeasure to night time neurobehavioural impairment. Our results show that in the absence of melatonin suppression, performance is worse during the follicular phase than during the luteal phase, consistent with the findings under CR conditions with no light exposure and no melatonin suppression. In contrast, when nocturnal light exposure suppressed melatonin, it improved neurobehavioural performance in women in the follicular phase to similar levels as observed in women in the luteal phase. Comparing performance in women the luteal phase with and without light-induced melatonin suppression did not show a significant difference between the groups, however. This lack of a difference may reflect a “floor” effect since performance did not appear to be impaired in either of the two groups (i.e. suppression and no-suppression groups), as

would be expected with being awake at an adverse circadian phase, based on the CR data. These results suggest that the use of a light intervention would effectively reduce neurobehavioural impairment associated with sleep loss and working during adverse circadian phase in both menstrual phases, but particularly so in the follicular phase when performance is more impaired. Additionally, these results underscore the importance of assessing menstrual phase also when interpreting the results of light exposure on neurobehavioural performance. A recent investigation has shown differential sensitivity to the acute alerting effects of light between men and women (Chellappa, Steiner, Oelhafen, & Cajochen, 2017), although they did not explore the effects of menstrual phases in detail in their cohort of women. The current study design, in which women were exposed to different wavelengths and irradiances of light as part of another study, precluded analysis of the optimum light intensity and wavelength needed to improve performance for each menstrual phase. Therefore, while our preliminary findings suggest potential differences in sensitivity to the acute alerting effects of light, future research should investigate light sensitivity using the same light exposure in both menstrual phases.

We also examined differences in body temperature and hormones between menstrual phases during prolonged wakefulness. As has been shown previously in several studies (Baker, Waner, et al., 2001; Cagnacci et al., 2002; Cagnacci et al., 1996; Cagnacci et al., 1997; Driver et al., 1996; Wright & Badia, 1999), women in the luteal phase of the menstrual cycle had significantly higher CBT, particularly during the night when body temperature reaches its nadir (Figure 2). This finding is consistent with the blunting of the amplitude in the CBT rhythm during the luteal phase (Cagnacci et al., 2002; Cagnacci et al., 1996; Cagnacci et al., 1997; Coyne, Kesick, Doherty, Kolka, & Stephenson, 2000; Kattapong et al., 1995; Lee, 1988; Shechter et al., 2010). Also consistent with previous studies (Brzezinski et al., 1988; Cagnacci et al., 1996; Delfs et al., 1994; Shechter, Lespérance, Ng Ying Kin, & Boivin, 2012; Shechter et al., 2010; Wright & Badia, 1999), we did not find a difference in melatonin secretion between the menstrual phases. In our analysis of the reproductive hormones, we found differences between the groups that were consistent with the differences known to characterise the two menstrual phases (Landgren, Unden, & Diczfalusy,

1980). Thus, the differences observed in the hormones during CR provide objective confirmation of menstrual phase classification in the current study.

In order to determine the factors influencing the differences in performance between the menstrual phases we examined the relationship between temperature, performance and reproductive hormones. These analyses showed that performance was significantly associated with CBT, such that higher temperatures were associated with better performance. Therefore, the differences observed in performance between the menstrual phases may be driven by differences in acute levels of CBT. Since the initial suggestion that the rhythm in reaction time is dependent on the 24-h rhythm in CBT (Kleitman, Titelbaum, & Feiveson, 1938), research has since shown that performance on a wide range of cognitive and neurobehavioural tasks follows the 24-h rhythm in CBT [e.g. (Cajochen, Khalsa, Wyatt, Czeisler, & Dijk, 1999; Dijk et al., 1992; Johnson et al., 1992; Monk et al., 1983; Wright et al., 2002; Wyatt, Ritz-De Cecco, Czeisler, & Dijk, 1999)]. While much of this research has been conducted in men, performance for women in both menstrual phases was observed to follow closely with the rhythm in CBT in the current study (Figure 2). The results of the light exposure in the current study further support a role for CBT in modulating performance between the menstrual phases. We found that the improvement in performance observed in the follicular phase in response to light that was sufficient to suppress melatonin was accompanied by an increase in temperature. Furthermore, the order of groups from best to worst performers was identical to the order of groups from highest to lowest temperature (Figure 4). Taken together, these results suggest that differences in CBT between the menstrual phases may account for the differences in performance. While we show that light exposure both increases temperature and improves performance in follicular women, evaluation of other possible countermeasures that manipulate temperature [e.g. heating and cooling techniques (Fronczek et al., 2008)] is warranted.

Given the association between temperature and performance, we next examined the relationship between temperature and reproductive hormones. Although we originally applied a linear regression model which showed only progesterone and the progesterone/estradiol ratio to be associated with temperature, use of a non-linear quadratic regression model previously found to fit

well with these data (Cagnacci et al., 2002; Cagnacci et al., 1997) explained more variance in CBT than did the linear model for both progesterone (linear: 32%; quadratic: 45%) and the progesterone/estradiol ratio (linear: 38%; quadratic: 54%). Together these results indicate that the association between temperature and both progesterone and the progesterone/estradiol ratio is not a simple dose-response linear relationship, but rather that higher concentrations of progesterone result in a plateau or reduction in temperature, which is represented by the flattening of the regression curve at higher progesterone concentrations.

Following our finding of an association between temperature and both progesterone and the progesterone/estradiol ratio, we next examined progesterone and the progesterone/estradiol ratio as potential biomarkers of performance. Despite the association between these hormonal markers and temperature, we did not find a relationship between performance and progesterone or the progesterone/estradiol ratio during the CR. Nevertheless, we also examined the effects of the light exposure on progesterone and the progesterone/estradiol ratio. The expectation that progesterone and the progesterone/estradiol ratio, like CBT, would increase in response to night time light exposure in the suppression group relative to the non-suppression group was not observed, however, as previously reported for 45-minutes of daytime white light exposure (Danilenko & Sergeeva, 2015). We also examined the relationship between other hormones associated with the menstrual cycle and performance, although, none of these measures showed a relationship with performance, or an effect of light. Therefore, our findings suggest that progesterone and the progesterone/estradiol ratio, while associated with CBT, are not effective biomarkers of performance although they may still play a role in cognitive function.

Although our findings show an association between progesterone and CBT, our data suggest that, unlike performance, progesterone does not appear to follow the same 24-h rhythm as CBT (Figure 2). While speculative, it may be that a certain concentration of progesterone once reached is able to alter the thermoregulatory set-point, but that fluctuations in progesterone throughout the day do not directly result in acute fluctuations in CBT. Therefore, if this is the case, we would not expect progesterone or the progesterone/estradiol to be an effective biomarker of performance

impairment, in that a single sample taken may not provide an indication of current level of impairment. Nevertheless, progesterone levels may be considered as a marker of potential vulnerability to performance impairment, as women with low levels of circulating progesterone indicative of the follicular phase would be expected to most vulnerable.

Although our study provides novel evidence showing that exposure to light is an effective countermeasure to mitigate vulnerability to cognitive performance impairment in the follicular phase, our study has limitations. First, all the women in the current study were young, healthy women who were naturally cycling. Future investigations should determine the effects of hormonal contraception on temperature and performance as ~30% of US women of reproductive age currently use one form of hormonal contraception (Daniels, Daugherty, & Jones, 2014). Several studies have shown that oral contraception (OC) use increases CBT to levels higher than the follicular phase (Baker, Mitchell, & Driver, 2001; Baker, Waner, et al., 2001; Kattapong et al., 1995; Tenaglia, McLellan, & Klentrou, 1999; Wright & Badia, 1999) and in accordance with these changes in CBT, one study has shown that cognitive throughput in OC users is greater than women in the follicular phase (Wright & Badia, 1999). Given only this one investigation into the effects of OC on performance during sleep loss, this area requires further inquiry. Considering the changes in CBT associated with progestin containing OC, the use of OC may be protective against sleep loss induced performance impairment, although it is important to keep in mind that not all progestins increase body temperature (Cagnacci et al., 2004). Furthermore, consideration of our findings in the context of menopause is important. Our data provide evidence for vulnerability to performance impairment in young women which has implications for female shift workers, however, approximately 30% (McMenamin, 2007) of the US female shift working population are of an age where they are likely to have experienced menopause (Gold et al., 2001; Nichols et al., 2006). Increasing age is associated with a decrease in the amplitude of the circadian CBT rhythm (Campbell & Murphy, 1998; Czeisler et al., 1992; Dijk, Duffy, Kiel, Shanahan, & Czeisler, 1999) and the CBT rhythm in older women, like women in the luteal phase, has been shown to be blunted compared to both young women and young men (Cagnacci, Soldani, & Yen, 1995; Czeisler et al.,

1992). Consistent with our data, showing less impairment in the luteal phase where the amplitude in CBT is blunted, older adults show less neurobehavioural impairment than do young individuals in response to sleep loss (Blatter et al., 2006; Duffy, Willson, Wang, & Czeisler, 2009; Silva, Wang, Ronda, Wyatt, & Duffy, 2010). While this has not been compared directly for young and old women, given the results of the current study, we may expect that menopause, like the luteal phase, could be protective against neurobehavioural impairment resulting from sleep loss. Lastly, given the small sample size of the current study, some of our analyses may have been statistically underpowered. For example, while we observed that melatonin decreased in response to sufficient light in the luteal phase, the difference between suppression and non-suppression groups was not statistically significant ($p= 0.58$), despite a clear suppression of melatonin (Figure 4).

In summary, our results support previous research identifying menstrual phase, namely the follicular phase, as a marker of vulnerability to performance impairment associated with sleep loss. While this finding has important implications for women working extended shifts and during the night, our results also provide evidence that light exposure is an effective countermeasure that recovers performance in the follicular phase. Together, our results suggest that menstrual phase is an important consideration when investigating the effects of sleep loss and light on performance in women and should be monitored and reported in future studies.

4.5. Acknowledgements

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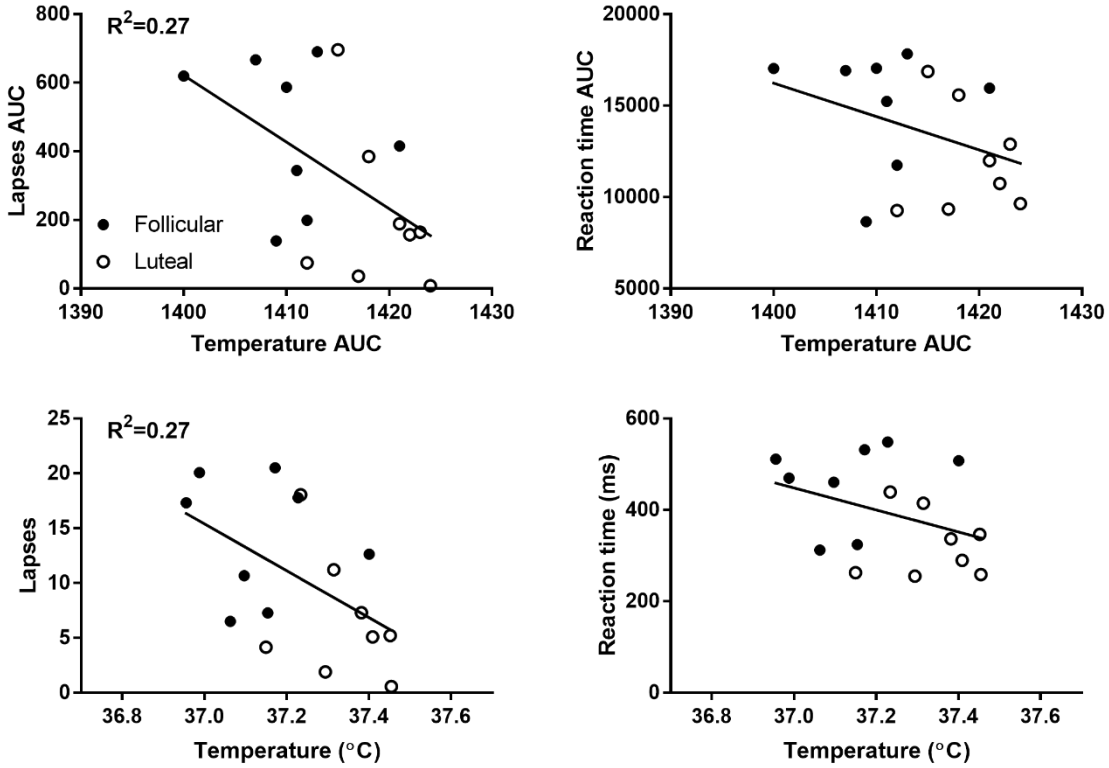
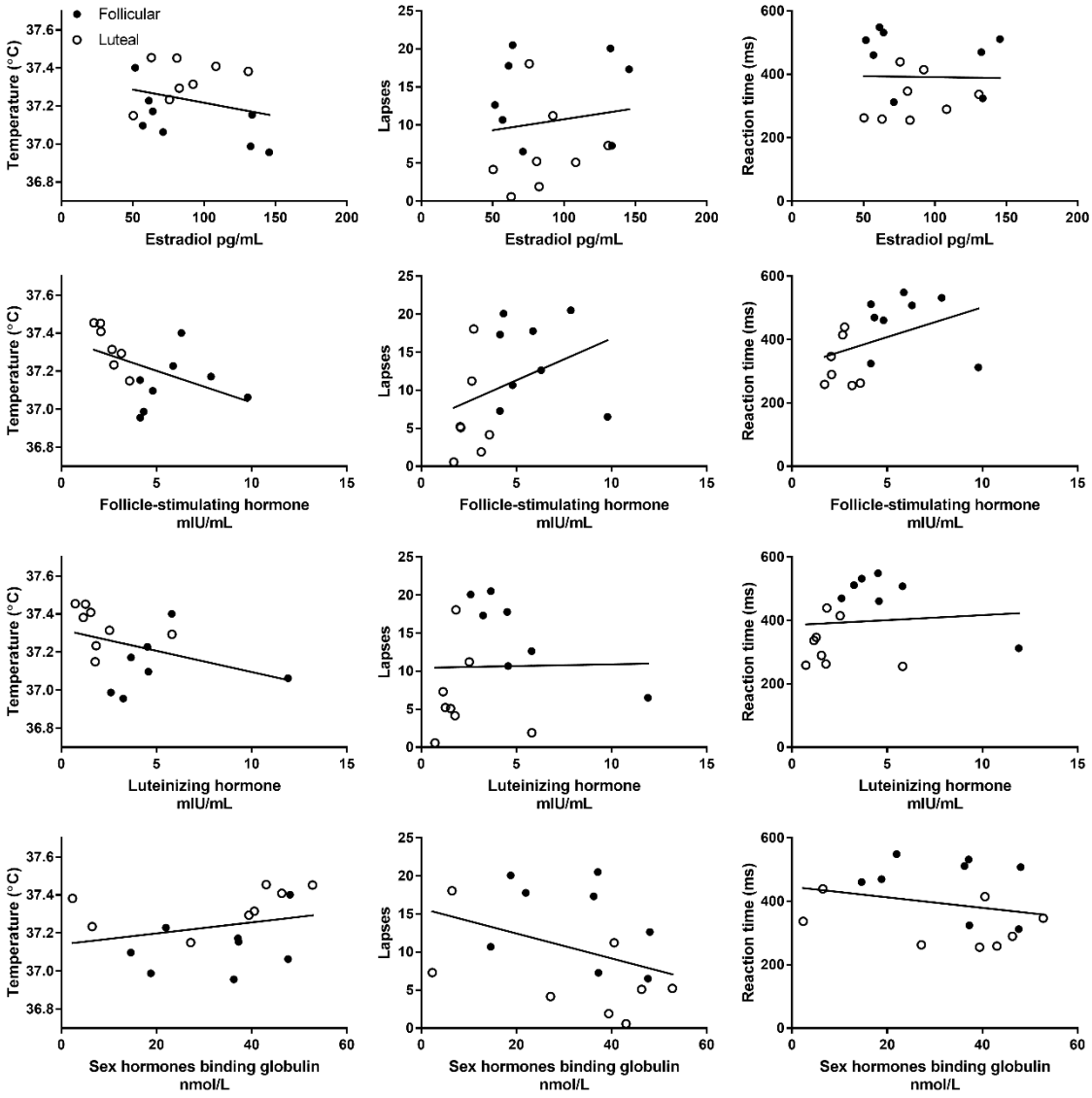
Chapter 4 Additional information supplement

4.7. Supplemental material prepared for submission to the *Journal of Clinical Endocrinology & Metabolism*

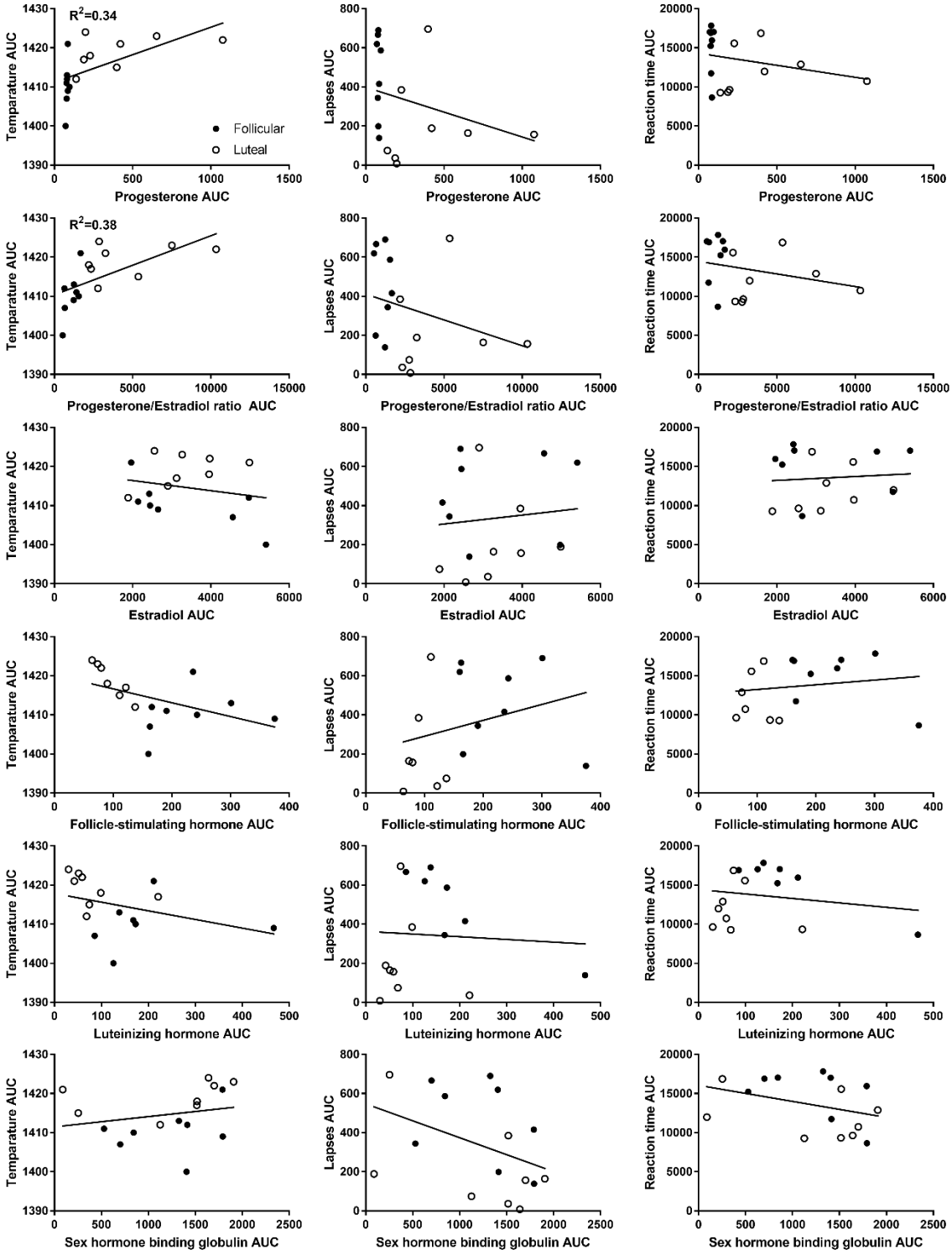
The following material consists of figures depicting analyses conducted in the above report that were not included in the main manuscript.



