THESIS

THE EFFECT OF CIRCADIAN REGULATION AND SLEEP DISRUPTION ON METABOLIC

HOMEOSTASIS

Submitted by

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ABSTRACT

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HOMEOSTASIS

Sleep and circadian disruption are ubiquitous in modern society. While the National

Sleep Foundation recommends adults sleep 7-9 hours per night, the average sleep duration of

American adults has decreased from ~8.8 hours to ~6.8 hours over the last century, with 1 in 3

people report sleeping fewer than 6.5 hours per night during the work week. People who sleep

fewer than 6 hours per night have a three-fold risk of impaired fasting glucose than those

sleeping at least 8 hours per night. Laboratory studies report that as little as one night of

insufficient sleep impairs insulin sensitivity, which is a common risk factor for obesity and

diabetes. Circadian misalignment is common in people who work non-standard hours, including

evening, night, or rotating shifts, and is associated with increased fasting glucose and insulin

concentrations, as well as impaired insulin sensitivity. Moreover, circadian misalignment can

also occur as a consequence of insufficient sleep. With more than 35% of adults reporting

insufficient amounts of sleep, chronic and acute circadian misalignment are likely even more

prevalent than commonly recognized.

Sleep and circadian disruption are associated with increased mortality rates and health

problems, including obesity and diabetes. However, the molecular mechanisms by which these

impairments occur are not known. Thus, the overall goal of this dissertation was to determine

the circadian rhythms of substrate oxidation and hormonal regulators of energy balance as well

as to identify molecular alterations associated with insufficient sleep, including skeletal muscle

lipid accumulation and altered gene expression, and their relation with insulin sensitivity.

The primary findings are that in healthy, young, lean participants 1) carbohydrate and lipid oxidation as well as ghrelin and peptide YY have circadian rhythms as identified by a constant routine protocol and 2) insufficient sleep induces skeletal muscle lipid accumulation and altered gene expression as well as impaired insulin sensitivity. Together, these studies indicate that sleep and circadian disruption may impair insulin sensitivity via dysregulated lipid metabolism.

ACKNOWLEDGEMENTS

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CHAPTER I – INTRODUCTION TO SLEEP AND CIRCADIAN PHYSIOLOGY

History of sleep research

In ancient times, it was believed that the soul left the body during sleep and that the brain turned off resulting in no experiences during sleep. There are numerous biblical references to sleep, and even Shakespeare refers to sleep in his play Macbeth (Shakespeare, 1992):

> 35 Me thought I heard a voice cry, "Sleep no more!
> Macbeth does murder sleep" – the innocent sleep,
> Sleep that knits up the raveled sleave of care
> The death of each day's life, sore labor's bath,
> Balm of hurt minds, great nature's second course,
> 40 Chief nourisher in life's feast.

In the 1800s, the first systematic studies of sleep deprivation were performed. Total sleep deprivation was found to be fatal in rats (Everson et al., 1989a; Everson et al., 1989b) and dogs (de Manacéïne, 1894) after just a few days. Patrick and Gilbert were the first to identify performance impairments in humans after sleep deprivation (Patrick and Gilbert, 1896). In 1909, Ishimori extracted an unknown sleep-inducing substance from the brain of sleep-deprived dogs (Ishimori, 1909) and soon after, Legendre and Pieron transferred cerebral spinal fluid from sleep deprived animals into not deprived animals. They found that when dogs were injected with this still unknown substance extracted from the brains of sleep deprived dogs, sleep was induced (Legendre and Piéron, 1910; 1913).

The first recording of sleep using an electroencephalogram in humans was in 1939 (Davis et al., 1938) and shortly after, rapid eye movement (REM) and non-rapid eye movement (NREM) sleep cycles were characterized (Aserinsky and Kleitman, 1953; 1955; Dement and Kleitman, 1957a; b). In the 1960s, Randy Gardner set the record for the longest duration without sleep – 264 hours or 11 full days! Interestingly, when he did finally sleep, he only slept for 14 hours and 40 minutes (Gulevich et al., 1966), suggesting that humans cannot recover sleep lost

during deprivation. The 1960s had a boom of interest in sleep including narcolepsy, a disorder in which patients experience sleep-onset REM sleep; an interest in sleep, epilepsy, and abnormal movements; benzodiazepines and sleep lab studies to define hypnotic efficacy; and the discovery of sleep apnea.