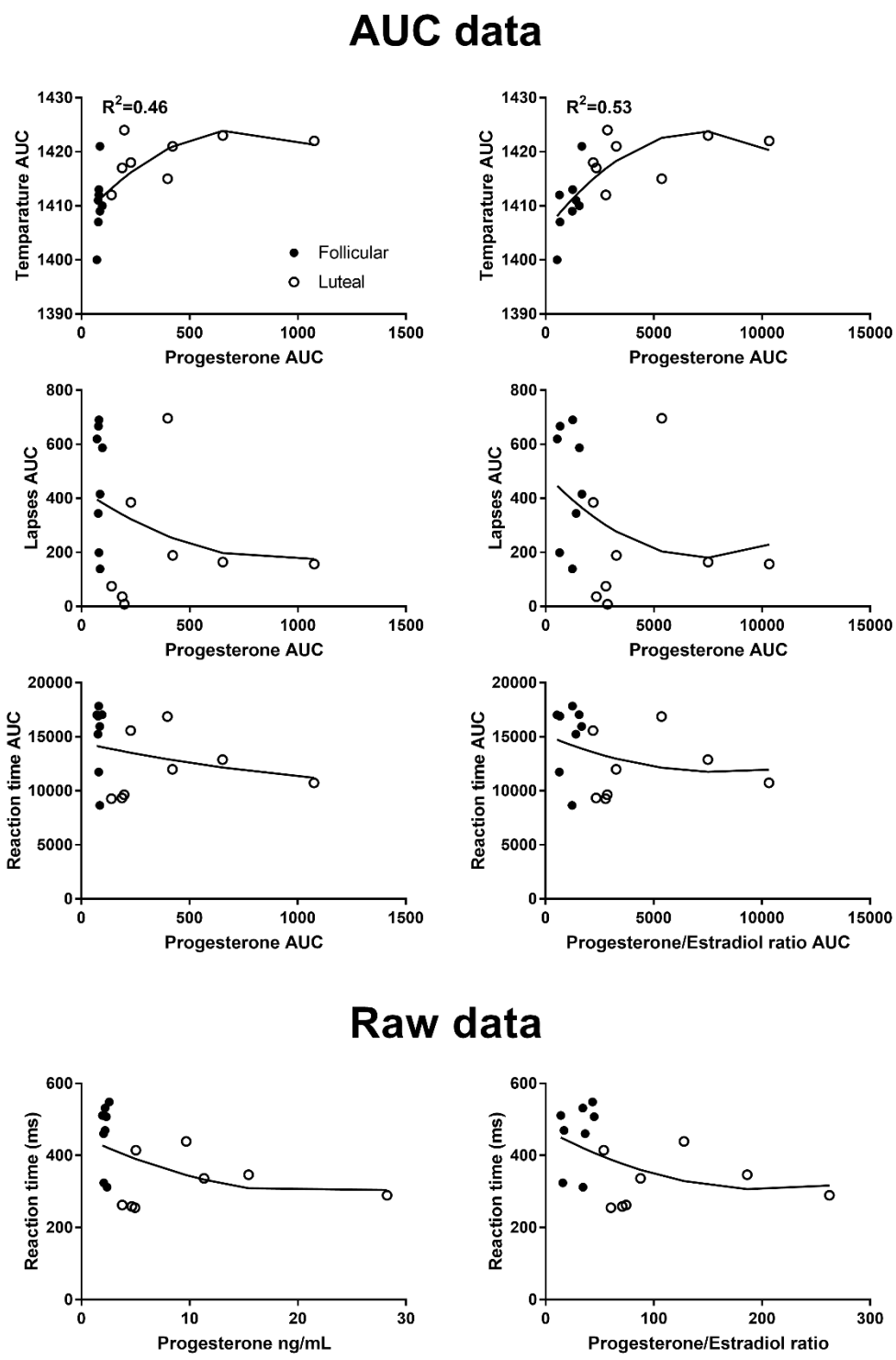
SI Figure 1. Linear regression analyses of the relationship between core body temperature (CBT) and aPVT performance for women in the follicular (closed circles) and luteal (open circles) phase of the menstrual cycle. Results of the analysis of the area under the curve data (AUC) are shown in the top panel for lapses (left) and reaction time (right), and results for the raw data are shown in the bottom panel. Temperature was a significant predictor of lapses, but not reaction time for both the AUC and raw data analyses.



SI Figure 2. Linear regression analyses of raw, unadjusted data examining the relationship between reproductive hormones (estradiol, FSH, LH and SHBG) and, CBT (left), lapses (middle) and reaction time (right) for women in the follicular (closed circles) and luteal (open circles) phase of the menstrual cycle. There was not a relationship between any reproductive hormones, and temperature or performance.



SI Figure 3. Linear regression analyses of AUC data examining the relationship between reproductive hormones (progesterone, progesterone/estradiol ratio, estradiol, FSH, LH and SHBG) and, CBT (left), lapses (middle) and reaction time (right) for women in the follicular (closed circles) and luteal (open circles) phase of the menstrual cycle. There was not a relationship between any reproductive hormones, and temperature or performance.

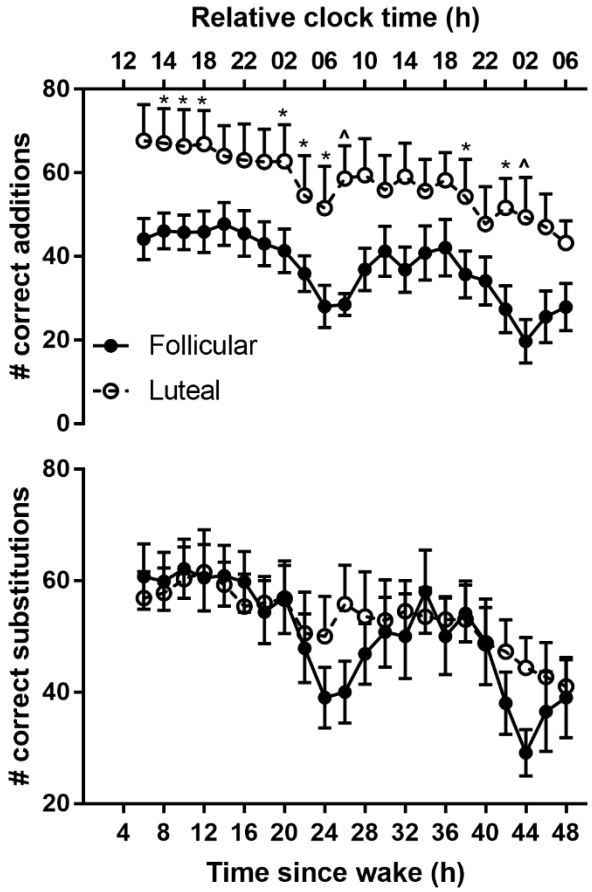


SI Figure 4. Non-linear quadratic regression analysis of AUC data examining the relationship between progesterone and the progesterone/estradiol ratio, with CBT (top), lapses (middle) and reaction time (bottom) for women in the follicular (closed circles) and luteal (open circles) phase of the menstrual cycle. Results for the analysis of raw reaction time, not included in the manuscript, are shown in the bottom panel.

4.8. Effects of menstrual phase on other performance tasks

The addition and the digit symbol substitution task (DSST; described in **Chapter 2**) were completed every 2-h during the CR, but not during the light exposure. The mean number of correct additions and correct substitutions in each testing session were calculated for the addition and DSST tasks, respectively. As with the analysis of other performance data in Chapter 4, the first 5-h of data for the addition and DSST tasks were excluded. Linear mixed model analyses were performed to compare performance on the addition task and the DSST whereby time and group were modelled as fixed factors, subject was modelled as a random factor, and time and subject were modelled as repeated factors. An autoregressive covariance structure was chosen, and degrees of freedom were calculated using the Kenward-Rogers method.

As seen in SI Figure 5, for both tasks performance deteriorated as time awake increased [Addition task: ($F_{21,241}$= 8.86, p< 0.0001); DSST: ($F_{21,244}$= 9.15, p< 0.0001)]. For the addition task, but not the DSST, there was a significant main effect of menstrual phase on the number of correct additions ($F_{1,14}$= 5.14, p< 0.04), such that women in the follicular phase made significantly fewer correct additions compared to women in the luteal phase. For both the addition ($F_{21,241}$= 1.71, p< 0.03) and DSST ($F_{21,244}$= 2.09, p< 0.01) tasks, there was a significant interaction between time awake and menstrual phase, whereby performance was most impaired following the CBT minimum. Likely due to insufficient statistical power, post-hoc comparison for the DSST were not significant, and following FDR correction none of the post-hoc comparisons were significant for the addition task. SI Figure 5, shows significant post-hoc comparisons for the addition task prior to FDR correction.



SI Figure 5. The mean ± SEM of performance on the addition and DSST tasks are shown for women in the follicular (solid circles, solid line) and luteal (open circle, broken line) phases of the menstrual cycle during the CR. Significance for post-hoc t-tests with no FDR correction is denoted by *($p<0.05$), ^ ($p<0.01$), and # ($p< 0.001$). Raw data are plotted.

Chapter 4 Concluding remarks

In this chapter, as has previously been demonstrated (Vidafar et al., under review; Wright & Badia, 1999), menstrual phase was confirmed to be a biological determinant of vulnerability to cognitive performance impairment during sleep loss. As has previously been reported, women in the follicular phase performed more poorly on the PVT (Vidafar et al., under review), such that they had slower reaction times and had more lapses of attention. Furthermore, investigation of other performance tasks showed that women in the follicular phase made less correct additions and less substitutions on the addition task and DSST, respectively, compared to women in the luteal phase. The finding that performance was also impaired on the DSST and addition task extends the findings of Vidafar et al. (under review) to show that vulnerability to performance impairment in the follicular phase extends beyond neurobehavioural performance. While a previous study has also shown impairment on tasks measuring cognitive throughput (Wright and Badia, 1999), to date an examination of whether these difference in performance during sleep loss extend also to more complex and cognitively demanding tasks, such as those that require executive control, has not been conducted. Unlike measures of simple attention and processing speed, executive functions appear to be more robust to sleep loss (Lim & Dinges, 2010), therefore future research might examine whether this biological factor (i.e. menstrual phase), like the *BDNF* Val66Met polymorphism described in **Chapter 3**, also results in greater impairment to executive functions during sleep loss or whether the impairment is limited to those cognitive functions that require simple attention and processing speed.

Although in this study the follicular phase was associated with impaired performance during sleep loss, a possible countermeasure (i.e. light) was investigated to determine whether the intervention was equally effective in improving performance during both menstrual phases. With exposure to light at night that was sufficient to suppress melatonin, it was found that the impairments observed in the follicular phase of the menstrual cycle were recovered. While the light exposure improved performance significantly for women in the follicular phase, there was only a

small benefit of light exposure observed in the luteal phase. The suitability of a photic countermeasure in the luteal phase therefore warrants consideration as the phase-shifting effects of light may outweigh the small benefit of improving performance, depending on the time of exposure. For example, exposure to light late into the biological night in a woman in the luteal phase who is on a night shift rotation may lead to further circadian disruption, while the light offers only a small improvement in alertness and cognitive performance.

In this chapter the female reproductive hormones that drive the menstrual cycle, in particular progesterone and the progesterone/estradiol ratio, were investigated as potential biomarkers of performance impairment. Although the expected relationship between temperature and performance, and temperature and progesterone were observed, performance was not significantly associated with either progesterone or the progesterone/estradiol ratio. The data did, however, trend in the expected direction, such that with greater statistical power this association may have been significant. The observation that light had no effect on progesterone or the progesterone/estradiol ratio, however, suggests that neither progesterone nor the progesterone/estradiol ratio are likely to be effective markers of current performance impairment as a true biomarker would be expected to change in response to stimuli, such as light, that alter alertness state. This finding therefore, highlights a drawback of using a targeted approach to identifying biomarkers of alertness, in that targeting specific biochemicals based on their association with a phenotype or genotype associated with enhanced vulnerability to sleep loss may not yield an effective biomarkers of alertness state.

While the expected biomarkers were not found to be viable candidates, we did find that temperature was associated with performance, and was altered by light exposure. Our findings showed that temperature was a significant correlate of performance. Temperature may therefore be a suitable biomarker, however, given that temperature is alerted by many factors, for example food intake, posture, exercise and ambient temperature (Dauncey, 1983; Gonzàlez-Alonso et al., 1999; Iwase, Cui, Wallin, Kamiya, & Mano, 2002; Kräuchi, Cajochen, & Wirz-Justice, 1997), it is

unlikely to have the sensitivity required for a biomarker of alertness. Temperature may, however, be a valuable addition to systems that integrate streams of physiological data (e.g. EEG, heart rate, etc) to monitor alertness. Although temperature may not be a viable biomarker on its own, the finding that temperature may be driving the differences in performance between the menstrual phases makes it a target for interventions.

In this chapter light at night was shown to alter both temperature and performance suggesting that a photic countermeasure may improve night-time performance, especially in the follicular phase. Since the effect of light at night on temperature appears to be mediated by melatonin (Cagnacci, Soldani, & Yens, 1993; Strassman, Qualls, Lisansky, & Peake, 1991), a photic countermeasure that induces the greatest melatonin suppression would likely be most effective. Therefore, in an operational setting, the use of blue-enriched polychromatic light [e.g.; (Sletten et al., 2017)], which induces greater melatonin suppression than polychromatic light with warmer colour temperatures (Brainard et al., 2015; Chellappa et al., 2011), may be a suitable intervention for reducing performance impairment in female night-shift workers. Although light exposure during the day has also been shown to induce an alerting effect (Rahman et al., 2014), the mechanism underlying this effect is not well understood in the absence of melatonin, and furthermore, light during the day does not increase CBT (Rüger, Gordijn, Beersma, De Vries, & Daan, 2006). Therefore, if the mechanism underlying the effects of light on performance in women in the follicular phase is the effect of light on temperature, then daytime light exposure may not be an effective countermeasure to mitigate daytime performance impairment in this particular group. Based on the result of the current study, however, the impairment in performance in women in both menstrual phases was greatest during the biological night when the nadir in CBT occurs, suggesting that interventions to mitigate these impairments are likely more necessary during the night than during the day. Nevertheless, where photic countermeasures are not practical or effective other means of manipulating temperature may also be viable. This hypothesis requires further investigation, however.

Although this chapter shows that the difference in performance between the menstrual phases appeared to be driven by differences in temperature, sleep was not examined in the current study and therefore the possibility that the differences in performance observed across the menstrual cycle could be partly explained by differences in sleep cannot be ruled out. Despite sleep not being examined in the current study, the literature shows that sleep may be poorer in the luteal phase of the menstrual cycle such that performance would be expected to be worse in this phase also. In the current study, however, it was found that women in the luteal phase performed better, not worse, than women in the follicular phase suggesting that differences in sleep between the menstrual phases may not account for the differences in performance. It is well established that self-reported sleep quality changes during the menstrual cycle whereby women report greater sleep disturbance during the premenstrual (late-luteal) phase of their cycle (Baker & Driver, 2004; Baker et al., 2012; Brown, Morrison, Calibuso, & Christiansen, 2008; Kravitz et al., 2005; Romans et al., 2015). Despite this difference in self-reported sleep, studies examining sleep objectively using PSG have shown little difference in sleep time, efficiency and architecture across the menstrual cycle [for review see (Baker & Driver, 2007)]. Consistently, studies have shown no effect of menstrual cycle phase on NREM sleep and a small decrease in the percentage of REM sleep during the luteal phase (Baker, Driver, Rogers, Paiker, & Mitchell, 1999; Baker, Waner, et al., 2001; Driver et al., 1996; Lee, McEnany, & Zaffke, 2000; Shechter et al., 2010). Importantly however, SWA, a marker of the sleep homeostat, does not appear to be altered during nocturnal sleep episodes, suggesting that sleep homeostatic mechanisms are likely not affected by menstrual phase (Baker et al., 1999; Baker, Waner, et al., 2001; Driver et al., 1996; Lee et al., 2000; Shechter et al., 2010). Despite there not being an effect of menstrual phase on nocturnal SWS, one study has shown that self-reported daytime sleepiness and the incidence of SWS in daytime naps is higher in women in the luteal phase compared to the follicular phase (Shibui et al., 2000). Taken together, these results suggest that, while sleep is largely unaltered by menstrual phase, subjective sleep quality is poorest and daytime sleepiness is highest during the luteal phase of the menstrual cycle such that it is unlikely

that sleep is contributing the finding of the current study showing that performance is better in women in the luteal phase of the menstrual cycle.

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Chapter 5

Circadian and wake-dependent changes in the human plasma polar metabolome during prolonged wakefulness

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Preface to Chapter 5

In **Chapter 4**, based on the associated between menstrual phase and vulnerability to alertness impairment during sleep loss, female reproductive hormones, particularly progesterone and the progesterone/estradiol ratio, were targeted as biomarkers of alertness state. While it appears that neither progesterone nor the progesterone/estradiol ratio are viable biomarkers of performance impairment during acute sleep deprivation, these biomarkers, had they been found to be useful, would only be applicable to naturally cycling women of reproductive age. Therefore, in **Chapter 5**, an untargeted metabolomics approach is used to identify biomarkers of alertness state that may be applicable to both men and women.

The study in the current chapter aimed to identify metabolites that changed with increased time awake and circadian phase, two main factors that modulate alertness. While previous research has typically measured moderately and non-polar metabolites (Ang et al., 2012; Chua, Shui, Cazenave-Gassiot, Wenk, & Gooley, 2015; Chua et al., 2013; Dallmann, Viola, Tarokh, Cajochen, & Brown, 2012; Davies et al., 2014; Kasukawa et al., 2012), in this study, highly polar compounds were targeted using hydrophilic interaction liquid chromatography (HILIC). Polar compounds, including amino and other organic acids, carbohydrates, polar lipids, nucleotides and nucleosides, are essential biochemicals in a number of important biological pathways. Furthermore, these metabolites play a role in some pathways shown to be affected by inadequate sleep and circadian misalignment in transcriptomic studies (Archer et al., 2014; Möller-Levet et al., 2013). In addition to addressing the core aim of identifying circadian and wake-dependent polar metabolites, this study also aimed to investigate individual differences in circadian and wake-dependent metabolites and assess the concordance between group- and individual-level analyses of these data.

Chapter 5 includes an article formatted for and submitted to the *Proceedings of the National Academy of Sciences of the United States of America*, submitted in March 2018. While the publication discusses the results in the context of biomarker development and implications for metabolic disease related to sleep and circadian disruption, these analyses led to the identification

of 18 candidates for future investigation as biomarkers of alertness. Details of candidates are shown in the Additional Information Supplement (Section 5.9.) of this chapter.

Chapter 5 Manuscript submitted for publication

Circadian and wake-dependent changes in human plasma polar metabolites during prolonged wakefulness

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Summary

Establishing circadian and wake-dependent changes in cellular metabolism are critical for understanding and treating human disease or adverse states associated with circadian misalignment or extended time awake. Here, we have assessed endogenous, self-sustained circadian rhythms and wake-dependent changes in plasma metabolites at both the group- and individual-levels. Thirteen healthy, young participants (4 females) completed a 40-h constant routine. Four-hourly plasma samples were analyzed by hydrophilic interaction liquid chromatography (HILIC)-LC-MS allowing the detection of 1,821 metabolite signals, including many polar metabolites involved in central carbon metabolism and macromolecule synthesis. Group-averaged and individual participant metabolite profiles were fitted with a non-linear regression model with cosinor (circadian) and linear (wake-dependent) components. In group-level analyses, ~22% of metabolites were rhythmic and ~7% were linear, whereas in individual-level analyses, ~14% of profiles were rhythmic and ~4% were linear. Compared to individual profiles, group-level analyses produced both false positives (i.e. metabolites significant at the group-level, but not significant in a single individual) and false negatives (i.e. metabolites significant in at least half of individuals, but not significant at the group-level). Owing to large inter-individual variation in the timing of rhythms and the magnitude and direction of linear change, estimates of acrophase and slope values also differed between group- and individual-level analyses. These findings extend previous work by identifying circadian- and wake-dependent changes in several novel polar metabolites and have important implications for biomarker development and our understanding of sleep and circadian regulation of metabolism.

Keywords

metabolomics; HILIC; circadian; sleep deprivation; biomarker

5.1. Introduction

Circadian rhythms, endogenously generated cycles of approximately 24 hours, govern many patterns of behavior and physiology including sleep/wake cycles, cognition, feeding patterns, hormone secretion, gene expression and cellular processes. Given the circadian system's control over so many biological processes, it is unsurprising that disruption to this endogenous clock and its outputs is associated with adverse health outcomes. Shift workers, for example, whose circadian rhythms are often chronically misaligned from their sleep-wake cycle (Dumont, Benhaberou-Brun, & Paquet, 2001; Roden, Koller, Pirich, Vierhapper, & Waldhauser, 1993), have an increased risk of developing serious diseases including obesity, diabetes, cardiovascular disease, stroke and some cancers (Gu et al., 2015; Karlsson, Alfredsson, Knutsson, Andersson, & Torén, 2005; Schernhammer et al., 2001). Moreover, experimentally-induced circadian disruption in controlled laboratory settings has shown that misalignment of circadian and behavioral cycles leads to acute cardiometabolic dysfunction in humans (Buxton et al., 2012; Morris, Purvis, Hu, & Scheer, 2016; Scheer, Hilton, Mantzoros, & Shea, 2009). A direct influence of the circadian system on metabolic homeostasis has been demonstrated in rodents, whereby knocking out core clock genes significantly alters metabolism (Paschos et al., 2012; Sadacca, Lamia, DeLemos, Blum, & Weitz, 2011; Turek et al., 2005). Furthermore, studies have demonstrated 24-h rhythms in the hepatic, serum and plasma metabolomes of rodents (Abbondante, Eckel-Mahan, Ceglia, Baldi, & Sassone-Corsi, 2016; Eckel-Mahan et al., 2012; Minami et al., 2009), prompting investigation of circadian control of the metabolome in humans.

Metabolomic analysis of human plasma samples collected during a normal day with an 8:16h sleep/wake cycle, or during sleep deprivation has shown 24-h oscillations in addition to wake-dependent increases or decreases in metabolites from a wide variety of chemical classes (Ang et al., 2012; Davies et al., 2014). Studies conducted under constant routine (CR) conditions, the gold-standard method for assessing endogenous circadian rhythms (Duffy & Dijk, 2002), have also described 24-h rhythms and increases or decreases over time awake in the human metabolome (Chua et al., 2015; Chua et al., 2013; Dallmann et al., 2012). Analysis of individual metabolomic

profiles, however, has shown substantial inter-individual differences in the timing and abundance of rhythmic lipids and in the magnitude and direction of change in lipids that increase or decrease with time awake (Chua et al., 2015; Chua et al., 2013). Despite this variability, many of the studies published to date have only conducted group-level analyses, which given the underlying inter-individual variation, may not accurately describe circadian and wake-dependent control of metabolite levels. Furthermore, previous studies have focused on metabolites that are resolved on reverse phase LC matrixes (i.e. lipids, fatty acids, acyl carnitines, some amino acids and carbohydrates) and have not detected changes in more polar compounds, such as nucleotides, nucleosides, organic acids, amino acids, and carbohydrates, which are important intermediates in central carbon metabolism and are reflective of changes in macromolecule synthesis, the urea cycle, and pathways of energy (i.e. glycolysis and the Krebs cycle).

Polar metabolites have been identified as biomarkers of cancers (Chen et al., 2009; Urayama, Zou, Brooks, & Tolstikov, 2010), diabetes (Wang et al., 2011), Alzheimer's disease (Ibáñez et al., 2012), myocardial ischemia and infarction (Naz et al., 2015; Sabatine et al., 2005), and osteoarthritis (Zhai et al., 2010). With single-point assessments of polar metabolites potentially being used as biomarkers of a variety of disease states, it is important that the circadian variation and effect of inadequate sleep on these compounds is well understood. Variation in a metabolite's concentration at different times of day or variation induced by sleep loss has implications for the timing and interpretation of clinical diagnostic tests and the efficacy of treatments. Improved understanding of circadian- and wake-dependent control of metabolism will also contribute to understanding the etiology of cardiometabolic diseases and may inform future development of interventions and chronotherapies to treat such disorders. In the current study, we sought to characterize circadian- and wake-dependent changes in polar metabolites using HILIC-LC-MS over 40-h of continuous wakefulness under highly controlled conditions. Changes to metabolite levels were subsequently assessed using both group- and individual-level analyses to observe the degree of concordance between these analysis approaches.

5.2 Results

Circadian and wake-dependent modulation of plasma polar metabolites was investigated in 13 healthy adults (4 females) aged 20-32 years (Table 1), who underwent a 40-h CR protocol (Figure 1). The final dataset of metabolites included 99 metabolites identified based on their accurate mass and coelution with authentic metabolite standards, in addition to 1,722 unidentified metabolite features that constituted the untargeted matrix and were detected in all participants. Ten of 13 participants had missing data points in the targeted matrix resulting in 14% (18 samples; total n=112) missing data, and 12 of 13 participants had missing data points in the untargeted matrix resulting in a total of 16% (21 samples; total n=109) missing data. Further information on missing samples can be found in the SI Materials and Methods.

We assessed the proportion of plasma metabolites in the targeted and untargeted matrices that changed in a rhythmic, linear or combined rhythmic and linear fashion over the 40-h of extended wakefulness. Results of these analyses are shown in SI Figures S1 and S2. Representative examples of metabolites that exhibited rhythmic, linear and combined rhythmic and linear changes in plasma levels at the group-level are shown in Figure 2B for the targeted matrix and in Figure 3B for the untargeted matrix.

5.2.1. Analysis of metabolites at the group-level

Group-level analysis of the 99 identified metabolites, showed that 21 metabolites were significantly rhythmic, and four that were significantly linear. In those that were rhythmic, nearly all (90%) had a peak time (acrophase) during the biological night (defined as Dim Light Melatonin Onset (DLMO) plus 10 hours). Of the four linear metabolites, two increased and two decreased with time awake. In addition to the 21 rhythmic only and four linear only metabolites, seven of the 99 metabolites showed both rhythmic and linear changes. Five increased, and two decreased with time awake. All of these metabolites had acrophases during the biological night. The 28 rhythmic metabolites (including combined rhythmic and linear metabolites), comprised 16 amino acids, 6 organic acids, 2 nucleotides, 2 carbohydrates and derivatives, and a single vitamin and peptide.

Table 1. Demographic characteristics of study participants.

Demographics	M ± SD or No. (%)
N	13
Age (years)	25.00 ± 4.31
Males	9 (69%)
Body mass index (kg/m^2)	22.00 ± 2.14
Dim light melatonin onset time (decimal time)	20.91 ± 1.47
Wake time (decimal time)	07.19 ± 0.73
Bed time (decimal time)	23.19 ± 0.73
Morningness Eveningness Questionnaire score	37.92 ± 2.66

Note: Participant excluded from the analysis is not included in table

Pathway analysis (Xia & Wishart, 2016) showed enrichment of the phenylalanine and tyrosine metabolism pathway (See SI Figure S3 and Table S1). The overall 11 linear metabolites (including combined rhythmic and linear metabolites), comprised 6 amino acids, 2 organic acids, and a single vitamin, xenobiotic and nucleoside. While the arginine and proline metabolism pathway showed enrichment, this was no longer significant following false discovery rate correction (See SI Figure S4 and Table S2). The time-course of all metabolites from the targeted matrix are shown in Figure 2A.

Group-level analysis of the untargeted data showed a similar proportion of metabolites (~23%) to those in the targeted matrix were significantly rhythmic. A wide range in acrophase times were observed with many of the metabolites peaking during the daytime (59%). Group-level analysis of the untargeted matrix also showed that ~7% of metabolites were significantly linear, of which most (66%) of these decreased with time awake. Approximately 3% of metabolites showed a combined rhythmic and linear pattern of change. Just over half (51%) of these metabolites decreased over time and the majority (67%) of these peaked during the day. Of the metabolites showing combined rhythmic and linear trends that increased, the majority (72%) had an acrophase during the night. The time-course of metabolites from the untargeted matrix that were significantly rhythmic, linear or combined rhythmic and linear are shown in Figure 3A.

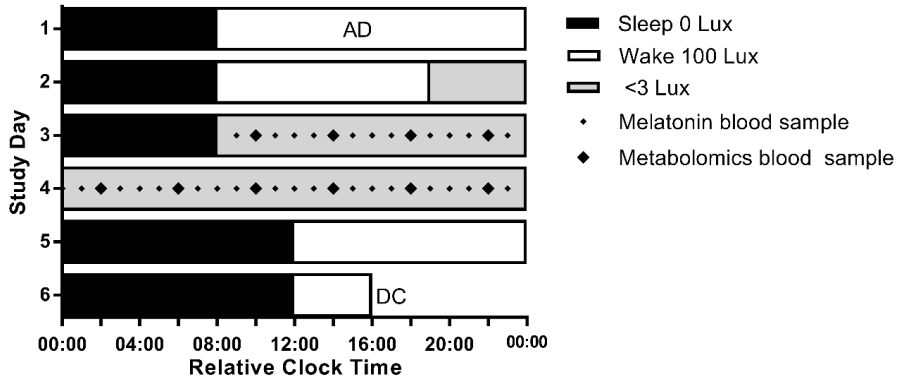

Figure 1. Participants completed a 6-day laboratory protocol. The protocol consisted of (i) two baseline days (8:16 sleep/wake based on average sleep time two weeks before admit [AD]), (ii) a 40-h constant routine, and (iii) two recovery days with up to 12-h sleep opportunities before discharge (DC). White bars represent wake episodes in 100 lux, black bars represent sleep episodes in 0 lux, and grey bars represent a DLMO assessment on day 2 and the 40-h CR in <3 lux ambient light. During the CR protocol, black diamonds represent blood samples, with larger diamonds representing samples used in the current metabolomics analysis. The protocol is shown in relative clock time with a relative bedtime of midnight. Study events were scheduled relative to each individual's pre-study self-selected wake time.

5.2.2. Analysis of metabolites at the individual-level

Following group-level analysis, we next analyzed individual participant metabolite profiles (i.e. single metabolite profiles over time for each participant), including the 1,287 (99 x 13 participants) targeted profiles and 22,386 (1,722 x 13 participants) profiles from the untargeted matrix. Results of these are shown in SI Figure 2. Of the profiles from the targeted matrix, ~10% were significantly rhythmic and over half (64%) of these rhythmic profiles peaked during the day. Profiles that were significantly linear accounted for ~5% of all analyzed profiles within the targeted matrix, and most (63%) of these showed an increase with time awake. Approximately 3% of individual profiles in the targeted matrix showed a combined linear and rhythmic pattern of change. Of these, over half (56%) increased with time awake, and 70% had acrophases during the night. In metabolites that decreased, however, there was an even spread of acrophases throughout the day and night.

The identified rhythmic compounds detected at the individual-level comprised mainly amino (29%) and organic acids (22%), although a number of nucleotides and nucleosides (16%) and

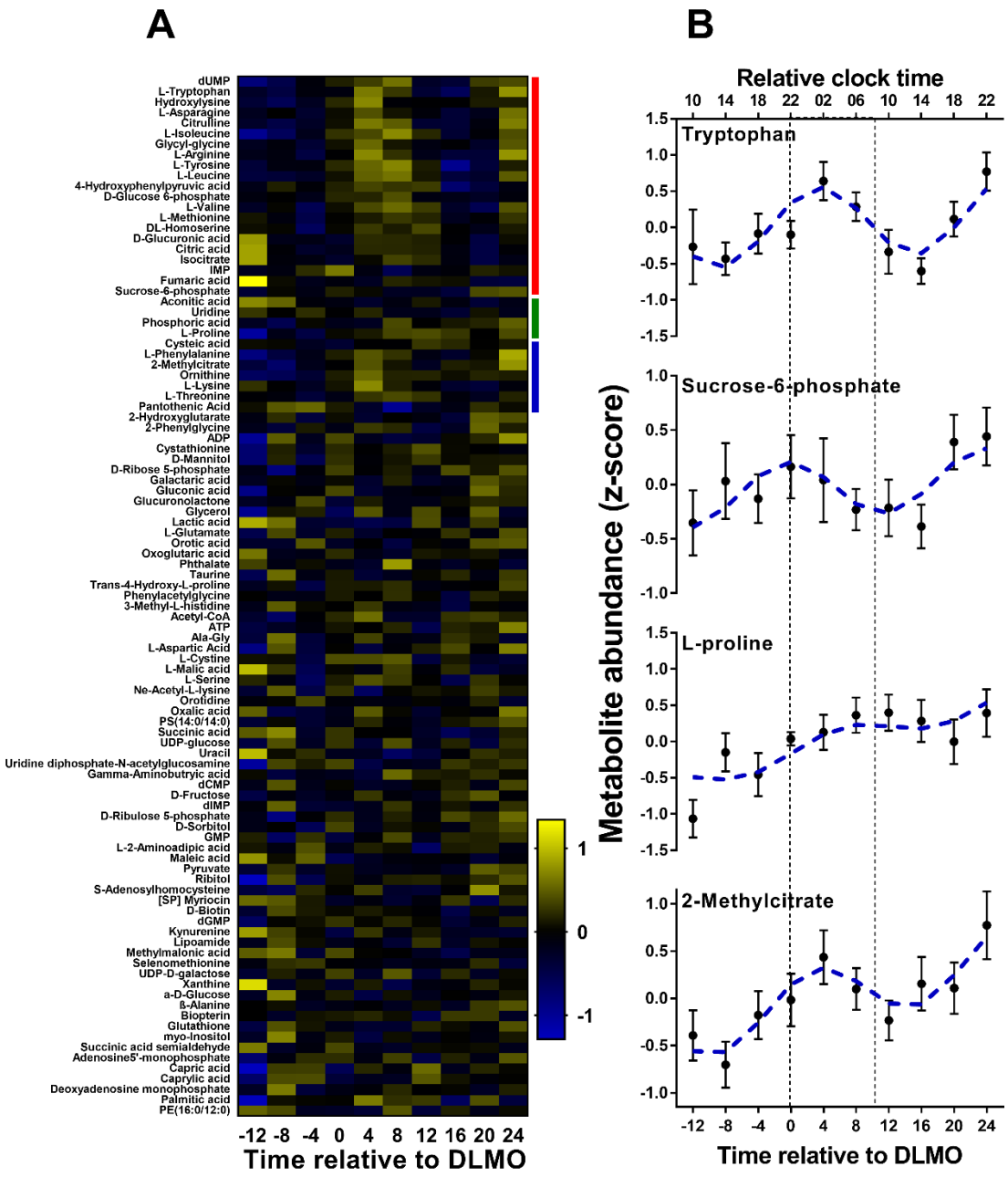

Figure 2. (**A**) Time course of metabolite concentrations (z-score area under the peak) for all identified metabolites in the targeted matrix. Significant metabolites are marked by the coloured bar to the right of the heatmap (red – rhythmic; green – linear; blue – combined rhythmic and linear). Data is represented relative to DLMO (time 0), the time at which plasma melatonin reached 5pg/mL. **(B)** Examples of significant profiles are shown for tryptophan (*top*: night peaking rhythmic, not linear), sucrose-6-phosphate (*middle, upper:* day peaking rhythmic, not linear), L-proline (*middle, lower:* linear increasing, not rhythmic), and 2-methylcitrate (*bottom:* night peaking rhythmic with linear increase). Data are plotted relative to DLMO, and by relative clock time, with relative bedtime at midnight. Errors bars represent SEM. The area between the dashed lines represent the 'biological night', defined as DLMO plus 10 hours, and the blue dashed line represents the predicted fit of the model.

carbohydrates and derivatives (14%) were also rhythmic. Amino acids had acrophases mainly during the evening and throughout the night. Similarly, organic acids predominantly peaked during the biological night, whereas carbohydrates and their derivatives had acrophases throughout the day and night. Nucleotides and nucleosides, however, tended to peak during the morning hours, in the first half of the day. Pathway analysis of these rhythmic compounds showed that the urea and Krebs cycle pathways were significantly enriched (See SI Figure S5 and Table S3). Similar to the rhythmic metabolites, linearly changing metabolites comprised mainly amino acids (29%), organic acids (29%), carbohydrates (16%), and nucleotides and nucleosides (9%). The amino and carboxylic acids showed both increases (amino: 55%; carboxylic: 52%) and decreases (amino: 45%; carboxylic: 48%), whereas most of the carbohydrates increased (69%) and all nucleosides and nucleotides increased with time awake. Significantly enriched pathways for the linear metabolites included the Krebs and urea cycles, malate-aspartate shuttle, beta-alanine metabolism and galactose metabolism (See SI Figure S6 and Table S4).

Of the 22,386 individual untargeted profiles, ~14% were significantly rhythmic and ~4% showed a linear change with time awake. Similar to the group-rhythmic metabolites, most (61%) of the significant individual-level rhythmic profiles peaked during the day. There was a near-even split in the direction of linear change, with just over half (54%) of metabolites decreasing with time awake. Of all the significant individual profiles, ~4% were combined rhythmic and linear. These profiles tended to show an increase (57%) with time awake and peaked mostly during the day (79% of those increasing, and 55% of those decreasing).

5.2.3. Comparison of group- and individual-level analyses

We next examined the level of concordance in group- and individual-level analyses. As seen in Figure 4A, the proportion of significant (rhythmic, linear or both rhythmic and linear) metabolites was decreased overall in the individual-level analysis (21%) compared to the group-level analysis (33%). This decrease appeared to be driven mainly by a reduction in significantly rhythmic metabolites; however, the proportion of linear metabolites also decreased slightly in the individual-

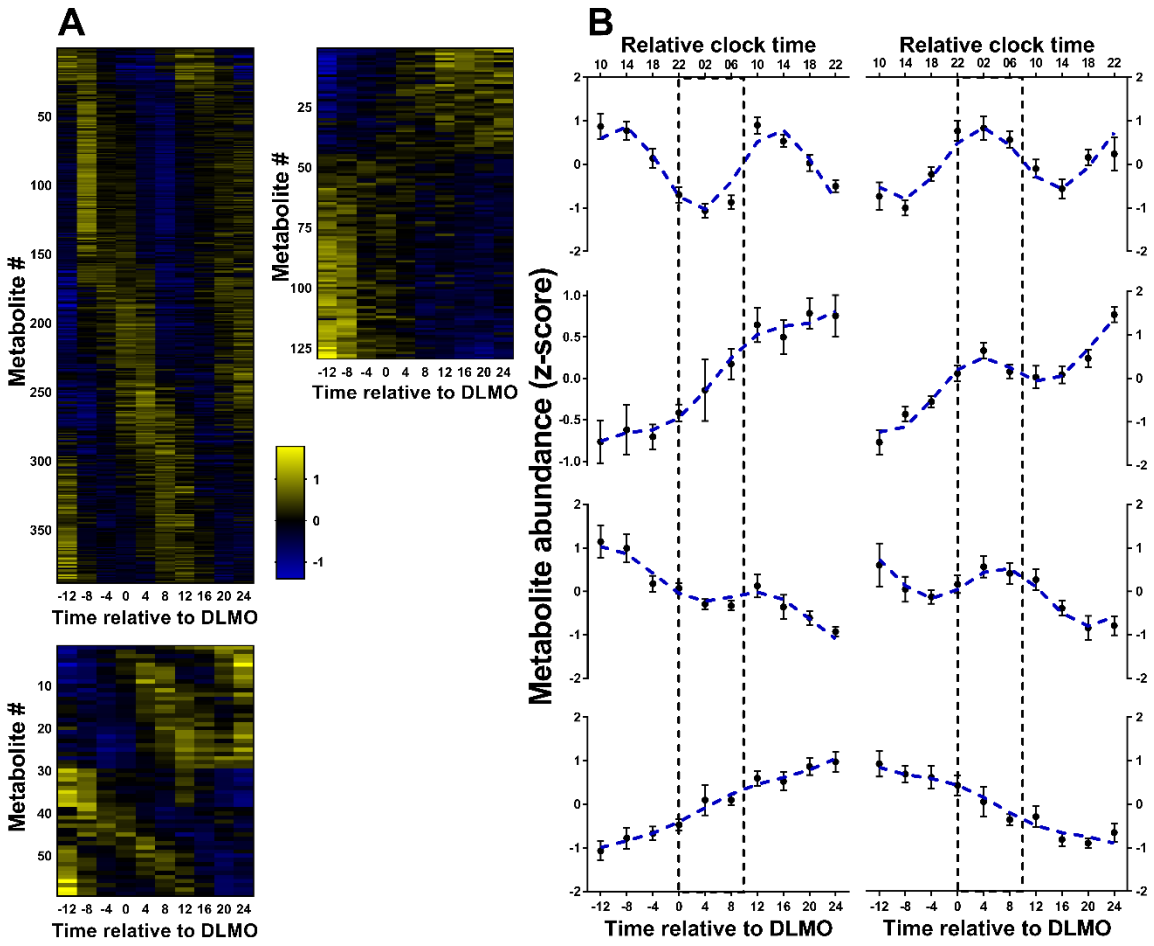

Figure 3. Group-level analysis of the untargeted metabolite matrix. **(A)** The time course of metabolites significantly rhythmic (*top left*), linear (*top right*), and combined rhythmic and linear (*bottom*) in group-level analysis. Data are represented relative to DLMO (time 0), the time plasma melatonin reached 5pg/mL. **(B)** Metabolites showing rhythmic and linear trends during sleep deprivation from the untargeted matrix. Examples include day peaking rhythmic, not linear (*top left*), night peaking rhythmic, not linear (*top right*), day peaking rhythmic with linear increase (*middle, upper left*), night peaking rhythmic with linear increase (*middle, upper right*), day peaking rhythmic with linear decrease (*middle, lower left*), night peaking rhythmic with linear decrease (*middle, lower right*), linear increase, not rhythmic (*bottom left*), and linear decrease, not rhythmic (*bottom right*). Data are plotted relative to DLMO, and by relative clock time, with relative bedtime at midnight. Errors bars represent SEM. The area between the dashed lines represent the 'biological night', defined as DLMO plus 10 hours, and the blue dashed line represents the predicted fit of the model.

level analysis of untargeted profiles. Figure 4 also shows the percentage of metabolites showing rhythmic (Figure 4B, including combined rhythmic and linear metabolites) and linear (Figure 4C, including combined rhythmic and linear metabolites) changes for each individual participant. The

proportion of overall significant metabolites differed between participants, with some participants having less than 10% of metabolites showing rhythmic and linear changes during prolonged wakefulness (Figure 4A).

As seen in Figure 4B and 4C, there were differences between participants in which metabolites were significant, and there were no metabolites that were significantly rhythmic or linear for all participants. While not significant in all participants, there were metabolites that were relatively consistent across some participants (i.e. significant in at least n=6), although, acrophase and slope estimates often differed substantially between participants for many of these metabolites. This inter-individual variability likely contributed to our observation of 'false negatives', which we defined as metabolites that were significant in almost half of the individual participant profiles (n= 6/13), but not significant at the group-level. This false negative rate was ~21% (6/28) and ~17% (4/23) for rhythmic and linear metabolites, respectively. The observed inter-individual variability in acrophase also contributed to some group acrophase estimates not being representative of the timing of rhythms in individual participants, even when these metabolites were significant at both the group- and individual-level (Figure 4B). For linear metabolites, however, group estimates of the direction of change generally reflected changes at the individual-level, although the magnitude of change was often decreased in the group estimate relative to individual participant profiles (Figure 4C). Overall metabolites with consistent profiles between individuals were more likely to be significant at the group-level (Figure 4D), and metabolites that had either a wide range in acrophases for rhythmic metabolites, or opposing slope directions for linear metabolites, were not significant at the group-level, despite being significant in approximately half of the participants (Figure 4E). Surprisingly, we also observed a group of metabolites ('false positives') that were significant at the group-level but were not significant in a single individual, as seen in the examples shown in Figure 4F. The false positive rate was ~6% (28/447) and 15% (29/188) for rhythmic and linear metabolites, respectively. This rate increased to ~66% for rhythmic and ~68% for linear metabolites when all metabolites significant in less than a third of participants (n= 3/13) were included.