In the 1970s, the first sleep medical centers were established and in the 1980s and 1990s, there was an explosion of sleep research. Studies investigating the importance of sleep in health and disease began in the 2000s and since then, research has continually shown the importance of sleep and the associated detriments when not enough sleep is received, or if sleep is received at the wrong time of day.

Sleep physiology

Sleep is a behavior characterized by six key characteristics: i. behavioral quiescence – period of decreased activity; ii. reduced responsiveness to stimuli during quiescent state; iii. homeostatic regulation of quiescent state; iv. quiescence and reduced responsiveness must be rapidly reversible (separate from paralysis or coma); v. rebound – increased sleep-state after sleep deprivation; vi. minimal movement, sleep posture, reduced responsiveness to stimulation, and quickly reversible (Campbell and Tobler, 1984; Allada and Siegel, 2008; Joiner, 2016).

Sleep is not only found in humans or mammals but it is believed that sleep-like behaviors are ubiquitous among many organisms. A recent study found that jellyfish show sleep-like behavior which is the first evidence of an organism without a central nervous system to sleep, suggesting the evolutionary benefit for sleep (Nath et al., 2017). Interestingly, with all the research being conducted in the field of sleep, it is still unknown why we sleep. It is clear that physiological detriments occur if we do not sleep, but the reason(s) for why we sleep is yet unknown. The National Sleep Foundation recommends healthy human adults receive 7-9 hours of sleep every night (Hirshkowitz et al., 2015).

History of circadian research

Sleep is regulated by two separate physiological systems known as the two process model of sleep regulation (Borbely, 1982). Process S is the sleep homeostasis regulatory arm whereas Process C is the circadian physiology (Borbely, 1982). Circadian physiology is the 24-hour daily rhythms that keep the body's internal rhythms in sync with the external environment. "Circadian" was termed by Franz Halberg in 1959 and translates from Latin to circa = around, approximately; diem = day; circadian means "around a day".

Circadian research also has a long history (McClung, 2006). In the 4th century, Androsethenes noticed diurnal leaf movements of tamarind tree (Bretzl, 1903). In the 13th century, Chinese medical texts observed diurnal processes in humans (Lu, 2002). In 1729, the endogenous circadian oscillation in heliotrope plant was observed to persist even in constant darkness (de Mairan, 1729). Patrick and Gilbert also recorded changes in sleepiness during extended sleep deprivation (Patrick and Gilbert, 1896). In 1918, it was discovered that animals are capable of maintaining 24h activity patterns in the absence of external cues (Szymanski, 1918). The discovery of the core clock genes led to the identification that the *Period* gene can disrupt the circadian clock of flies (Konopka and Benzer, 1971). The isolation of the *Period* gene and the discovery of the *Timeless* gene in flies help discovery the negative feedback loop that makes up the core circadian clock (Bargiello et al., 1984; Zehring et al., 1984; Vosshall et al., 1994). Details of this 24h transcription/translation autoregulatory negative feedback loop has since been expanded (Dunlap, 1999; Young and Kay, 2001; Reppert and Weaver, 2002; Lowrey and Takahashi, 2004). The 2013 discovery of a rooster's crow having a circadian component expanded the field beyond humans and laboratory research animals (Shimmura and Yoshimura, 2013). With the 2017 Nobel Prize in Physiology or Medicine awarded to Young, Rosbash, and Hall for their discoveries of the molecular mechanisms controlling circadian rhythms, the applicability and interest in circadian physiology skyrocketed (NobelPrize.org).

Circadian physiology

Circadian rhythms are controlled by a few core clock genes including *Period (Per)*, *Cryptochrome (Cry)*, *Bmal*, and *Clock* (Bargiello et al., 1984; Zehring et al., 1984; Yi et al., 2021). *Per* and *Cry* make up the positive arm of the molecular clock as the activation of their expression induces the expression of *Bmal* and *Clock*. *Bmal* and *Clock* are then the negative arm as their activation inhibits *Per* and *Cry* expression. The positive and negative arms of the molecular clock have antiphasic gene expression and act in an autoregulatory, transcription/translation negative feedback loop. Most cells of the brain and body express core clock genes (Andreani et al., 2015; Takahashi, 2017). Half of all proteins coding for genes show circadian dependent transcription in at least one tissue in mammals (Zhang et al., 2014). While the specific genes that cycle vary across species and tissues (Panda et al., 2002; Ko and Takahashi, 2006), the core clock genes are consistent throughout the body and between species (Reppert and Weaver, 2001; Allada and Chung, 2010). Clock gene cycling occurs in most nucleated mammalian cells except for the thymus and testis (Hastings et al., 2003; Alvarez and Sehgal, 2005).