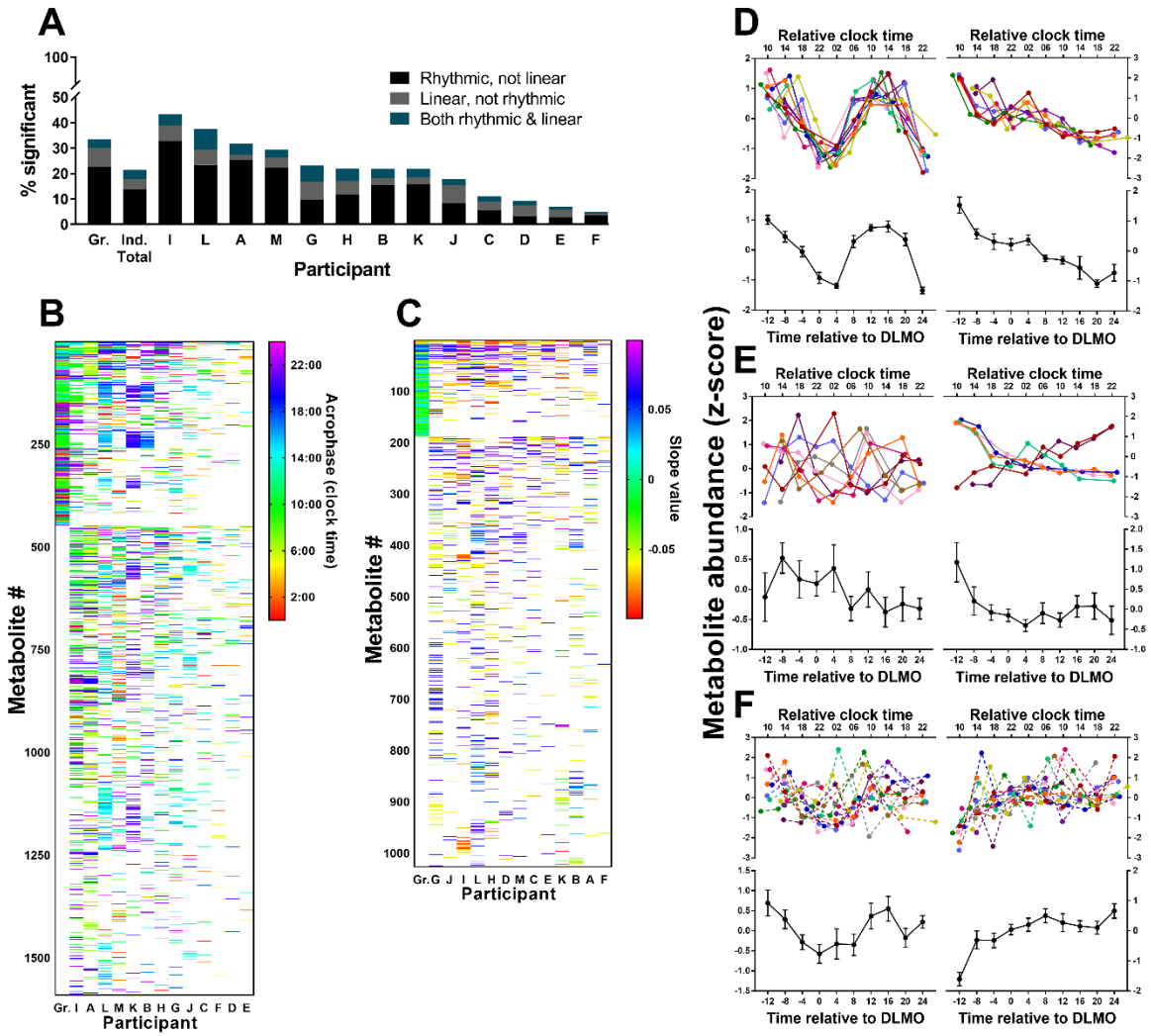

Figure 4. Comparison of group- versus individual-level analyses. **(A)** The percentage of metabolites that were significantly rhythmic (black), linear (grey), or combined rhythmic and linear (blue) at the group-level (Gr.), overall for the individual-level analysis (Ind. total), and for each individual participant (A-M). **(B)** Acrophase and **(C)** slope values are shown for significant metabolites (including combined rhythmic and linear metabolites) at the group-level (Gr.) and in individual participants (A-M). Metabolites are ordered based on the number of significant cosinor or linear fits across participants, with group-significant metabolites shown first. Participants are ordered from left to right based on the greatest number of significantly rhythmic or linear metabolites at the individual-level. **(D-F)** Examples of true positives, false negatives and false positives. **(D)** Metabolites that were significantly rhythmic (*left*) or linear (*right*) in group- and individual-level analyses (true positives). **(E)** Metabolites significantly rhythmic (*left*) or linear (*right*) in almost half of individuals, but were not group-significant (false negatives). **(F)** Metabolites that were significantly rhythmic (*left*) or linear (*right*) in group-level, but not individual-level analyses (false positives; non-significance denoted by broken lines in individual-level plots). Individual participant profiles are shown in colour and the group mean (±SEM) for that metabolite is shown below in black.

5.3. Discussion

Our study presents the first evidence of circadian- and wake-dependent modulation of highly polar metabolites over the course of 40-h of extended wakefulness. We describe rhythmic and linear changes in plasma metabolites at both the group- and individual- level. Due to large inter-individual differences observed in both circadian- and wake-dependent metabolites, our findings highlight the importance of data being examined at both the group-and individual-level for biomarker discovery work. For a biomarker discovery program, aiming to identify biomarkers of an abnormal state, the biomarker should ideally have utility at the individual-level. Targeting or rejecting a metabolite based on group-level data may therefore lead to inconclusive results or a missed signal of interest. With this in mind, while our data support earlier findings demonstrating rhythmic and/or linear changes in the human plasma metabolome during sleep deprivation (Ang et al., 2012; Chua et al., 2015; Chua et al., 2013; Dallmann et al., 2012; Davies et al., 2014), they also suggest caution when interpreting results from analyses of grouped data.

Previous studies using reverse phase-LC-MS to detect changes in plasma metabolite levels, indicated that ~13-40% of the lipids and apolar metabolites preferentially detected by this platform are under circadian control (Ang et al., 2012; Chua et al., 2013; Dallmann et al., 2012; Kasukawa et al., 2012). We show that a similar proportion of polar metabolites detected using HILIC-LC-MS exhibited circadian rhythmicity in our group-level analyses (~22% or ~26% including metabolites that were combined rhythmic and linear). Furthermore, the timing of the peak of metabolite rhythms in our targeted matrix was consistent with previous reports that have shown amino acids to peak predominantly in the evening and during the biological night (Ang et al., 2012; Dallmann et al., 2012; Kasukawa et al., 2012). Thirteen (of 28, including those showing a combined rhythmic and linear pattern) metabolites found to be rhythmic in our targeted group of compounds were rhythmic in at least one other study (Ang et al., 2012; Davies et al., 2014; Kasukawa et al., 2012). Overall, our analyses using HILIC-LC-MS detected 16 previously unreported rhythmic metabolites, which included organic acids and the amino acids serine, threonine, and asparagine.

Following our group-level analysis, we also analyzed individual participant profiles. These analyses showed that ~14% (~18% including combined rhythmic and linear metabolites) of individual participant metabolite profiles were rhythmic. Similarly, despite the difference in the classes of measured metabolites, Chua et al. (Chua et al., 2013) reported that ~18% of lipid metabolite profiles were rhythmic, suggesting that a similar proportion of lipid and polar metabolites are under circadian control. Based on the identified metabolites from the targeted matrix, the rhythmic metabolites in the current study were predominantly amino and organic acids, such that pathways involving these classes of metabolites, including the Krebs and urea cycles, showed enrichment. While some metabolites involved in the urea and Krebs cycles had acrophases during the biological night, most had acrophases during the day, consistent with the diurnal peak in urea concentration (Kamperis, Hagstroem, Radvanska, Rittig, & Djurhuus, 2010; Kräuchi & Wirz-Justice, 1994) and energy expenditure reported in the literature (Jung et al., 2011). Furthermore, the timing of amino acid rhythms in the current study is broadly consistent with previous research showing that transcripts associated with gene expression and RNA metabolism tend to peak during the night (Archer et al., 2014; Arnardottir et al., 2014; Möller-Levet et al., 2013), such that that the timing of amino acids observed in the current study coincides with the timing of protein synthesis.

As has been reported previously for plasma lipids (Chua et al., 2013), none of the polar metabolites were consistently rhythmic across all individuals and we observed a large degree of inter-individual variation in the timing of rhythms between participants. As seen in Figure 3B, some participants appeared to have a similar timing of rhythms across most metabolites (e.g. acrophase estimates for participant K were mostly during the evening hours), suggesting that some individuals may have a particular phase predominance in their metabolic profile. A similar finding was observed in plasma lipids, whereby participants could be clustered into morning and evening phenotypes based on the peak times of lipid rhythms (Chua et al., 2013). Further characterization of the range of inter-individual variability in metabolites within and between individuals is necessary if these are to be used as potential biomarkers. The wide range of individual phases observed in metabolites is not surprising, however, when the inter-individual variation in well-established

circadian markers is taken into consideration. Melatonin, the gold standard marker of circadian phase, when measured under dim light conditions exhibits an ~5-h range of phase (5.85-h in the current sample) and phase angles (DLMO time relative to sleep) in young healthy individuals [e.g. (Sletten, Vincenzi, Redman, Lockley, & Rajaratnam, 2010; Wright, Gronfier, Duffy, & Czeisler, 2005)]. Even within individuals, there is also variation in internal phase relationships, for example, between melatonin and temperature (Shanahan & Czeisler, 1991), not only because of methodological variance but also likely intrinsic differences in internal circadian organization. It is therefore important to interpret potential new circadian markers with similar expectations, i.e., that substantial inter-individual variation will exist, and biomarkers are not likely to exhibit identical timing between, or even within individuals, but that does not preclude their use as circadian biomarkers.

Given large inter-individual differences in metabolites, we sought to compare the results of our group- and individual-level analyses. Overall, metabolites that had consistent profiles between participants tended to be significant at the group-level, while those with a large spread of acrophases were typically not significant at the group-level (false negatives). Furthermore, we observed that 28 of 447 metabolites were significant at the group-level despite not being significant in a single individual ('false positives'). These findings are important given the widespread use of group-level analyses in previous studies assessing circadian rhythms in the metabolome (Ang et al., 2012; Dallmann et al., 2012; Davies et al., 2014; Giskeødegård, Davies, Revell, Keun, & Skene, 2015).

In addition to identifying rhythmic metabolites, we also identified approximately 7% (11% including combined rhythmic and linear metabolites) of metabolites that showed a linear increase or decrease with time awake in group-level analyses. The proportion of linearly changing metabolites in the current study is similar to the proportion of metabolites that showed an increase or decrease in response to acute sleep deprivation [~12%; (Dallmann et al., 2012)], and sleep restriction to 5.5.-h time-in-bed for 8 nights [~4%; (Bell et al., 2013)]. Despite similarity in the proportion of metabolites increasing or decreasing, metabolites that changed linearly in the current

study are not consistent with the metabolites that changed in response to chronic (5-8 nights) sleep restriction (Bell et al., 2013; Weljie et al., 2015), such that a number of metabolites that were altered by sleep restriction did not show a wake-dependent change in the present study. This difference suggests that biomarkers signaling sleep deficiency due to acute sleep deprivation may not be the same as those sensitive to chronic sleep loss. Another possible explanation is that the 24-h rhythm of some metabolites was shifted due to the sleep restriction protocol [as seen in melatonin (Lo et al., 2012; Rogers & Dinges, 2008)], such that the change attributed to sleep restriction may represent measurement at a different phase of the rhythm. This may be the case for some of the metabolites identified as markers of sleep restriction, for example tryptophan, phenylalanine, and isoleucine, as these metabolites have been shown to be rhythmic, both in the current study and in previous research (Ang et al., 2012; Davies et al., 2014; Kasukawa et al., 2012). Further investigation is required to determine whether the metabolites that show wake-dependent increases or decreases in response to sleep deprivation are also altered by sleep restriction, or whether there are different mechanisms resulting in a different set of metabolites showing change in response to the sleep deprivation versus sleep restriction.

In our analysis of individual participant metabolite profiles, we found that 4% (~8% including combined rhythmic and linear metabolites) of metabolite profiles changed linearly. Based on the targeted analyses, these were mainly amino and organic acids, as well as a smaller number of carbohydrates. Enrichment analysis showed that the linearly changing metabolites were related to energy metabolism in the glycolysis and Krebs cycle pathways. While some of the metabolites in these pathways decreased, the majority increased with time awake and this is consistent with the reported increase in energy expenditure during sleep deprivation (Jung et al., 2011; McHill et al., 2014). The urea cycle pathway also showed enrichment, with majority of the metabolites involved in this pathway showing an increase with time awake, which is consistent with the increase in urea in response to sleep loss (Kamperis et al., 2010; Kant, Genser, Thorne, Pfalser, & Mougey, 1984).

As with the rhythmic metabolites, there was inter-individual variation in the patterns of change of linear metabolites, such that the magnitude, and in some cases the direction of change,

differed between participants (Figure 4C). While these different responses between participants may indicate differential vulnerability to the metabolic consequences of sleep loss, confirmation requires further investigation to determine whether these inter-individual responses to sleep loss are stable and trait-like. Our finding of inter-individual variation in linearly changing metabolites is consistent with the large inter-individual differences reported in lipids showing wake-dependent changes (Chua et al., 2015), although our results suggest that polar metabolites are less likely to change with time awake than lipid species (27% lipid *vs* 8% polar). In comparing linear metabolites in our group- and individual-level analyses, we observed that analysis of metabolites at the group-level produced both false positive and false negative results, as with our analysis of rhythmic metabolites. We observed that 29 of the 188 metabolites detected as significantly linear at the group-level were not significantly linear in a single participant. This discrepancy highlights the importance of using both group- and individual-level analyses, as had we only conducted a group-level analysis, significant resources may have been used in trying to identify and validate these 'false positive' metabolites that lack utility as a biomarker at the individual-level. There were a small number of unidentified linear metabolites from the untargeted matrix, however, that showed strikingly consistent changes across majority of the participants (e.g. right panel of Figure 4D). Metabolites such as these may be useful as biomarkers of sleep drive, however, further work to identify these metabolites and validate our results is required.

Our study has two main strengths. First, our data are novel in that our analytical approaches allowed detection of a broad range of polar and non-polar metabolites, extending the range of metabolites that had previously been detected. While circadian- and wake-dependent changes have been previously described in moderately polar metabolites under CR conditions (Dallmann et al., 2012), our findings, showing large inter-individual differences in the circadian phase of polar metabolites, suggest that these prior data were potentially confounded by the pooling of samples from multiple participants at the same clock time. Second, our study is the first to employ both group- and individual-level analyses to examine 24-h rhythms and wake-dependent changes in polar metabolites. Differences between group- and individual-level analyses have only been

investigated in plasma lipids (Chua et al., 2015; Chua et al., 2013), while other studies that have measured both moderately and non-polar compounds have reported data at the group-level (Ang et al., 2012; Bell et al., 2013; Dallmann et al., 2012; Davies et al., 2014; Weljie et al., 2015).

This study comprises the first step within a larger biomarker discovery program, where the current study was designed to produce proof-of-concept data within a small, but highly controlled study. The small sample size, however, means that the current study may have been underpowered to detect some rhythmic or linear changes, particularly those with low amplitudes or shallow slopes. Despite this, our sample size is commensurate to previous metabolomics studies (Ang et al., 2012; Dallmann et al., 2012; Davies et al., 2014). As with previous studies, participants in the current study were all young and extremely healthy, and the laboratory conditions were highly controlled during the CR protocol. While at this stage in the biomarker development process it is important to first identify the presence of any circadian and wake-dependent changes in an homogenous sample under highly controlled conditions, these findings will need to be validated in other populations and in less controlled, applied settings including circadian misalignment, sleep restriction, and in field settings. Moreover, while we identified metabolites that changed linearly during sleep deprivation it is difficult to ascertain whether these metabolites are directly under the control of the sleep homeostat and are truly wake-dependent, or perhaps represent something else, for example, a build-up of certain metabolites from the hourly meals given during the CR. Similarly, the use of a CR protocol makes it difficult to uncouple the contribution of the circadian system and the sleep homeostat. Future studies might employ a forced desynchrony protocol to allow for a more comprehensive investigation of the individual contribution of the homeostatic and circadian processes on the abundance of specific metabolites.

To our knowledge this is the first study to characterize circadian- and wake-dependent changes in highly polar plasma metabolites. Our results describe circadian- and wake-dependent control of the polar metabolome and highlight the importance of analyzing these types of data at both the group- and individual-level. We showed that analysis at the group-level resulted in inaccurate measures of the abundance and time-course of both rhythmic and linearly changing

metabolites. Underlying inter-individual differences in circadian- and wake-dependent modulation of the metabolome will also likely be an important consideration for future biomarker development programs using metabolomics.

5.4. Materials and methods

5.4.1. Study protocol

Participants completed a 6-day in-laboratory study (Figure 1). Upon waking on Day 3, participants commenced a 40-h CR. During the CR, participants remained awake under constant supervision in dim light conditions (<3 lux), in a semi-recumbent posture and received identical hourly snacks. Plasma samples collected during the CR were used for metabolomics analysis and assay of plasma melatonin. See SI Materials and Methods for further detail.

5.4.2. Metabolomics and statistical analysis

Metabolomics analysis was performed on plasma samples collected at 4-h intervals starting 2-h post wake. Circadian and wake-dependent changes in plasma metabolites were assessed using a non-linear regression model comprised of a cosinor function with a linear component. Details of the metabolomics analysis and all statistical analyses are given in SI Materials and Methods.

5.5. Acknowledgements

We thank the study participants, and the technicians, staff and students of the Monash University Sleep and Circadian Medicine Laboratory who aided data collection. We also thank Caroline Beatty for recruitment and research support; William McMahon and Jinny Collett for research assistance in the study; the study physicians Dr Simon Joosten and Dr Eric Kuo; Sylvia Nguyen for conducting psychiatric screening; Veronica Lui for assistance with metabolomics sample extraction; Dr Shadab Rahman for assistance with statistical analysis; and Kellie Hamill for logistical help. The authors also acknowledge the assistance of the Adelaide Research Assay Facility for providing plasma melatonin analysis, and Dr Saravanan Dayalan and Sean O'Callaghan at Metabolomics Australia for assistance with metabolomics analyses. This research was supported by the CRC for Alertness, Safety, and Productivity.

Chapter 5 Additional information supplement

5.6. Supplemental material submitted for publication to *Proceedings of the National Academy of Sciences of the United States of America*

The following material consists of a detailed methodology and supplementary figures and tables that were not included in the main manuscript.

5.6.2. Participants

Fourteen healthy adults (13 following exclusion of n=1, 4 females, 24.74 ± 4.09 years) completed a 6-day in-laboratory study. Participants were free from medical, psychiatric or sleep disorders, had not engaged in night- and/or shift-work in the past three years, or travelled across more than one time zone in the past three months. Two weeks prior to the laboratory study, participants maintained a self-selected 8:16 sleep-wake schedule, which was confirmed by wrist actigraphy (Actiwatch Spectrum, MiniMitter Inc, Bend, OR) and sleep diaries. The use of prescription and over-the-counter medications, supplements, recreational drugs (also exclusionary if consumed in the previous month based on self-report), nicotine, caffeine, and alcohol were not permitted from 3 weeks prior to admission until completion of the study. Urine drug screening, and a pregnancy test for women, was conducted prior to laboratory admission. All participants provided informed consent and the study was approved by the Monash University Human Research Ethics Committee (CF14/2790 – 2014001546).

5.6.3. Method details

5.6.3.1. Study Protocol

Participants were continuously monitored for 6-days in an environment free of time cues. There was no access to windows, clocks, live TV, or newspapers, and participants were supervised by technicians trained not to reveal time of day. Women were studied during their follicular phase, with admit occurring immediately after their last menses, to minimize differences between women due to menstrual phase. The study started with two baseline nights with sleep scheduled at the same

time as participants' self-selected sleep in the two weeks prior to admission. Full polysomnography was recorded on the first night to confirm no presence of sleep disorders, including restless legs syndrome, periodic limb movements, and sleep disordered breathing. During baseline days, participants were fed three main meals and three snacks per day.

Upon waking on Day 3, participants commenced a 40-h CR. During the CR, participants remained awake under constant supervision in dim light conditions (<3 lux), in a semi-recumbent posture (head of bed at 45˚), and received identical hourly snacks (quarter sandwich, 60ml water and 40ml apple juice). The calorie content of hourly snacks was ~1.1 x the average resting energy expenditure (REE) for all participants (1796 ± 236 cal/day) and the macronutrient content adhered to the recommendations of the Australian Dietary Guidelines 2013 (National Health and Medical Research Council, 2013). REE for each participant was calculated as (Mifflin et al., 1990):

$$REE_{Male} = 9.99 * \ weight\ (kg) + 6.25 * \ height\ (cm) - 4.29 * \ age + 5$$

$$REE_{Female} = 9.99 * \ weight\ (kg) + 6.25 * \ height\ (cm) - 4.29 * \ age - 161$$

Participants had the choice of four sandwich options that were approximately equivalent in calorie (1804 ± 99 cal/ day) and macronutrient content (~20% protein, ~33% fat, ~46% carbohydrate). Each participant received only one of the sandwich options for the duration of the CR.

5.6.3.2. Lighting

During baseline and recovery days, maximum ambient light during wake episodes was ~100.9 ± 18.2 lux when measured in the horizontal plane and ~44.0 ± 13.9 lux when measured in the vertical plane at the height of 182cm. On baseline night 2 and during CR, lights were dimmed to ~2.8 ± 0.5 lux in the horizontal plane and ~1.2 ± 0.3 lux in the vertical plane when measured at 182cm. During scheduled sleep episodes, ambient lighting was turned off. The room lighting was generated from ceiling-mounted 4100 K fluorescent lamps (Master TL5 HE 28W/840 cool lights, Philips Lighting, Amsterdam, Netherlands) that were covered with neutral density filters (3-stop LEE Filters,

Lightmoves, Noble Park, Australia). Illuminance measures (J17 Lumacolor photometer, Tektronix, Beavertown, USA) were taken daily in four locations around the room, positioned directly under light panels.

5.6.3.3. Blood sample collection and processing

Plasma samples were collected during the CR via an indwelling intravenous cannula, inserted into the forearm or antecubital vein approximately 1-h after wake. Blood was collected hourly for plasma melatonin assay, and additional blood was collected every 2-h for metabolomics analysis starting 2-h post wake. At each collection, whole blood was collected in a syringe and aliquoted into a blood tube spray coated with K^2EDTA. Samples were immediately centrifuged at 4˚C, or stored in a fridge at 4˚C for up to ~30 minutes until processing. Samples were spun at 1,300 x g for 10 minutes and plasma was aliquoted into 500µL fractions and temporally stored on dry ice before transfer to permanent storage at -80˚C within 4 to12-h.

Of the total 546 scheduled blood collections (39 samples x 14 participants), 529 were collected successfully (3.11% missing samples) and assayed for melatonin. For the metabolomics analysis, up to ten 4-h samples per participant were analyzed at times 2, 6, 10, 14, 18, 22, 26, 30, 34 and 38-h post-wake. Of the 140 possible samples, 18 (13.6%) were missing due to either a missed collection (n= 1 sample) or had moderate to severe haemolysis (orange to red in colour; n=17 samples). To be included in the metabolomics analysis, participants could not have more than 70% missing blood samples, and no more than two consecutive missing samples. To avoid excluding two individuals, a 4-h sample was replaced with a successful collection occurring 2-h before or after the sample that required replacement— for example, a sample collected at 32-h was used to replace a missing sample at 34-h. With these replacements, a total of 124 samples were included in the final metabolomics analysis from 14 participants.

Of the 124 plasma samples analysed using LC-MS, five samples were lost in both the targeted and untargeted matrices post-analysis due to mis-injection into the LC-MS (n=119 samples). Retention time drifts (>2mins) resulted in the exclusion of an additional three samples from the untargeted matrix following XCMS analysis (n=116), but not from the targeted matrix

which was manually integrated such that the retention time window could be widened to incorporate these metabolites (n=119). One male participant was excluded entirely from further analysis, as this additional loss of samples resulted in three consecutive missing time-points in the middle of their data series making it difficult to interpret the model fits. Following removal of this participant, 10 of 13 participants had missing data points in the targeted matrix resulting in 14% (18 samples; total n=112) missing data, and 12 of 13 participants had missing data points in the untargeted matrix resulting in a total of 16% (21 samples; total n=109) missing data.

5.6.3.4. Marker of the circadian clock

Total blood plasma melatonin was determined at the Adelaide Research Assay Facility (ARAF; University of South Australia, Adelaide, Australia) by reverse-phase C-18 column extraction of 500µl plasma, followed by double antibody radioimmunoassay using standards and reagents supplied by Buhlmann Laboratories (RKMEL-2, Buhlmann Laboratories AG, Schönenbuch, Switzerland). The sensitivity of the assay using 500µl of extracted plasma was 1.0pg/ml. Samples were assayed in duplicate and the intra-assay coefficient of variation of the assays was 7.61%. The inter-assay coefficient of variation of the low concentration quality control was 11.03%, and the inter-assay coefficient of variation of the high concentration quality control was 13.08%.

To determine circadian phase, Dim Light Melatonin Onset (DLMO) was defined as the time at which plasma melatonin levels reached 5pg/ml in the first cycle of the CR, calculated by interpolating between two adjacent samples (Lewy, Cutler, & Sack, 1999). For two participants, DLMO was calculated from the second cycle due to missing samples in the first 24-h cycle. The biological night was defined as DLMO plus 10 hours (DLMO+10) and split according to first half (first 5-h) and second half (second 5-h) of the night. The biological day was defined hours the 14-h between DLMO+10 and DLMO. The biological day was further broken down into two equal 7-h halves.

5.6.3.5. Metabolomics analysis

Metabolomics analysis was performed on plasma samples collected at 4-h intervals starting 2-h post wake (Metabolomics Australia, Bio21 Molecular Science and Biotechnology Institute,

University of Melbourne, Parkville, Australia). Samples were thawed on ice and 20µL of plasma was aliquoted for analysis by LC-MS. An additional 20µL from each sample was pooled to generate a plasma quality control (PQC) sample, from which aliquots were taken in preparation for extraction with plasma samples. Plasma samples and PQCs were extracted using 180µL acetonitrile/methanol (1:1 v/v) solution containing 2µM ^{13}C-sorbitol, 2µM $^{13}C^{15}N$-AMP, and 2µM $^{13}C^{15}N$-UMP as internal standards. Samples were vortexed for 30 seconds, sonicated for 5 minutes at 4˚C, then incubated for 10 minutes at 4˚C (in an Eppendorf Thermomixer). Samples (prepared in batches of 24, with a PQC every 10 samples) were centrifuged (4,500 x g, 10 minutes, 4˚C) and 180uL of the supernatant was transferred into a glass vial. An aliquot of each sample of the extracts (10µL) was pooled to create a pooled biological quality control (PBQC) sample.

Samples (10µL) were resolved on a ZIC®-pHILIC column (5µm particle size, 150 x 4.6mm, Merck SeQuant®) connected to an Agilent 1260 (Santa Clara, CA, USA) HPLC system running a 29.5-minute gradient with mobile phases 20mM ammonium carbonate (pH 9.0; Sigma-Aldrich; Solvent A) and 100% acetonitrile (solvent B) at a constant flow rate of 300µL/min. The elution gradient started at a composition of 80% solvent B and decreased to 30% solvent B in 18.5-min for 6.5-min. Extracted plasma volumes of 7µL were injected onto the column (maintained at 25˚C). Metabolites were detected by electrospray ionization using an Agilent 6545 Q-ToF MS system (Santa Clara, CA, USA) in negative ionization mode. The instrument was cleaned and calibrated weekly to ensure a mass accuracy of ±0.2ppm. Detailed Q-Tof MS parameters can be found in Stewart, et al. (2017). Samples were analysed in the same analytical batch and randomized by participant and time, with a QC every 5 samples. PQCs were run every tenth sample to monitor any batch preparation effects and PBQCs were run every tenth sample to monitor instrument performance during the run. Solvent blanks were analysed every 24 samples to monitor background. Five mixtures of authentic standards (234 metabolites) were also run to generate a library for the targeted analysis.

Metabolite identification for the targeted analysis (targeted matrix) was based on accurate mass, retention time and MS/MS fragmentation patterns for metabolites in the standard mixtures.

Relative abundances based on area under the metabolite peak were obtained using MassHunter Quantitative Analysis B 0.7.00 (Agilent). Metabolites with low quality chromatographic peaks (<10,000 area count) and peaks not reliably detected across samples were excluded resulting in the detection of 99 (of 234) metabolites [Level 1 confidence according to the Metabolomics Standard Initiative (Fiehn et al., 2007)]. The untargeted matrix containing 1,722 metabolite features was generated by XCMS *centWave* algorithm (Tautenhahn, Bottcher, & Neumann, 2008) to detect molecular features in the raw files and the features list was further refined in CAMERA (Kuhl, Tautenhahn, Böttcher, Larson, & Neumann, 2012) to group related features by annotating isotope and adduct peaks.

5.6.4. Statistical analysis

To reduce biological variability between participants and timepoints, raw area count data were normalized relative to the median metabolite abundance for each individual sample. Given differences in the relative concentration of metabolites between individuals, the median normalized data were z-scored in order to scale the data prior to analysis. Data were z-scored relative to the mean and standard deviation of each participants' scores for a single metabolite. Each time-point was then expressed relative to DLMO, where DLMO was defined as time 0. Grouped data were averaged across 4-h phase bins to align the data points relative to each participant's internal circadian time. Data were fitted with a non-linear regression model (Chua et al., 2015) that was comprised of a cosinor function with a linear component:

$$y = A\ cos\left\{2\pi\left(\frac{t-\emptyset}{\tau}\right)\right\} + Ct + D$$

In the model, A is the amplitude of the sinusoid, τ is the period set at 24-h, t is time, $\emptyset$ is the acrophase of the sinusoid, and C and D are the slope and y-intercept of the linear component, respectively. Fitting of the model was conducted in SAS 9.4 using the *proc nlin* procedure. The model was fitted to the 99 and 1,722 metabolite profiles averaged within phase bins from the targeted and untargeted matrix (group-level analysis), and to each individual participants' metabolite profiles from the targeted and untargeted matrix (individual-level analysis). The cosinor

and linear components of the regression were considered significant if the amplitude and slope, respectively, were significantly different from 0. Where the regression model detected a significant nadir, acrophase time was calculated as the peak 12-h later. Given the exploratory nature of the study, p-values were set at 0.05. The model estimates for all analyses, including amplitude, acrophase and slope estimates are shown in SI Tables 2-5.

Pathway enrichment analysis was conducted in MetaboAnalyst 3.0 (http://www.metaboanalyst.ca) using the Enrichment Analysis module. This analysis provides a p-value for the overall likelihood that a metabolite set or pathway is involved based on the metabolites entered, and also indicates the degree of enrichment (fold enrichment), which is representative of how many metabolites within a specific pathway are present in the metabolite set entered into the analysis. For example, if two out of four metabolites in a pathway are present then that pathway will show a greater fold enrichment than a pathway that has two out of 10 metabolites that are present. Pathway enrichment analysis was conducted separately for linear and rhythmic metabolites at the group and individual level. Metabolites that were combined rhythmic and linear were included in both analyses. The results of the pathway enrichment analyses generated in MetaboAnalyst 3.0 are shown in SI Figures 3-6 and SI Tables 1-4.

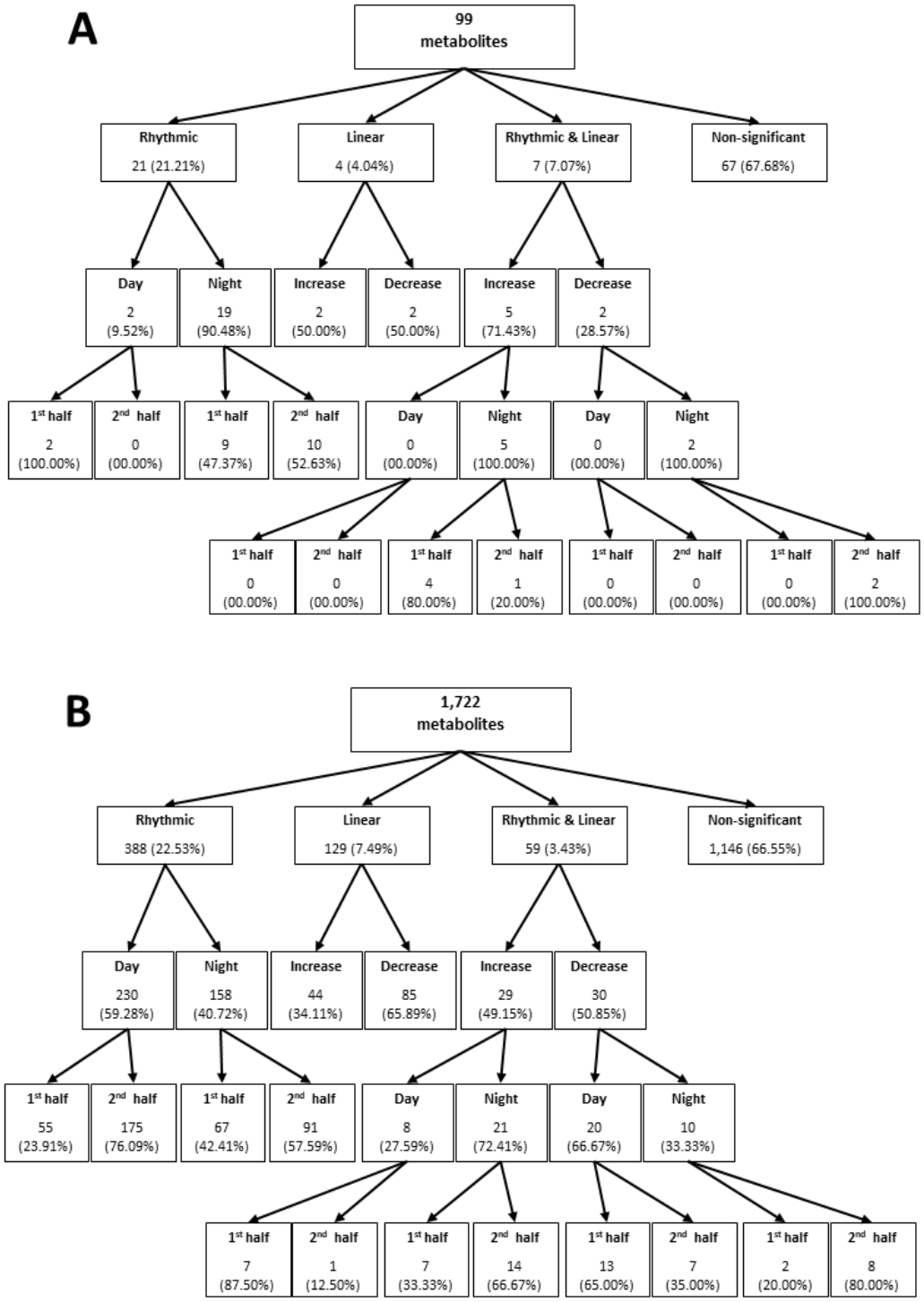

Figure S1. Results of the group-level analyses for the (**A)** targeted and (**B**) untargeted matrices showing the proportion of rhythmic, linear, combined rhythmic and linear, and non-significant metabolites. Rhythmic metabolites are further classified by acrophase (day/night and $1^{st}/2^{nd}$ half of day or night), and linear metabolites are classified according to the direction of linear change (increasing or decreasing).

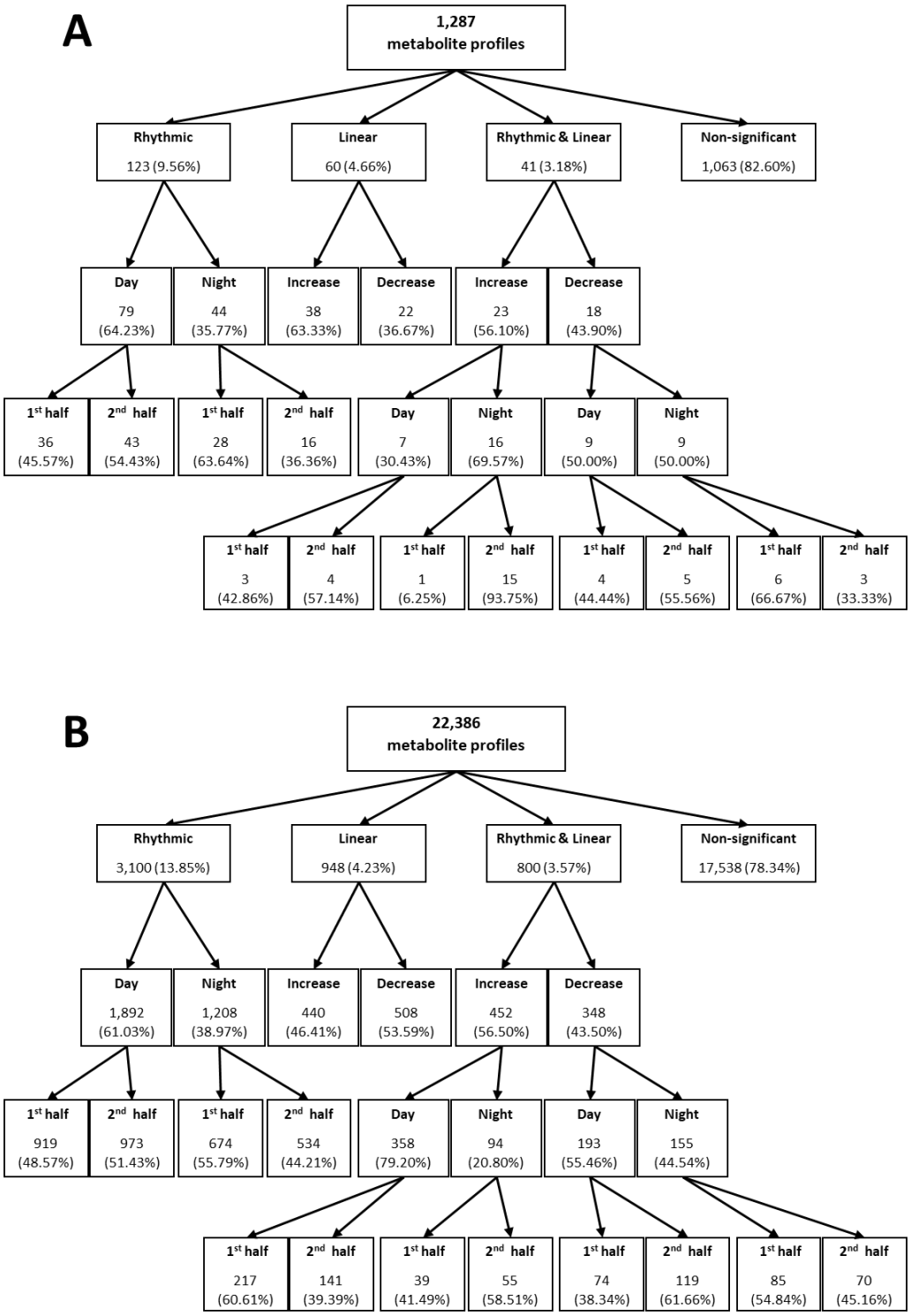

Figure S2. Results of the individual-level analyses for the (**A)** targeted and (**B**) untargeted matrices showing the proportion of rhythmic, linear, combined rhythmic and linear, and non-significant metabolite profiles. Rhythmic metabolites are further classified by acrophase (day/night and 1st/2nd half of day or night), and linear metabolites are classified according to the direction of linear change (increasing or decreasing).

Table S1. Results of the pathway enrichment analysis of identified group-rhythmic (including combined rhythmic and linear) metabolites from the targeted matrix. The table shows the pathway involved, the total number of metabolites in that pathway, the number of significantly rhythmic metabolites in that pathway and the raw and false discovery rate (FDR) adjusted p-values. Results were generated by MetaboAnalyst 3.0.

Pathway	Total metabs. in pathway	# sig. metabs. in pathway	Raw p	FDR adjusted p
Phenylalanine and tyrosine metabolism	13	4	0.000623	0.0492
Urea cycle	20	4	0.00358	0.141
Aspartate metabolism	12	3	0.00633	0.167
Arginine and proline metabolism	26	4	0.00957	0.189
Citric acid cycle	23	3	0.0396	0.625
Starch and sucrose metabolism	14	2	0.0789	1
Valine, leucine and isoleucine degradation	36	3	0.119	1
Biotin metabolism	4	1	0.13	1
Inositol metabolism	19	2	0.134	1
Tyrosine metabolism	38	3	0.134	1
Catecholamine biosynthesis	5	1	0.159	1
Methionine metabolism	24	2	0.195	1
Taurine and hypotaurine metabolism	7	1	0.216	1
Nucleotide sugars metabolism	9	1	0.269	1
Pantothenate and CoA biosynthesis	10	1	0.294	1
Betaine metabolism	10	1	0.294	1
Beta-alanine metabolism	13	1	0.365	1
Lysine degradation	13	1	0.365	1
Mitochondrial electron transport chain	15	1	0.408	1
Ammonia recycling	18	1	0.467	1
Propanoate metabolism	18	1	0.467	1
Pentose phosphate pathway	18	1	0.467	1
Glycolysis	21	1	0.521	1
Galactose metabolism	25	1	0.585	1
Glycine, serine and threonine metabolism	26	1	0.599	1
Gluconeogenesis	27	1	0.613	1
Tryptophan metabolism	34	1	0.699	1
Pyrimidine metabolism	36	1	0.72	1
Purine metabolism	45	1	0.798	1

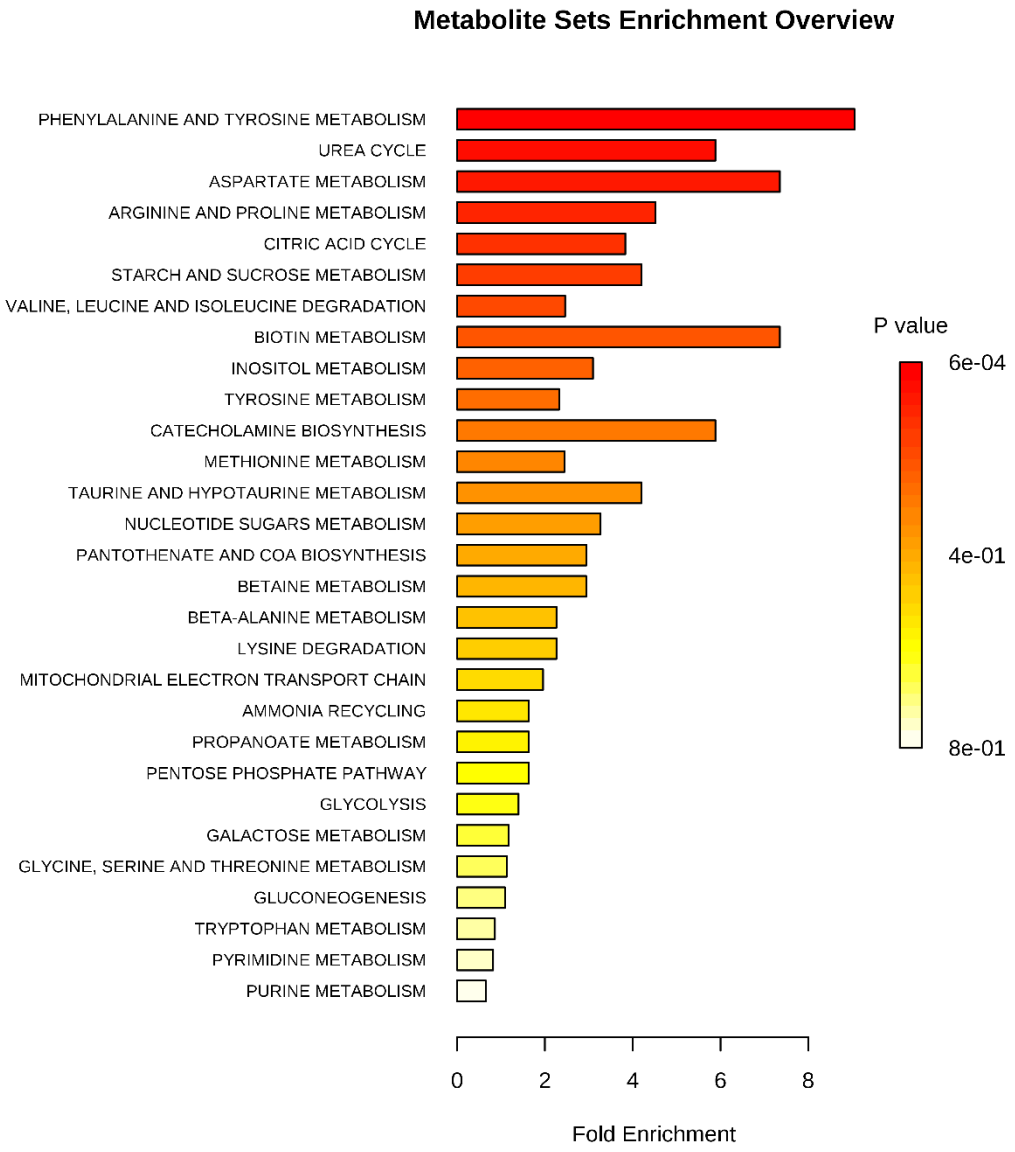

Figure S3. Pathway enrichment analysis of metabolites that were significantly rhythmic (including combined rhythmic and linear metabolites) in group-level analyses. Unadjusted p-values are represented. Generated by MetaboAnalyst 3.0.

Table S2. Results of the pathway enrichment analysis of identified group-linear (including combined rhythmic and linear) metabolites from the targeted matrix. The table shows the pathway involved, the total number of metabolites in that pathway, the number of significantly linear metabolites in that pathway and the raw and false discovery rate (FDR) adjusted p-values. Results were generated by MetaboAnalyst 3.0.