In laboratory animal studies, tissues such as the brain, skeletal muscle, and liver, can be extracted and processed to identify core clock gene expression in these tissues. However, non-invasive techniques have been discovered which allow accurate circadian measurements to occur in living human participants, including the three circadian phase markers in humans – melatonin, cortisol, and core body temperature (Klerman et al., 2002; Wright, 2008). Melatonin is a marker of circadian phase when light is controlled (Benloucif et al., 2005). Melatonin was the first hormone isolated from the pineal body (Alberti, 1958). Melatonin is low during the day, begins to rise a few hours before habitual bedtime, and is highest at night. However, a limitation of melatonin is that it is influenced by light (Wurtman et al., 1963). Thus, even a brief light pulse can suppress melatonin release and mask circadian melatonin expression. In order to prevent light exposure from influencing melatonin expression, dim-light melatonin onset (DLMO) is

measured to assess the phase of the melatonin rhythm by measuring melatonin concentration in frequently sampled (hourly) saliva, urine, or blood. Core body temperature is another circadian phase marker in humans that is lowest during the biological night and highest during the biological day. Analysis of core body temperature for circadian phase requires constant posture, activity, sleep, and temperature as each of these may alter core body temperature and mask the endogenous circadian rhythm (Zulley et al., 1981; Benloucif et al., 2005). Cortisol is the third circadian phase marker in humans that is released in a pulsatile manner with a peak at the beginning of the biological morning and decreases throughout the day. Cortisol is also a major stress hormone so analysis for circadian phase requires constant activity and minimal, constant stress (Saba et al., 1963; Adam et al., 2017). While circadian phase markers are key measurements, investigators must be aware of the external factors which impact melatonin, core body temperature, and cortisol to ensure these external factors are not influencing the circadian rhythmicity of these markers.

Using melatonin as a circadian phase marker, the mean intrinsic circadian period in humans was measured to be 24.15 hours (Czeisler et al., 1999a; Wyatt et al., 1999; Wright et al., 2001; Wright et al., 2006; Gronfier et al., 2007; Duffy et al., 2011). Note that this is slightly longer than our sun-based 24-hour day. Thus, if the average human lived on their 24.15-hour day, their internal rhythms would soon be out of sync with the natural light/dark cycle of our world. Therefore, we need something to keep our bodies in sync with the natural light/dark cycles. Light is the primary zeitgeber (German for "time-giver") which resets the central circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Rusak and Zucker, 1979; Klerman et al., 1998; Roenneberg and Merrow, 2000). Historically, the sun was the primary source of light. However, in modern societies, the invention of natural lighting has enabled us to receive light at all times of the day and night which is constantly resetting our internal, endogenous clocks ultimately producing internal rhythms that are out of phase to the external world (sleeping during the day, eating at night). While light is the primary zeitgeber,

other zeitgebers also exist including auditory stimulus (Menaker and Eskin, 1966; Goel, 2005), food (Mistlberger, 1994; Challet et al., 1998; Damiola et al., 2000; Stokkan et al., 2001), exercise (Mrosovsky, 1996; Buxton et al., 2003; Barger et al., 2004), temperature (Hoffmann, 1968; Liu et al., 1998; Rajaratnam and Redman, 1998), and social interactions (Mrosovsky, 1988; Duffy et al., 1996; Levine et al., 2002). There are also tissue-specific zeitgebers, i.e., the lungs respond to oxygen, the liver responds to feeding, and the kidney has a mixed response to oxygen and feeding (Manella et al., 2020; Manella et al., 2021).