Pathway	Total metabs. in pathway	# sig. metabs. in pathway	Raw p	FDR adjusted p
Arginine and proline metabolism	26	2	0.0443	1
Biotin metabolism	4	1	0.0525	1
Taurine and hypotaurine metabolism	7	1	0.0902	1
Pantothenate and CoA biosynthesis	10	1	0.127	1
Beta-alanine metabolism	13	1	0.162	1
Phenylalanine and tyrosine metabolism	13	1	0.162	1
Lysine degradation	13	1	0.162	1
Urea cycle	20	1	0.238	1
Citric acid cycle	23	1	0.269	1
Glycine, serine and threonine metabolism	26	1	0.299	1
Pyrimidine metabolism	36	1	0.39	1

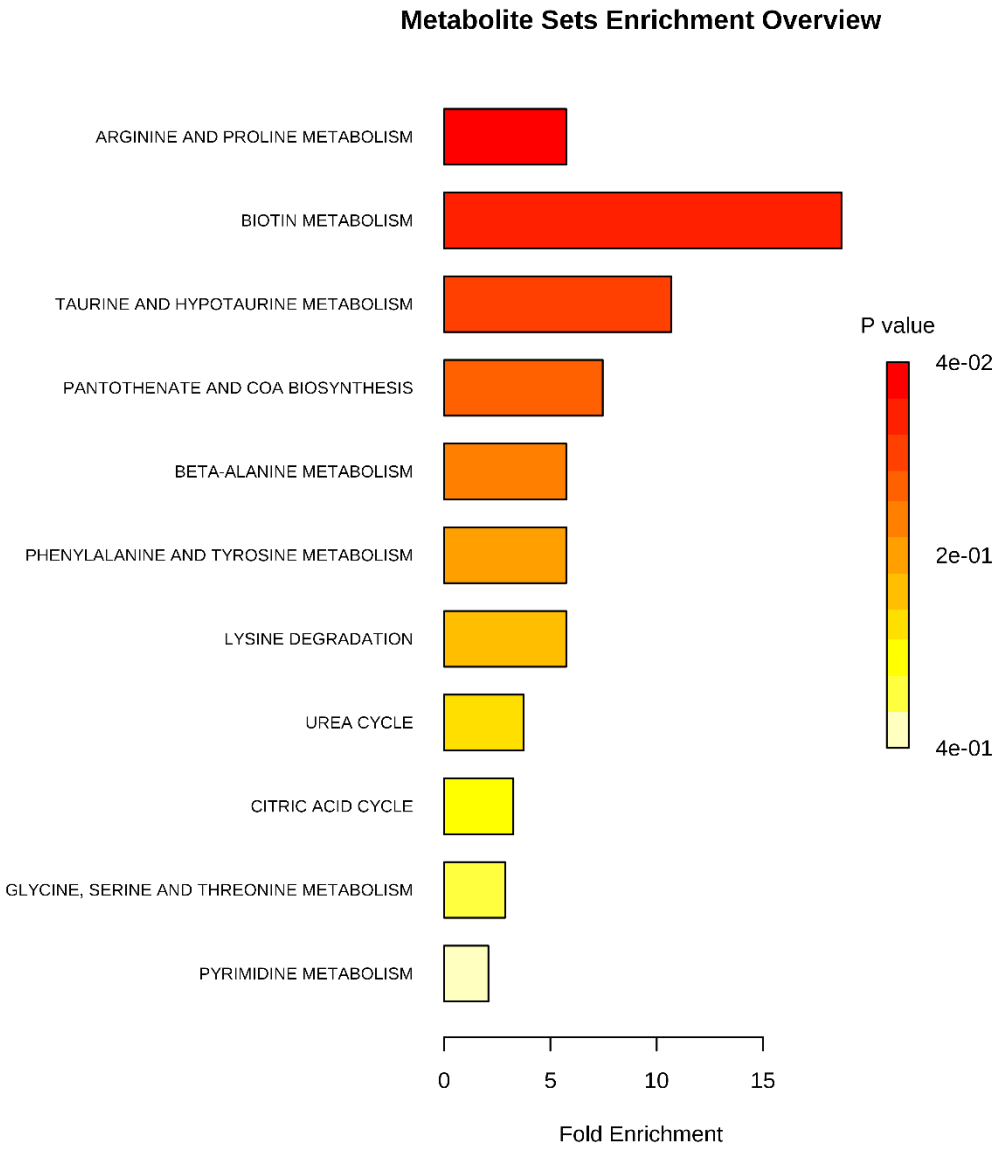

Figure S4. Pathway enrichment analysis of metabolites that were significantly linear (including combined rhythmic and linear metabolites) in group-level analyses. Unadjusted p-values are represented. Generated by MetaboAnalyst 3.0.

Table S3. Results of the pathway enrichment analysis of identified individual-level rhythmic (including combined rhythmic and linear) metabolites from the targeted matrix. The table shows the pathway involved, the total number of metabolites in that pathway, the number of significantly rhythmic metabolites in that pathway and the raw and false discovery rate (FDR) adjusted p-values. Results were generated by MetaboAnalyst 3.0.

Pathway	Total metabs. in pathway	# sig. metabs. in pathway	Raw p	FDR adjusted p
Citric acid cycle	23	9	0.000107	0.00847
Urea cycle	20	8	0.000226	0.00892
Methionine metabolism	24	6	0.0201	0.371
Galactose metabolism	25	6	0.0244	0.371
Phenylalanine and tyrosine metabolism	13	4	0.0275	0.371
Gluconeogenesis	27	6	0.035	0.371
Starch and sucrose metabolism	14	4	0.0358	0.371
Nucleotide sugars metabolism	9	3	0.0453	0.371
RNA transcription	9	3	0.0453	0.371
Biotin metabolism	4	2	0.0469	0.371
Purine metabolism	45	8	0.0536	0.385
Ammonia recycling	18	4	0.0819	0.477
Glutamate metabolism	18	4	0.0819	0.477
Arginine and proline metabolism	26	5	0.0905	0.477
Aspartate metabolism	12	3	0.0964	0.477
Insulin signalling	19	4	0.0965	0.477
Intracellular signalling through prostacyclin receptor and prostacyclin	6	2	0.104	0.481
Beta-alanine metabolism	13	3	0.117	0.487
Lysine degradation	13	3	0.117	0.487
Glycolysis	21	4	0.129	0.5
Taurine and hypotaurine metabolism	7	2	0.136	0.5
Beta oxidation of very long chain fatty acids	14	3	0.139	0.5
Mitochondrial electron transport chain	15	3	0.163	0.559
Malate-aspartate shuttle	8	2	0.171	0.562
Propanoate metabolism	18	3	0.239	0.703
Pentose phosphate pathway	18	3	0.239	0.703
Betaine metabolism	10	2	0.243	0.703
Valine, leucine and isoleucine degradation	36	5	0.249	0.703
Inositol metabolism	19	3	0.266	0.724
Pyruvate metabolism	20	3	0.293	0.771
Glucose-alanine cycle	12	2	0.317	0.808

Intracellular signalling through FSH receptor and follicle stimulating hormone | intracellular signalling through LHCGR receptor and luteinizing hormone/choriogonadotropin	4	1	0.329	0.812
Catecholamine biosynthesis	5	1	0.393	0.817
Excitatory neural signalling through 5-HTR 4 and serotonin | excitatory neural signalling through 5-HTR 7 and serotonin | excitatory neural signalling through 5-HTR 6 and serotonin	5	1	0.393	0.817
Corticotropin activation of cortisol production	5	1	0.393	0.817
Vasopressin regulation of water homeostasis	5	1	0.393	0.817
Intracellular signalling through histamine H2 receptor and histamine	5	1	0.393	0.817
Intracellular signalling through PGD2 receptor and prostaglandin D2	5	1	0.393	0.817
Pyrimidine metabolism	36	4	0.45	0.89
Alanine metabolism	6	1	0.451	0.89
Intracellular signalling through adenosine receptor A2A and adenosine | intracellular signalling through adenosine receptor A2B and adenosine	7	1	0.503	0.97
Butyrate metabolism	9	1	0.594	1
Pantothenate and CoA biosynthesis	10	1	0.633	1
Ketone body metabolism	10	1	0.633	1
Tyrosine metabolism	38	3	0.718	1
Glycine, serine and threonine metabolism	26	2	0.725	1
Oxidation of branched chain fatty acids	14	1	0.755	1
Sphingolipid metabolism	15	1	0.778	1
Amino sugar metabolism	15	1	0.778	1
Fructose and mannose degradation	18	1	0.837	1
Tryptophan metabolism	34	2	0.851	1
Fatty acid metabolism	29	1	0.947	1
Steroid biosynthesis	31	1	0.957	1
Bile acid biosynthesis	49	1	0.993	1

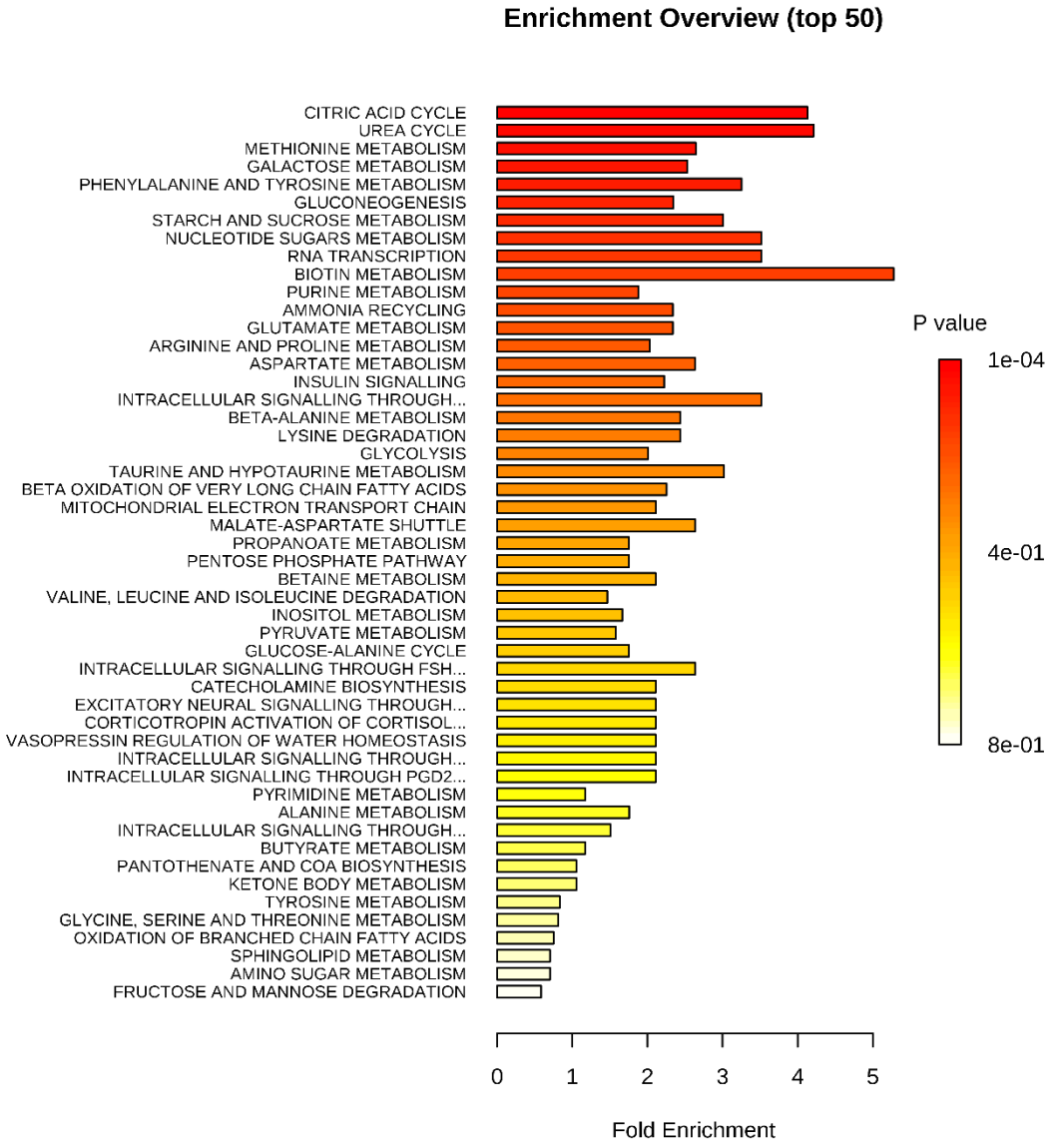

Figure S5. Pathway enrichment analysis of metabolites that were significantly rhythmic (including combined rhythmic and linear metabolites) in individual-level analyses. Unadjusted p-values are represented. Generated by MetaboAnalyst 3.0.

Table S4. Results of the pathway enrichment analysis of identified individual-level linear (including combined rhythmic and linear) metabolites from the targeted matrix. The table shows the pathway involved, the total number of metabolites in that pathway, the number of significantly linear metabolites in that pathway and the raw and false discovery rate (FDR) adjusted p-values. Results were generated by MetaboAnalyst 3.0.

Pathway	Total metabs. in pathway	# sig. metabs. in pathway	Raw p	FDR adjusted p
Citric acid cycle	23	10	2.41E-06	0.00019
Urea cycle	20	8	5.83E-05	0.0023
Malate-aspartate shuttle	8	4	0.00195	0.0337
Beta-alanine metabolism	13	5	0.00207	0.0337
Galactose metabolism	25	7	0.00213	0.0337
Alanine metabolism	6	3	0.00793	0.104
Ammonia recycling	18	5	0.0101	0.107
Glucose-alanine cycle	12	4	0.0108	0.107
Arginine and proline metabolism	26	6	0.0126	0.11
Gluconeogenesis	27	6	0.0152	0.12
Starch and sucrose metabolism	14	4	0.0194	0.139
Nucleotide sugars metabolism	9	3	0.028	0.184
Propanoate metabolism	18	4	0.0467	0.263
Glutamate metabolism	18	4	0.0467	0.263
Insulin signalling	19	4	0.0557	0.293
Aspartate metabolism	12	3	0.0618	0.305
Pyruvate metabolism	20	4	0.0656	0.305
Phenylalanine and tyrosine metabolism	13	3	0.0759	0.318
Glycolysis	21	4	0.0764	0.318
Beta oxidation of very long chain fatty acids	14	3	0.0914	0.361
Mitochondrial electron transport chain	15	3	0.108	0.406
Methionine metabolism	24	4	0.114	0.408
Cysteine metabolism	8	2	0.126	0.434
Pentose phosphate pathway	18	3	0.164	0.539
Betaine metabolism	10	2	0.183	0.559
Inositol metabolism	19	3	0.184	0.559
Biotin metabolism	4	1	0.281	0.822
Valine, leucine and isoleucine degradation	36	4	0.315	0.888
Catecholamine biosynthesis	5	1	0.338	0.89
Glycine, serine and threonine metabolism	26	3	0.338	0.89
Intracellular signalling through prostacyclin receptor and prostacyclin	6	1	0.391	0.995
Taurine and hypotaurine metabolism	7	1	0.439	1

Purine metabolism	45	4	0.483	1
Butyrate metabolism	9	1	0.525	1
Pyrimidine metabolism	36	3	0.553	1
Glutathione metabolism	10	1	0.563	1
Pantothenate and CoA biosynthesis	10	1	0.563	1
Ketone body metabolism	10	1	0.563	1
Tyrosine metabolism	38	3	0.591	1
Histidine metabolism	11	1	0.598	1
Lysine degradation	13	1	0.66	1
Glycerolipid metabolism	13	1	0.66	1
Oxidation of branched chain fatty acids	14	1	0.687	1
Amino sugar metabolism	15	1	0.712	1
Fructose and mannose degradation	18	1	0.776	1
Fatty acid metabolism	29	1	0.912	1
Steroid biosynthesis	31	1	0.926	1
Tryptophan metabolism	34	1	0.943	1

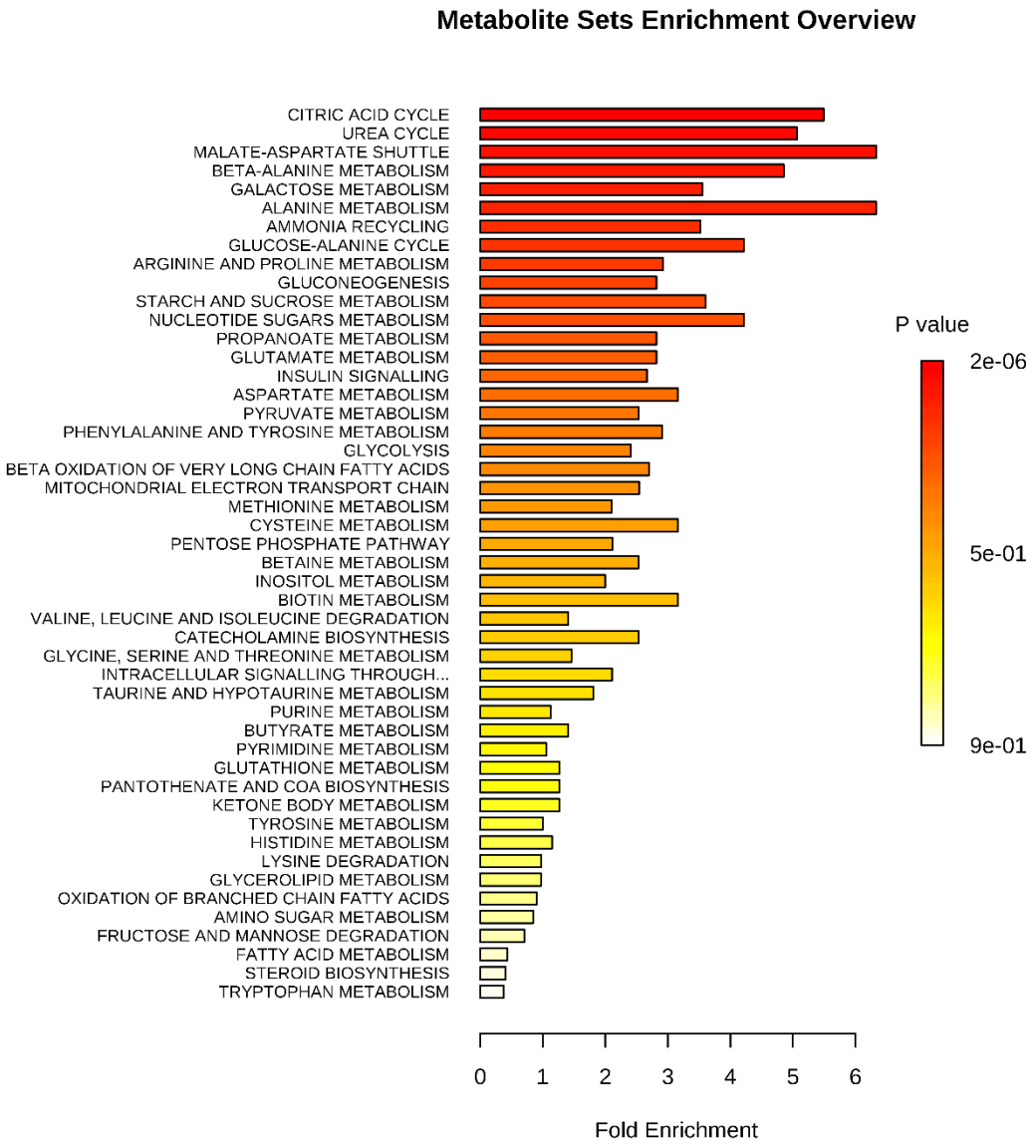

Figure S6. Pathway enrichment analysis of metabolites that were significantly linear (including combined rhythmic and linear metabolites) in individual-level analyses. Unadjusted p-values are represented. Generated by MetaboAnalyst 3.0.

5.7. Biomarker candidates

This study comprises the first stage within a larger biomarker development program where this proof-of-concept data will be used to inform further biomarker detection and validation with a greater number of participants and more frequent sampling. From the analyses conducted in the submitted manuscript in this chapter, 18 metabolite candidates were determined as putative biomarkers of alertness. Only candidates that showed a linear (wake-dependent) or both a rhythmic and linear change were included in these analyses as ideally a biomarker of alertness should represent the sleep homeostat, or the combination of the homeostatic and circadian influences on alertness, which is often seen in the profile of laboratory standard markers of alertness, such as neurobehavioural performance.

The work flow for reducing the candidates to the final 18 metabolites is shown in SI Figure 7A. From the overall 1,722 metabolites analysed, 997 metabolites were significantly linear, or both rhythmic and linear in at least one individual. This number was reduced to 27 metabolites by including only those metabolites that were significant in at least half of the participants (SI Figure 7B). From these 28 candidates, metabolites that had a consistent direction of change (e.g. increased in all participants) comprised the final 18 candidates, which included 12 metabolites that showed a consistent linear decrease and 6 that showed a consistent linear increase. As can be seen in SI Figure 7C, while the direction of change was consistent between participants, the magnitude of change (i.e. the slope value), differed between individual participants. SI Figure 7D shows an example of an increasing and a decreasing metabolite from the final 18 candidates. The median normalised z-scored data, which was used in the analysis, are represented at the group- and individual-level, however, the raw median normalised data are also represented to show differences in abundance between individuals, which is not demonstrated in the z-score transformation.