Circadian researchers have developed a few circadian protocols in order to accurately assess the circadian system by limiting the influence of all the potentially relevant external factors. The in-laboratory constant routine protocol eliminates, makes constant, or equally distributes external factors that influence the circadian system across the 24h day in order to measure circadian rhythmicity (Mills et al., 1978; Czeisler et al., 1999b; Broussard, 2017). During a constant routine, a minimum of 24h is required to assess a full circadian cycle. Sleep and physical activity are eliminated during the protocol; temperature, posture, and dim lighting are made constant throughout the protocol; and caloric intake is equally distributed across the protocol by providing isocaloric hourly meals. Another common circadian protocol is the in-laboratory forced desynchrony in which participants live on a day length longer or shorter than the typical 24h day – frequently 20h or 28h – for multiple weeks (Kleitman, 1939; Broussard, 2017). With the altered day length of this protocol, wake to sleep ratios are maintained (2:1; e.g. 18h and 40min scheduled wake and 9h and 20 min scheduled sleep for 28h day) however, the clock time of the sleep opportunity will gradually shift across all hours of the 24h day. Therefore, by the end of the forced desynchrony protocol, the sleep opportunity (and therefore all wake activities) have all occurred at every clock time across the 24h day. Thus, physiological events under circadian control will not be dependent on the environmental light-dark cycle but rather maintain a 24h rhythm. Using rigorously controlled in-laboratory constant routine, forced

desynchrony, or other circadian protocols, investigators can identify circadian rhythmicity of activities or factors of interest.

Impact of sleep and circadian disruption on metabolic homeostasis

Insufficient sleep is a novel and independent risk factor for metabolic syndrome. Spiegel et al. (Spiegel et al., 1999) brought together the separate fields of sleep, circadian rhythms, and metabolism with the first discovery that sleep and circadian disruption impaired metabolic outcomes. This finding launched the intertwining of the fields of sleep, circadian rhythms, and metabolism. This field emerged from the unique progression of a first experimental paper leading to larger, observational, and population studies, instead of the more typical direction of observational findings leading to experimental studies. This was the first paper to show that in a highly controlled inpatient study on young, healthy participants, 6 days of insufficient sleep of 4 hours per night significantly lowered glucose tolerance (Spiegel et al., 1999). Since this discovery, numerous inpatient and outpatient studies have replicated these findings.

Objectives, Specific Aims, and Hypotheses

While much research has been done in the fields of sleep, circadian, and metabolic physiology, many questions still remain regarding mechanisms, pathways, and specific factors involved in the metabolic impairments associated with sleep and circadian disruption. Thus, the overall objective of this thesis was to examine the effects of circadian regulation and sleep disruption on metabolic physiology. Chapter 2 will introduce a specific circadian protocol, the constant routine, in order to identify circadian rhythms in metabolic factors.

Study 1 (Chapter 2)

Circadian rhythms in energy expenditure, substrate oxidation, and concentrations of appetite-related hormones have been identified under fasting and non-fasting conditions. However, these variables have rarely been measured simultaneously in the same participants to evaluate relationships. We therefore used a 26h constant routine protocol to investigate the

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circadian rhythms in substrate oxidation and concentrations of hormonal regulators of energy balance.

Aims and Hypotheses – Determine the impact of a 26h constant routine protocol on substrate oxidation and hormonal regulators of energy balance. We hypothesize that substrate oxidation will have circadian rhythmicity with fat oxidation highest during the biological night and carbohydrate oxidation highest in the biological day. We further hypothesize that ghrelin, leptin, and PYY will have circadian rhythms with peaks in the biological evening for ghrelin and a peak in the biological morning for PYY and leptin.

After an introduction to the methods of measuring whole body and tissue-specific insulin sensitivity (Chapter 3), we propose some considerations for metabolic estimates in people with sleep and circadian disruption (Chapter 4). Chapter 5 will describe results from an inpatient study designed to identify potential mechanisms by which insufficient sleep impairs skeletal muscle insulin sensitivity.

Study 2 (Chapter 5)

Previous research consistently reports that insufficient sleep impairs insulin sensitivity; however, the mechanisms by which this occurs are still unknown. Considering skeletal muscle is the largest metabolic tissue responsible for ~80% postprandial glucose uptake in humans (Defronzo et al., 1981), we therefore investigated potential mechanisms by which insufficient sleep impairs skeletal muscle insulin sensitivity (Conceptual Model - Fig 1.1).

Aims and Hypotheses – Determine the impact of insufficient sleep on skeletal muscle lipid accumulation, RNA expression, and mitochondrial function. We hypothesize that insufficient sleep leads to lipid accumulation, altered RNA expression, and mitochondrial dysfunction. We further hypothesize that these changes will be related to impaired *in vivo* skeletal muscle-specific insulin sensitivity as assessed by the hyperinsulinemic euglycemic clamp.