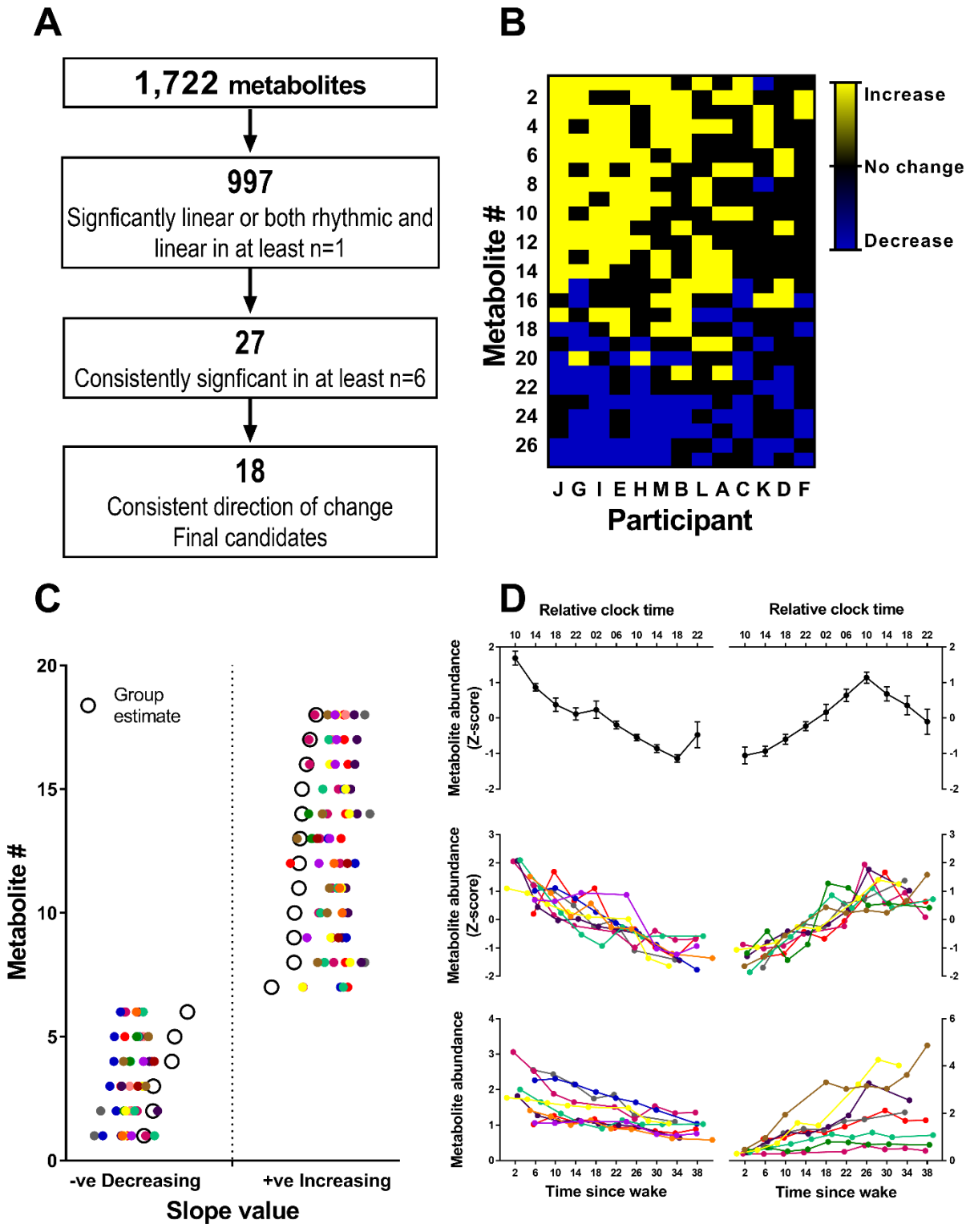

Figure S7. Biomarker of alertness candidates. (A) Work flow demonstrating the steps taken to reduce the list of biomarker candidates. (B) Metabolites showing a linear or both rhythmic and linear change in at least 6 participants. (C) Final 18 metabolite candidates significant in at least 6 participants and showing a consistent direction of change between participants. The black, unfilled circles represents the slope value for the group-level analysis and the coloured dots represent slope estimates for individual participants. (D) An example of a decreasing (*left*) and an increasing (*right*) metabolite included in the final 18 candidates. Median normalised, z-scored data used in the analysis are shown for the group- (*top*) and individual-level (*middle*) analyses, and the raw median normalised data (*bottom*) are also shown for these candidates.

Chapter 5 Concluding remarks

In this chapter circadian and wake-dependent changes in the human plasma polar metabolome during prolonged wakefulness are described. While circadian and wake-dependent control of the plasma metabolome has previously been described (Chua et al., 2015; Chua et al., 2013; Dallmann et al., 2012; Davies et al., 2014; Kasukawa et al., 2012), the present study expands this work by validating polar metabolites assessed in previous studies. Furthermore, this study also identified circadian rhythmicity and/or increases or decreases with time awake in several novel polar metabolites that have not previously been detected. As discussed in the manuscript, this work has three main implications, each of which will be discussed below.

First, this work may provide insights for future biomarker development. While the identification of metabolite candidates for further investigation as biomarkers of alertness was the purpose of thus study, these data also have implications for developing biomarkers of disease. With more research using 'omics' techniques to identify biomarkers of specific disease states and drug targets for therapeutic intervention (Ibáñez et al., 2012; Munger et al., 2008; Urayama et al., 2010; Vastag, Koyuncu, Grady, Shenk, & Rabinowitz, 2011; Wang et al., 2011), it is important that circadian and wake-dependent changes in the metabolome are well defined. Endogenous rhythmic changes over the course of the day and changes related to prior sleep/wake behaviour may lead to strikingly different results depending on when a sample is collected. For example, changes as large as two standard deviations, which were observed for some rhythmic metabolites across 24-h in the current study, could be misinterpreted as abnormal and thus change the clinical interpretation of a test. Therefore, metabolites showing clear sleep and circadian modulation may not be suitable as single time point biomarkers of disease and for the purpose of future biomarker development it is important that these metabolites are identified. The identification of metabolites that do not show sleep and circadian modulation is equally as important, however, as these metabolites may be ideal single time point biomarker of disease candidates since they do not appear to change with time-of-day or prior sleep/wake behaviour.

Second, the current data, as well as previous analyses of circadian and wake-dependent changes in the human metabolome, may also provide insight into the mechanisms via which sleep and circadian disruption affects many aspects of our health. Understanding which metabolic pathways are most vulnerable to such disruption may help to inform the underlying etiology of numerous disease states, especially metabolic diseases, associated with inadequate sleep and circadian misalignment. It is also crucial that we understand individual differences in metabolic responses as certain phenotypic responses may be indicative of vulnerability to sleep and circadian disruption and the effect such disruption has on metabolic and other adverse health outcomes. More work is required to determine the extent of these individual differences, particularly the reproducibility of these individual differences, and to identify phenotypes that may indicate specific metabolic responses to sleep and circadian disruption.

Lastly, the findings of this study also have methodological implications for future work investigating circadian and wake-dependent modulation of the human metabolome. The large inter-individual differences observed in both circadian and wake-dependent metabolites in the current study and in previous lipidomics work (Chua et al., 2015; Chua et al., 2013) suggest that group-level analyses may fail to detect underlying inter-individual differences that may have biologically significant implications. An international effort to develop biomarkers of sleep drive and circadian phase is underway (Mullington et al., 2016; Quan et al., 2011; von Schantz & Skene, 2015) and our findings highlight the importance of utilizing individual-level analyses in future studies aimed at developing such biomarkers. Despite the large inter-individual differences observed in the current study, 18 putative biomarker of alertness candidates were detected. The raw data presented for two of these final 18 candidates in SI Figure 7D are particularly promising as these appear to show different trajectories of change in response to increasing time awake and it may be that these different trajectories could represent individual behavioural responses to sleep loss. While these data appear promising, the current results will need to be replicated and biomarker candidates will require validation against established laboratory standard markers of alertness to determine whether changes in these metabolites truly represent changes in behavioural and physiological alertness.

Chapter 5 References

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Chapter 6

General discussion

6.1. Summary of main experimental findings

This program of work has focused on exploring different approaches to identifying biomarkers of vulnerability to alertness failure and biomarkers of alertness state, including potential genetic, endocrine and metabolic biomarkers. The studies presented in the experimental chapters aimed to 1) investigate the *BDNF* Val66Met polymorphism as a genetic marker of vulnerability to executive dysfunction, 2) investigate progesterone and the progesterone/estradiol ratio as novel biomarkers of a performance impairment associated with menstrual phase during sleep loss in naturally cycling women, and 3) characterise circadian and wake-dependent modulation of human plasma polar metabolites to establish biomarker candidates for future investigation.

The study presented in **Chapter 3** investigated the effects of the *BDNF* Val66Met polymorphism on a novel executive function measure, inhibitory control. Carriers of the *BDNF* Val66Met polymorphism showed impaired inhibitory control on the Stroop task, where they made more errors with increased time awake compared to Val/Val homozygotes. Furthermore, participants with the vulnerable Val/Met genotype also took longer to respond when they had to inhibit a prepotent response, particularly during the biological night. These results suggest that individuals with the *BDNF* Val66Met polymorphism may be vulnerable to the combined effects of inadequate sleep and adverse circadian phase on performance outcomes. The findings of this study are commensurate with previous research showing that the *BDNF* Val66Met polymorphism affects performance in the well-rested (Egan et al., 2003; Hariri et al., 2003; Miyajima et al., 2008; Raz, Rodrigue, Kennedy, & Land, 2009; Schofield et al., 2009) and sleep deprived state (Bachmann, Klein, et al., 2012). In a novel aspect of this study, however, this polymorphism was shown to affect not only short-term memory as previously shown (Bachmann, Klein, et al., 2012), but also inhibitory control.

The study presented in **Chapter 4** replicated previous findings showing menstrual phase as a biomarker of vulnerability to alertness failure (Vidafar et al., Under review; Wright & Badia, 1999), where women in the follicular phase were more vulnerable to neurobehavioural performance

impairment during prolonged wakefulness. In a novel aspect of the study, however, these differences in performance between the menstrual phases were shown to be recovered by night time light exposure. Therefore, light appears to be an effective countermeasure that can improve performance during the night in women, particularly when women are in the follicular phase. As progesterone is a key hormone in the menstrual cycle (Landgren, Unden, & Diczfalusy, 1980) and is thought to modulate temperature (Israel & Schneller, 1950), both progesterone and the progesterone/estradiol ratio were investigated as biomarkers of performance impairment in naturally cycling women. During prolonged wakefulness, neither progesterone nor the progesterone/estradiol ratio were associated with performance and light had no effect on either endocrine marker. It was found, however, that temperature had a significant relationship with performance during sleep loss and improvement in performance during light exposure was accompanied by increases in temperature. These results suggest that temperature may be responsible for differences in performance between the menstrual phases. Together, the results of this study highlighted the importance of measuring and reporting menstrual phase in studies investigating the effects of sleep loss and light on performance, and suggest that temperature manipulation, either through light or other means, may be a suitable countermeasure to reduce the vulnerability observed in women in the follicular phase of the menstrual cycle.

The study presented in **Chapter 5** investigated circadian and wake-dependent changes in plasma polar metabolites during prolonged wakefulness. It was found that polar metabolites exhibit both circadian and wake-dependent modulation. There were large inter-individual differences, however, in the abundance and timing of rhythmic metabolites and in the abundance and magnitude of change in metabolites increasing or decreasing with time awake. While the results of this study were similar to previous studies showing circadian and wake-dependent modulation of the plasma metabolome (Ang et al., 2012; Chua, Shui, Cazenave-Gassiot, Wenk, & Gooley, 2015; Chua et al., 2013; Dallmann, Viola, Tarokh, Cajochen, & Brown, 2012; Davies et al., 2014; Kasukawa et al., 2012), this study was also able to identify several novel polar metabolites that showed circadian rhythmicity or linear changes with time awake. Importantly, the results of this study provide

information for selecting candidates for future research using metabolomic approaches to develop biomarkers of disease and alertness, and also have implications for understanding the effects of sleep and circadian disruption on metabolism and how this may ultimately lead to the development of metabolic disease. As has been shown previously for plasma lipids (Chua et al., 2015; Chua et al., 2013), large inter-individual differences were observed between participants and this finding has implications for the use of group-level analyses in future biomarker discovery work. Despite the large inter-individual differences, however, 18 putative metabolite biomarkers were identified for future investigation.

6.2. Advantages and disadvantages of different biomarker approaches

This thesis has explored different methods to identify biomarkers of vulnerability to alertness failure and biomarkers of current alertness state. These different biomarker development approaches were chosen as they have been used previously in the search for biomarkers of alertness thus allowing for an examination of the efficacy of these different methods and the opportunity to further validate putative biomarkers previously identified using these methods. Furthermore, as discussed in Chapter 1, Section 1.1., given the large range of potential biomarker targets, this thesis examined three different types of biomarkers including genetic, endocrine and metabolic markers. The following section will discuss the advantages and disadvantages of each of these methods.

6.2.1. Targeted genetic approach to identifying biomarkers of vulnerability to alertness failure

As seen in **Chapter 3**, this thesis has explored a targeted approach to identifying a genetic marker of vulnerability to alertness failure by targeting the *BDNF* Val66Met polymorphism. This polymorphism was targeted as it has previously been implicated in vulnerability to cognitive dysfunction, particularly under conditions where functioning is already impaired, for example in psychiatric disorders and pathological aging (Boots et al., 2017; Cao et al., 2016; Da Rocha, Malloy-Diniz, Lage, & Correa, 2011; Egan et al., 2003; Y. Y. Lim et al., 2013; Raz et al., 2009; Rybakowski, Borkowska, Czerski, Skibińska, & Hauser, 2003; Rybakowski et al., 2006; Tükel et al., 2012). Furthermore, this polymorphism has also been shown to affect working memory

performance during sleep deprivation (Bachmann, Klein, et al., 2012). A targeted approach, which typically targets genes based on their involvement in the regulation of circadian rhythms or the sleep homeostat, or based on knowledge of their involvement in vulnerability to cognitive dysfunction, has also been used in previous research investigating genetic markers of vulnerability to sleep loss [reviewed in section 1.4; e.g. (Bachmann, Klaus, et al., 2012; Bodenmann et al., 2012; Bodenmann, Rusterholz, et al., 2009; Holst et al., 2017; Satterfield, Wisor, Field, Schmidt, & Van Dongen, 2015; Viola et al., 2007)]. This targeted approach is useful particularly given the small sample sizes often used in highly controlled laboratory studies investigating the effects of sleep and circadian disruption on cognition. Given that many different genes are likely to be associated with vulnerability to sleep loss, collection of genetic samples from all participants enrolled in these highly controlled sleep and circadian studies may facilitate future endeavours to conduct a genome wide association study (GWAS), which may allow for the detection of novel genetic markers. Considering the size of the genome, these studies require large cohorts of participants in order to acquire adequate power to detect genes that may influence specific behaviours or phenotypes of interest (Visscher, Brown, McCarthy, & Yang, 2012). Several studies using GWAS have already successfully identified novel genetic loci associated with various sleep disorders, and sleep timing and duration (Gottlieb et al., 2015; Hammerschlag et al., 2017; Hu et al., 2016; Jones et al., 2016; Lane et al., 2017; Ollila et al., 2014; Parsons et al., 2013; Scheinfeldt et al., 2015). Sample sizes in these studies ranged from approximately 5,000 to more than 100,000 participants. While a genomic approach to identifying genes associated with vulnerability to alertness failure will likely yield a number of novel genetic targets for investigation, these studies are not yet possible due to sample size restrictions. Therefore, targeting genes based on current evidence appears to be the best way to identify genetic biomarkers of vulnerability to sleep loss and to validate genes that have already been identified, as done in **Chapter 3**.

Although markers of global vulnerability to acute alertness failure and associated impairment are potentially important indicators for prevention of sleepiness mediated errors, accidents and injuries, a biomarker of alertness state would allow for greater sensitivity to detect

current alertness levels and predict alertness failure in the short-term, for example within a work shift or before a drive home. In this thesis two approaches were used to identify novel biomarkers of alertness state—1) targeting specific hormones that change with menstrual phase, which is associated with vulnerability to sleep loss, and 2) performing an untargeted screen of global changes within the metabolome to identify markers that are most sensitive to sleep loss and circadian phase.

6.2.2. Targeted and untargeted approach to identifying biomarkers of alertness state

Identification of biomarkers of alertness state based on a predefined characteristic or phenotype that is associated with vulnerability to sleep loss has been done previously in targeting immune-related biochemicals (Frey, Fleshner, & Wright, 2007; Hui, Hua, Diandong, & Hong, 2007; Sauvet et al., 2010; Shearer et al., 2001). A similar approach was also utilised in **Chapter 4**, where progesterone and the progesterone/estradiol ratio were targeted based on their dynamic changes throughout the menstrual cycle (Landgren et al., 1980) and their role in the regulation of temperature (Cagnacci, Arangino, Tuveri, Paoletti, & Volpe, 2002; Cagnacci, Volpe, Paoletti, & Melis, 1997; Israel & Schneller, 1950; Rothchild & Barnes, 1952; Stachenfeld, Silva, & Keefe, 2000). The advantage of using a targeted approach is that prior knowledge of the phenotype, and the biology and biochemistry underpinning the phenotype may allow for more swift development of appropriate countermeasures. Furthermore, as these phenotypes may already be well known and studied, as is the case for menstrual phase, there may already be commercially available assays developed to measure the biochemicals of interest. A targeted approach, however, while hypothesis driven, is very narrow in scope and may only be applicable to a small cohort of individuals, for example only naturally cycling women of reproductive age as in **Chapter 4**. Furthermore, targeting biomarkers related to already known phenotypes means that these biomarkers, due to their role in some other biological process, may not possess the specificity required for a biomarker of alertness. More importantly, while targeted biomarkers are selected based on a hypothesis, they may not be effective biomarkers at all, as was seen for progesterone and other female reproductive hormones in **Chapter 4**. Nevertheless, targeting specific biochemicals can help us to better understand the

effects of sleep and circadian disruption on many different biological processes, which may provide other important insights into the underlying processes linking inadequate and mistimed sleep to a number of adverse health outcomes (discussed further in Section 6.5). From a biomarkers development viewpoint, these studies are also important as they can help to rule out potential biomarker candidates for further study.

Given some of the limitations of a targeted approach to identifying biomarkers of alertness state, a broader approach appears to be more appropriate. One way to achieve this is to conduct an untargeted biomarker discovery program using an omics approach, for example metabolomics, as in **Chapter 5**. Use of omics technologies has been recommended in a recent workshop aiming to identify future opportunities for developing novel sleep and circadian biomarkers (Mullington et al., 2016). These untargeted omics approaches allow for the screening of hundreds to thousands of biochemicals from a single sample in order to detect markers that can best distinguish between the well-rested and sleep deprived states. With the inclusion of a diverse range of participants, using an untargeted approach also allows for the detection of biomarkers that are applicable to many different individuals, and not just associated with one specific group or phenotype. Furthermore, an untargeted approach allows for the detection of biomarkers involved in many different pathways that can each uniquely contribute to a panel of biomarkers to indicate alertness state. The main limitation of an untargeted approach, however, is that identification and characterisation of the biochemical(s) of interest and how these compounds interact within the body to affect sleep, circadian and other regulatory systems may be necessary, and this is likely to be costly and time-consuming processes. Similarly, development of a commercial assay may also be required if the biochemical of interest is unknown. As discussed in Section 1.5.2.1., determining the identities of metabolites from untargeted metabolomics experiments can be a costly and time-consuming process, particularly if the metabolite is an unknown compound that has not previously been identified. Despite these limitations, using an untargeted approach is likely the quickest and simplest way to develop a number of robust biomarkers that can contribute to a panel of biomarkers to measure and predict alertness state.

6.3. Systems level integration of biomarker development

In using different techniques to identify biomarkers of vulnerability to alertness failure and biomarkers of alertness state, this thesis has highlighted the complexity of determining current and predictive risk of alertness failure, and the multiple factors that can contribute to this risk. The complexity of this vulnerability underscores the necessity to incorporate information from all levels from the genome through to the resulting phenotype to determine an overall impression of vulnerability for any one individual. Combining the different approaches used in this thesis will allow for the determination of how multiple factors, including innate vulnerability dictated by genes or other biological factors, and current state-specific vulnerability dictated, for example, by prior sleep/wake history or working during the biological night, can contribute to an individual's current or future risk of alertness failure. For example, in **Chapter 3**, the combination of the vulnerable *BDNF* Val66Met polymorphism and the vulnerable follicular phase appeared to result in enhanced susceptibility to performance impairment during sleep loss in women. While the sample size precluded further analyses, this type of approach may lead to a better understanding of how these biomarkers, which are typically determined individually, interact to result in an overall susceptibility to alertness failure.

Future studies combining the different omics approaches (i.e. genomics, transcriptomics, proteomics, metabolomics and microbiomics), and investigating these in different groups (i.e. different age ranges, sexes and menstrual phases), may help us to understand how these multiple factors contribute to one individual's overall level of susceptibility to alertness failure due to sleep and circadian challenges. The use of individual omics approaches can give us an indication of what biochemicals are associated with sleep and circadian disruption (i.e. correlational), however, a systems biology approach that combines multiple omics platforms may provide further insight into the mechanisms or pathways via which inadequate sleep and circadian timing actually result in alertness failure [i.e. causal (Hasin, Seldin, & Lusis, 2017)]. Understanding these mechanisms and pathways could ultimately lead to a better understanding of how this disruption results in adverse

health outcomes and may also help with the development of interventions to counteract alertness failure. The use of a combined approach may also help us to better understand the inter-individual differences in response to sleep loss and adverse circadian phase, which have been observed previously in both behaviour and physiology (Chua, Yeo, Lee, Tan, Lau, Tan, et al., 2014; Rupp, Wesensten, & Balkin, 2012; Van Dongen, Baynard, Maislin, & Dinges, 2004), and more recently in omics studies [**Chapter 5**; (Arnardottir et al., 2014; Chua et al., 2015; Chua et al., 2013)]. Combining approaches may, for example, help us to understand the different metabolic phenotypes seen in response to sleep loss, as described in **Chapter 5** and previously for plasma lipids (Chua et al., 2013). These specific metabolic responses may be linked to different genotypes, and identification of these may help us to predict an individual's metabolic response to sleep and circadian disruption and thus, their risk to metabolic disease caused, for example, by shift work.