Finally, Chapter 6 will summarize the above chapters as well as provide limitations and future directions.
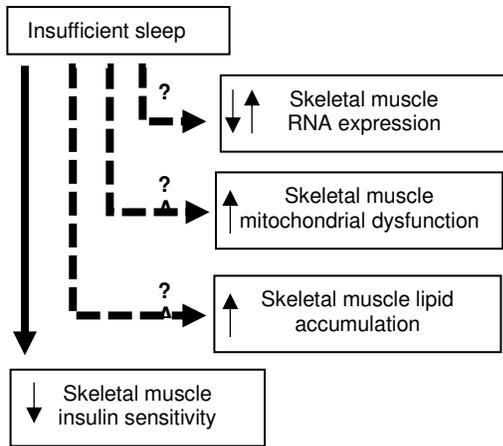
Fig 1.1. Conceptual Model

CHAPTER II – MANUSCRIPT I
CIRCADIAN RHYTHM OF SUBSTRATE OXIDATION AND HORMONAL REGULATORS OF
ENERGY BALANCE[1]

Summary

Objective

The circadian system provides an organism with the ability to anticipate daily food availability and appropriately coordinate metabolic responses. Few studies have simultaneously assessed factors involved in both the anticipation of energy availability (i.e., hormones involved in appetite regulation) and subsequent metabolic responses (such as energy expenditure and substrate oxidation) under conditions designed to reveal circadian rhythmicity.

Methods

Eight healthy adults (four females; age: 28.0 ± 2.3 years; BMI: 24.3 ± 2.9 kg/m^2) participated in a 26-hour constant routine protocol involving continuous wakefulness with constant posture, temperature, dim light, and hourly isocaloric snacks. Indirect calorimetry was performed every 3 hours for measurement of energy expenditure and substrate oxidation. Subjective hunger was obtained hourly using questionnaires. Saliva and plasma were obtained hourly to assess melatonin (circadian phase marker) and hormones (leptin, ghrelin, and peptide YY).

Results

Fat and carbohydrate oxidation was highest in the biological evening and morning, respectively. Subjective hunger ratings peaked during the middle of the biological day. Significant circadian rhythms were identified for ghrelin and peptide YY with peaks in the biological evening and morning, respectively.

Conclusions

These findings support a role for the circadian system in the modulation of nutrient oxidation, subjective measures of appetite, and appetitive hormones.

Study Importance

What is already known?

Previous research has identified circadian rhythms in energy expenditure, substrate oxidation, subjective ratings of hunger, and concentrations of appetite-related hormones under fasting and nonfasting conditions.

However, rarely are all of these variables measured simultaneously in the same participants to evaluate interrelationships.

What does this study add?

Using a highly controlled constant routine protocol (26 hours of wakefulness with constant posture, temperature, dim light, and hourly isocaloric snacks), we demonstrate endogenous circadian rhythms in substrate oxidation, subjective hunger ratings, ghrelin, and peptide YY in healthy adults.

These findings contribute to our understanding of how factors involved in energy balance are modulated by the circadian system.

How might these results change the direction of research?

Future studies are needed to determine how rhythms in energy metabolism differ between lean adults and adults with conditions such as obesity and diabetes.

It will also be important to know how rhythms in energy metabolism respond to therapeutic interventions such as weight loss and exercise as well as the potential implications of these responses for metabolic health.

Introduction

Energy balance refers to a physiologic state in which total daily energy expenditure (TDEE) (composed of resting energy expenditure [EE] and diet induced- and exercise-induced thermogenesis) is equal to energy intake (EI). Daily EE and EI are tightly regulated by homeostatic mechanisms to maintain energy balance (Schwartz et al., 2003). During the fasted state, the orexigenic hormone ghrelin is secreted from the gut, enters the plasma, and stimulates energy-sensing regions of the hypothalamus triggering EI. During the postprandial period, hormonal responses favor satiety and increased EE, marked by the release of anorexigenic humoral signals from the pancreas (insulin), adipose tissue (leptin), and the gastrointestinal tract (glucagon-like peptide 1, cholecystokinin, and peptide YY [PYY]) (Schwartz et al., 2003). In healthy individuals, the interplay among orexigenic and anorexigenic signals results in a remarkably stable body mass over time despite large day-to-day fluctuations in EI and EE (Schwartz et al., 2003).