6.4. Developing tailored countermeasures

6.4.1. Developing countermeasures targeted at individuals who are more vulnerable

In **Chapters 3** and **4** the *BDNF* Val66Met polymorphism and menstrual phase, respectively, were confirmed to be biomarkers of vulnerability to alertness failure, as previously reported (Bachmann, Klein, et al., 2012; Vidafar et al., Under review; Wright & Badia, 1999). The identification and validation of genes, phenotypes, or combinations of genes and/or phenotypes that result in enhanced susceptibility to alertness failure will aid in the implementation of targeted interventions for individuals who are most at risk. An example of a targeted intervention aimed at a specific population is the use of a driving curfew for young drivers in Massachusetts (Rajaratnam et al., 2015). Young and inexperienced drivers have been shown to be involved in a higher rate of motor vehicle accidents, particularly sleepiness-related accidents (McCartt, Ribner, Pack, & Hammer, 1996; Pack et al., 1995; Tefft, 2012) which led to the introduction of legislation that prohibited drivers younger than 18 years of age from driving unsupervised at night, and established a mandatory driver education program, which included education on drowsy driving. An investigation of the effects of this legislation showed that, relative to one year before its

introduction, there was an approximately 19% reduction in crash rates involving drivers aged below 18 years of age within the 5-year period following the change in legislation (Rajaratnam et al., 2015). A second example of an intervention targeted at a specific high-risk population is the introduction of a Sleep Health Program in firefighters, which included 1) education sessions that provided information on fatigue-related health hazards, the importance of sleep and sleep hygiene and advice on sleepiness countermeasures, such as naps and caffeine use; 2) voluntary sleep disorders screening; and 3) sleep disorders diagnosis and treatment (Sullivan et al., 2017). An investigation of the efficacy of the Sleep Health Program showed that the fire stations that were randomised to receive the intervention reported almost half the number of 24-hour pay periods coded as injury or disability days per firefighter compared to those stations that did not receive the intervention. Furthermore, those firefighters who attended the education sessions were 24% less likely to file an official injury report than those who did not attend (Sullivan et al., 2017). These studies provide evidence that tailored interventions targeted at specific high-risk populations can be effective. Therefore, by identifying who is most susceptible, allocation of resources, education and interventions can be prioritised to those who will benefit most.

6.4.2. Developing countermeasures based on the cause of vulnerability

While identifying biomarkers of vulnerability is important in order to identify who is more vulnerable, these biomarkers may also be important in identifying what is causing this vulnerability, or why some individuals display enhanced susceptibly. Understanding the biology underlying alertness failure may provide insight into the likely effectiveness of a range of sleepiness reduction strategies used by workers to maintain alertness during shift. For example, our understanding of how the circadian system and sleep homeostat regulate the metabolome, which was explored in **Chapter 5**, may influence our understanding of how meal-timing affects alertness (Grant, Dorrian, et al., 2017; Gupta et al., 2017) and metabolic outcomes (Buxton et al., 2012; Grant, Coates, et al., 2017; Morris, Purvis, Mistretta, & Scheer, 2016; Scheer, Hilton, Mantzoros, & Shea, 2009). While this type of investigation has not yet been conducted, characterisation of individual metabolic phenotypes in response to challenges to the circadian system and sleep

homeostat may help individuals to plan meals suited to their metabolic responses, which may ultimately help to improve alertness and reduce the risk of adverse metabolic outcomes.

Understanding the cause of enhanced vulnerability could also allow for implementation of countermeasures matched to the biological and physiological underpinnings of the vulnerability itself. Studies of the *ADORA2A* and *COMT* genes, for example, have already identified polymorphisms in these genes that modulate the effects of two common sleepiness countermeasures, caffeine and modafinil, on performance during sleep deprivation. These studies have found that caffeine and modafinil are not effective sleepiness countermeasures in individuals with specific *ADORA2A* (Bodenmann et al., 2012) and *COMT* (Bodenmann & Landolt, 2010; Bodenmann, Xu, et al., 2009) polymorphisms, respectively. Furthermore, the *PER3* polymorphism has been shown to modulate the effects of blue light on alertness levels and suppression of melatonin, such that the homozygous $PER3^{5/5}$ allele is associated with greater sensitivity to blue light (Chellappa et al., 2012). As discussed in **Chapter 3** (section 3.3 Concluding remarks) the *BDNF* Val66Met polymorphism may alter the effectiveness of interventions aimed at promoting or enhancing slow wave sleep, although, this requires further investigation. In **Chapter 4,** light was found to be an effective countermeasure particularly for vulnerable women in the follicular phase of the menstrual cycle. Given that differences in performance during sleep loss between menstrual phases may be driven by temperature, however, other interventions that incorporate manipulation of temperature [e.g. drinking or eating hot or cold beverages meals, or distal cooling or heating (Fronczek et al., 2008)] may also be useful sleepiness countermeasures for women in the follicular phase when photic countermeasures are not available or appropriate. Thus, understanding the source of vulnerability to alertness failure, and the effects of genotypes and phenotypes on common sleepiness countermeasures will allow for tailored interventions for managing alertness at the individual level.

6.5. Women in sleep and circadian research

The findings of **Chapter 4** and those previously reported (Vidafar et al., Under review; Wright & Badia, 1999), which show the follicular phase of the menstrual cycle is associated with enhanced vulnerability to sleep loss, suggest that, while women respond differently to sleep loss than men (Blatter et al., 2006; Santhi et al., 2016), women also respond differently to women depending on menstrual phase. Differences between the sexes is important given that many omics-based sleep and circadian studies to date have included only male participants [e.g. (Ang et al., 2012; Chua et al., 2015; Chua et al., 2013; Dallmann et al., 2012; Davies et al., 2014; Giskeødegård, Davies, Revell, Keun, & Skene, 2015; Kasukawa et al., 2012; Pellegrino et al., 2012)]. If a biomarker of alertness that is applicable to all individuals is to be determined, inclusion of women in future research aiming to identify such biomarkers is crucial.

Women, compared to men, have been shown to have a shorter circadian period (Duffy et al., 2011), altered timing and amplitude of the circadian temperature rhythm (Baker et al., 2001; Boivin, Shechter, Boudreau, Begum, & Ying-Kin, 2016; Kattapong, Fogg, & Eastman, 1995), a larger phase angle between sleep onset and DLMO (Cain et al., 2010), differences in sleep structure (Boivin et al., 2016; Chellappa, Steiner, Oelhafen, & Cajochen, 2017) and power density (Dijk, Beersma, & Bloem, 1989), differences in light perception (Chellappa et al., 2017), poorer performance and alertness in response to sleep loss (Blatter et al., 2006; Santhi et al., 2016), and different circadian and wake-dependent expression of various biochemical markers, for example plasma BDNF (Cain et al., 2017), melatonin (Cain et al., 2010; Gunn, Middleton, Davies, Revell, & Skene, 2016), gene transcripts (A. S. P. Lim et al., 2013), and markers of inflammation (Irwin, Carrillo, & Olmstead, 2010). These differences highlight the need to further investigate women in future studies identifying biomarkers of vulnerability to alertness failure and biomarkers of alertness state. This future research does not just need to address sex differences, but also differences across the menstrual cycle in women. Many of the studies showing sex differences in sleep and circadian outcomes did not control for menstrual phase (Cain et al., 2010; Chellappa et

al., 2017; Dijk et al., 1989; Duffy et al., 2011; Santhi et al., 2016) and in those studies that did, several different approaches were taken, including studying women only in the follicular phase (Blatter et al., 2006), requiring all female participants to be taking hormonal contraception (Gunn et al., 2016), and adjusting analyses for levels of progesterone and estrogen (Irwin et al., 2010). Few studies, however, directly compared women in different phases of the menstrual cycle (Baker et al., 2001; Boivin et al., 2016; Cain et al., 2017; Kattapong et al., 1995). In **Chapter 5**, women were specifically recruited in the follicular phase to reduce the impact on menstrual phase on the biomarkers, however, based on the findings of **Chapter 4**, further investigation should include women in both menstrual phases.

While identifying and characterising vulnerability in women is important to ensure adequate alertness management that can be tailored to the underlying causes of this vulnerability, care should be taken when presenting such findings. It is important that this information is presented in such a way that it cannot be used to discriminate against women, or any other group of individuals who are shown to be vulnerable to alertness failure. Rather, our understanding of enhanced vulnerability in these populations should be used to empower these groups by providing the necessary tools and education which can be used to better manage their alertness and ultimately their safety. Furthermore, this research, while important in identifying vulnerable individuals, also needs to provide practical solutions to mitigate this vulnerability. For example, in **Chapter 4**, while women in the follicular phase were shown to be more vulnerable to sleep loss, light exposure was explored as a possible countermeasure.

6.6. Implications for health and disease

While this thesis has predominantly focused on the short-term consequences of inadequate sleep (i.e. alertness failure and cognitive impairment), the research incorporated in this thesis also has implications for the long- term health outcomes associated with sleep and circadian disruption. In **Chapters 4** and **5**, the acute effects of sleep loss on female reproductive hormones and the metabolome were investigated and the findings of these studies may help us to better understand

how chronic exposure to sleep loss and/or circadian disruption might contribute to the development of adverse health outcomes. For example, our understanding of the effects of inadequate sleep on progesterone and other female reproductive hormones may help us to better understand disruption to menstrual cycles, negative pregnancy outcomes, infertility, and increased breast cancer risk in female shift workers (Chung, Yao, & Wan, 2005; Cone, Vaughan, Huete, & Samuels, 1998; Labyak, Lava, Turek, & Zee, 2002; Mínguez-Alarcón et al., 2017; Mozurkewich, Luke, Avni, & Wolf, 2000; Schernhammer et al., 2001; Schernhammer, Vitonis, Rich-Edwards, & Missmer, 2011). Similarly, the study in **Chapter 5**, along with previous metabolomics studies, provides an opportunity to identify metabolic pathways affected by inadequate and mistimed sleep. Identifying and understanding these pathways may give crucial insight into the relationship between sleep and circadian disruption and increased risk for obesity, diabetes and other cardiometabolic diseases (Depner, Stothard, & Wright, 2014). Moreover, these data may provide further insight into the function of sleep. By investigating the effects of inadequate sleep on many different biological outcomes we may improve our understanding of the role of sleep in maintaining homeostasis within the brain and body.

6.6.1. Differentiating short- and long-term vulnerability

Given that some individuals are more vulnerable than others to the acute effects of sleep loss on behaviour (Maire et al., 2014; Van Dongen et al., 2004), future research may investigate whether those individuals who are more vulnerable to the acute effects of sleep loss are also more vulnerable to the long term-health consequences. It is interesting to consider whether a poorer acute response to sleep loss, typically measured by behavioural outcomes (i.e. performance), is an adaptive response designed to encourage an individual to avoid inadequate sleep. Therefore, those individuals who are considered 'resilient' based on these acute behavioural responses may be exhibiting a maladaptive response that could potentially be more harmful with respect to the long-term outcomes of inadequate sleep and circadian misalignment. While this notion has not yet been investigated specifically, research examining heart rate variability as a marker of acute vulnerability to sleep loss has shown that vulnerable individuals (i.e. those who showed greater performance

impairment on the PVT) exhibited greater variability in heart rate relative to resilient individuals during extended wakefulness (Chua, Yeo, Lee, Tan, Lau, Cai, et al., 2014). Although this reduction in heart rate variability is associated with behavioural resilience to sleep loss, reduced heart rate variability has also been shown to predict an increased risk for future cardiac events in individuals who have not previously had a cardiac event (Tsuji et al., 1996), and is associated with a greater risk of mortality following myocardial infarction (Kleiger, Miller, Bigger Jr, & Moss, 1987; La Rovere, Bigger Jr, Marcus, Mortara, & Schwartz, 1998). Similarly, individuals who are behaviourally resilient to sleep deprivation have been found to show significant blunting of circadian rhythmicity in gene expression compared to individuals who are behaviourally vulnerable to sleep loss (Arnardottir et al., 2014). Although this reduction in rhythmicity appears to help individuals adapt behaviourally to the challenge of sleep loss, changes to circadian gene expression are also associated with a number of adverse health outcomes (Busik et al., 2009; Li et al., 2013; Martino et al., 2007; Naito, Tsujino, Fujioka, Ohyanagi, & Iwasaki, 2002; Paschos et al., 2012; Peliciari-Garcia, Bargi-Souza, Young, & Nunes, 2018; Sadacca, Lamia, DeLemos, Blum, & Weitz, 2011; Turek et al., 2005; Young, Razeghi, & Taegtmeyer, 2001). Together these findings may suggest that those individuals who appear to be resilient to sleep loss at the behavioural level, usually considered an advantage, may be more vulnerable to long-term health related outcomes associated with inadequate sleep. While this hypothesis is speculative, further research is required to determine whether vulnerability to sleep loss is consistent across both short-term behavioural outcomes, and long-term health outcomes related to sleep and circadian disruption.

Determining whether vulnerability is consistent across both short- and long-term outcomes is particularly important when identifying biomarkers of behavioural vulnerability to sleep loss as these biomarkers may be used in some instances to determine whether one individual is more suited to perform a high-risk, safety sensitive job than another. While individuals who are behaviourally resilient are the obvious candidates for such occupations, there may be a greater long-term risk of exposure to sleep and circadian disruption. If this is the case, it makes developing effective countermeasures for alertness failure even more important. Developing effective alertness

management strategies to combat the short-term effects of alertness failure, even in those individuals who are most vulnerable, will allow for these individuals to perform in high-risk, safety sensitive occupations, rather than relying only on those individuals who display behavioural resilience to sleep loss. Furthermore, identifying those who are most vulnerable to the long-term health-related effects of inadequate sleep may help to develop treatments to combat the adverse health consequences related to poor sleep and circadian misalignment associated with sleep disorders, shift work and jetlag.

6.7. Implications, future directions and limitations

As previously summarised, this thesis has investigated both a global biomarker of vulnerability to alertness failure and biomarkers of alertness state using genetic, endocrine and metabolic approaches. Implications and future directions of this research will be discussed separately below.

6.7.1. Biomarkers of vulnerability to alertness failure

Key findings from **Chapters 3** and **4** have confirmed, in accordance with previous research (Bachmann, Klein, et al., 2012; Vidafar et al., Under review; Wright & Badia, 1999), that the *BDNF* Val66Met polymorphism and menstrual phase, respectively, are biomarkers of vulnerability to executive dysfunction and performance impairment during sleep loss. These findings are important as they add to a growing literature of potential markers of vulnerability to sleep and circadian disruption. As discussed above, further work should combine different genetic and phenotypic markers to ascertain the additive and/or reductive effect of different combinations of biomarkers of vulnerability or resilience to sleep loss. Furthermore, the use of the rapidly developing omics technologies (e.g. GWAS) will help to identify novel biomarkers and confirm previously identified genetic markers of vulnerability to alertness failure. Future research identifying biomarkers of vulnerability should also focus on identifying how these markers result in vulnerability as this may lead to the ability to develop countermeasures appropriate not only to the environment, but also to the individual. Specific future directions for the work presented in this thesis include, investigation of the effects of the *BDNF* Val66Met polymorphism on cognition

following slow wave sleep enhancement, and the effects of body temperature modifications as a countermeasure to performance impairment during sleep loss in the follicular phase of the menstrual cycle.

6.7.2. Biomarkers of alertness state.

In **Chapter 4**, progesterone and the progesterone/estradiol ratio were investigated as potential biomarkers of performance impairment in naturally cycling, pre-menopausal women. While this research did not identify any viable biomarkers of alertness state, the findings of this study highlight the importance of including women in future research aiming to identify biomarkers of vulnerability to alertness failure and biomarkers of alertness state. Understanding the behavioural and physiological responses to sleep and circadian disruption unique to women is important for the effective management of alertness and future biomarker development. Importantly, the results of the research conducted in **Chapter 4** also ruled out possible biomarker candidates. While identifying and developing effective biomarkers of alertness state is the ultimate goal of this research, ruling out ineffective biomarkers for further study is also important given the time consuming and costly nature of these highly controlled biomarker studies.

In **Chapter 5**, circadian and wake-dependent control of the human plasma polar metabolome was investigated. This study identified a number of metabolites previously shown to be under circadian control, and also identified a number of novel highly polar compounds that showed rhythmic and linear changes during extended wakefulness. Moreover, the study in **Chapter 5** showed that there were large inter-individual differences in both rhythmic and linear metabolites, suggesting that the metabolic response to sleep loss is highly variable between individuals. These results have important methodological implications for future biomarker studies, indicating that it is important to examine such markers both at the group- and individual-levels. The results of the study in **Chapter 5** and other sleep and circadian omics studies are also important from the perspective of future development of biomarkers of disease. Information regarding how these potential biomarkers change with time-of-day or prior sleep/wake behaviour may be a crucial aspect of

biomarker candidate selection. The studies conducted in both **Chapters 4** and **5** may also contribute to our understanding of the contribution of sleep and circadian disruption to the etiology of female reproductive issues and metabolic disease.

Although the study conducted in **Chapter 5** determined 18 metabolite candidates that showed consistent wake-dependent changes across participants, further work is required to identify robust alertness state biomarker candidates. The study in **Chapter 5** was designed to provide proof-of-concept data showing circadian and wake-dependent changes in response to sleep loss in novel polar metabolites. The immediate next steps of this research will expand on this dataset to include a larger sample of participants. This second data set will be used to confirm the 18 putative candidates determined in **Chapter 5** and potentially identify new metabolite candidates that show promise as biomarker of alertness state. Subsequent research within this program of work will then need to determine the validity of these biomarkers by examining whether the metabolite candidates can accurately measure and predict laboratory standard markers of alertness, such as EEG derived microsleeps, PVT performance and ocular measures. Development of methodologies to measure these biomarkers on a targeted metabolomics platform that can provide quantitative information about metabolite concentrations, rather than just area under the curve data, will also be an important aspect of the ongoing project. These quantitative data will aid in the development of cut-off concentrations that can be used to indicate impairment. Furthermore, understanding how these biomarkers behave during a typical sleep/wake cycle, sleep restriction, circadian misalignment and during recovery following sleep loss will also be important if these are to be used in a real-world setting. This research will also need to be replicated in a different sample of participants to ensure the biomarkers are reliable across different individuals. If a valid and reliable biomarker or panel of biomarkers can be identified, the next steps will require taking these biomarkers out of the laboratory to investigate them in a real-world setting.

Biomarkers of alertness that can be successfully validated in real-world settings have several applications. First, such biomarkers would be useful as a roadside test to identify drowsy

drivers. Currently, there is no test to identify alertness that parallels tests for alcohol and drug use in drivers. As the role of alertness failure in motor vehicle accidents is likely underestimated, such a road-side biomarker of alertness would be very useful to reduce the impact of this preventable risk. Similarly, such biomarkers may also be used retrospectively, in a similar way to alcohol and drug testing, to determine whether drowsiness was a contributing factor to an accident. Second, these biomarkers may also be useful as fitness-for-duty tests. Testing alertness at the start of a shift will ultimately facilitate early detection and intervention, thus reducing the impact of alertness failure on workplace errors, accidents and injuries. Furthermore, such biomarkers may also be useful for continuous monitoring of individuals throughout a shift to ensure that fitness-for-duty is maintained throughout work. Lastly, biomarkers capable of indicating impaired alertness may also be useful in the diagnosis of sleep disorders and additionally in the assessment of the efficacy of interventions to treat such disorders.

Considering the many applications for a biomarker of alertness, it is likely that different biomarkers will need to be developed to suit specific applications. As highlighted in this thesis, biomarker discovery approaches differ depending on the final purpose of the biomarker and different biomarker targets are more suited to different applications. Because of the many different applications for biomarkers of alertness and the multitude of methodologies that can be used to develop such biomarkers, an important next step forward in this area of research will involve international collaboration to develop standards for the collection, analysis, interpretation and validation of these biomarkers. Already such collaboration at international meetings has resulted in the publication of white papers describing optimal workflows and the best evidence-based targets for future biomarker development initiatives [e.g. (Mullington et al., 2016)]. Consensus on the appropriate methodologies and targets for development of these biomarkers will ultimately improve the chances of successfully identifying and validating biomarkers of alertness, which, as described above, have several important applications.

6.7.3. Limitations

The limitations of each study are discussed in the relevant experimental chapters. This section will discuss broadly the limitations of the methodologies that were similar across all three studies, namely the highly controlled nature of these studies and long sleep deprivation protocols.

In a real-world setting, factors that have been specifically controlled for in the studies presented in this thesis (e.g. light, meal timing, temperature, and posture) would not be controlled, and sleep loss would be more likely occur in a more naturalistic way, for example recurrent nights of sleep restriction. For a biomarker of alertness to be effective it would need to be sensitive not only to acute sleep deprivation, but also sleep restriction, and other patterns of sleep and circadian disruption caused by sleep disorders, shift work or jetlag. Furthermore, an alertness biomarker would need to be robust to multiple individual and environmental factors that could alter its expression. Despite these requirements however, in order to confirm whether a gene or phenotype determines vulnerability to alertness failure or a biochemical marker represents alertness state these biomarkers need to be tested under conditions where other factors affecting alertness are controlled. Furthermore, the use of extreme sleep deprivation is also important as it increases the likelihood of finding a signal of interest which shows a clear response to sleep loss that other biochemicals may not. Importantly, these longer protocols also allow for the measurement of circadian rhythms in any alertness state biomarkers, which as discussed in Section 1.5.2.2. is an important factor to consider when selecting biomarker candidates. Therefore, these highly controlled sleep deprivation studies, while not representative of real-world sleep loss, are a necessary first step in biomarker discovery. Once biomarker candidates have been identified in a laboratory setting, however, they need to then be tested under real-world conditions.

6.8. Summary and conclusions

The results of this thesis contribute to a growing literature dedicated to identifying the biological determinants of vulnerability to alertness failure and biomarkers of alertness state. Specifically, this thesis has 1) confirmed the *BDNF* Val66Met polymorphism as a marker of vulnerability executive

dysfunction during sleep loss; 2) showed the effectiveness of a common sleepiness countermeasure (i.e. light) to mitigate vulnerability to performance impairment associated with the follicular phase of the menstrual cycle; and 3) contributed important novel data on sleep and circadian control of plasma polar metabolites, which will be used in future research to identify metabolite-based biomarkers of alertness state. With ongoing research, the studies within this thesis will contribute to the development and application of valuable alertness management strategies suited both to the environment and the individual. The ability to measure, monitor and predict alertness will ultimately lessen the economic burden of alertness mediated errors, accidents, and injuries in the workplace and on the roads. Most importantly, however, these developments have the capacity to substantially reduce the human cost of alertness failure.

Chapter 6 References

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