Many hormones (e.g., leptin, ghrelin, and PYY) (Hill et al., 2011; Markwald et al., 2013) and metabolic processes involved in the homeostatic regulation of energy balance exhibit distinct diurnal rhythmicity that is cued to the feeding-fasting cycle. However, some are also secreted in an anticipatory manner in part regulated by the circadian system (Espelund et al., 2005; McHill et al., 2018; Zitting et al., 2018). The circadian timekeeping system is designed to anticipate environmental changes (e.g., light, food availability) so that metabolic physiology and behavior are timed appropriately. Clock-driven rhythms have been identified in EE (Krauchi and Wirz-Justice, 1994; Spengler et al., 2000; Zitting et al., 2018), substrate oxidation (Zitting et al., 2018), subjective hunger (Owens et al., 1996; Waterhouse et al., 2004; Scheer et al., 2013; Sargent et al., 2016; McHill et al., 2018), leptin, and ghrelin (Shea et al., 2005; McHill et al.,

2018; Qian et al., 2019a), though results have been inconsistent. For example, carbohydrate oxidation has been shown to peak during the biological day with fat oxidation peaking during the biological night in one study (Zitting et al., 2018), but another failed to demonstrate a rhythm in substrate oxidation as measured by the respiratory quotient (RQ) (Spengler et al., 2000).

Furthermore, few previous studies have simultaneously measured variables relevant to energy balance in the same participants. To understand how the timing of factors involved in energy intake are temporally related to the timing of factors involved in nutrient oxidation, it is important to examine these variables in the same individuals. Moreover, there appears to be vast interindividual variability in the circadian profiles of variables associated with energy balance regulation.

Therefore, the present study utilized a highly controlled constant routine protocol to simultaneously evaluate group- and individual-level variability and rhythms in EE, substrate oxidation (i.e., RQ and calculated fat and carbohydrate oxidation), subjective hunger, and appetite-related hormones (leptin, ghrelin, and PYY) over one circadian cycle. The constant routine protocol evenly distributes or holds constant environmental and behavioral factors that impact outcomes of interest (e.g., dim light, continuous wakefulness, ambient thermoneutral temperature, hourly snacks, posture, and activity levels [bedrest]) (Duffy and Dijk, 2002). Under these conditions, it is possible to determine whether a given metabolic or physiologic variable possesses a circadian rhythm.

Given the discrepant reports between rhythms in EE and substrate oxidation, we aimed to verify circadian rhythmicity in these variables. We hypothesized that circadian rhythms in both ghrelin and leptin would oscillate in parallel with subjective ratings of hunger and fullness, respectively. We further hypothesized that the satiety signal PYY would display a circadian rhythm, which has not been assessed previously.

Methods

<u>Participants</u>

   Eight healthy participants (four females/four males) aged 28.0 (2.3) years (mean [SD]), with a normal to overweight BMI of 24.3 (2.9) kg/m² and percent body fat of 30.0% (5.9%), completed the study protocol. Men were 27.5 (2.6) years old with a BMI of 25.4 (1.6) and percent body fat of 27.1% (5.0%), and women were 28.5 (1.9) years old with a BMI of 23.2 (3.2) and percent body fat of 33.0% (5.3%). Specific inclusion and exclusion criteria are provided in the Supporting Information. One female participant had to discontinue the inpatient protocol early because of nausea and was excluded from the present analyses. Therefore, data for seven participants are presented in this manuscript. All study procedures were approved by the scientific and advisory review committee of the Colorado Clinical and Translational Sciences Institute and the Colorado Multiple Institutional Review Board.

<u>Run-in period</u>

   Prior to admission to the Clinical Translational Research Center (CTRC), participants completed a 7-day outpatient run-in protocol with an 8-hour sleep schedule based on habitual sleep/wake times and verified by wrist actigraphy (Actiwatch-2; Philips Respironics, Murrysville, Pennsylvania), sleep logs, and text messages of bed and wake times to an online form. Drugs, medications, and nicotine were proscribed during the 7-day home assessment prior to CTRC admission for the inpatient protocol. For 3 days prior to the inpatient protocol, vigorous exercise, caffeine, and alcohol were proscribed, and participants consumed an energy-balanced diet provided by the CTRC Metabolic Kitchen (TDEE = resting metabolic rate [RMR] × an activity factor of 1.4). Macronutrient composition consisted of 30% fat, 55% carbohydrate, and 15% protein. To control for potential effects of prior meal timing on circadian phase of variables of interest, the 3-day outpatient diet meals were consumed at 1.5 hours, 5.5 hours, 10.5 hours, and 14.5 hours after awakening for breakfast (30% of TDEE), lunch (30% of TDEE), dinner (30% of TDEE), and an evening snack (10% of TDEE). All three female participants were

studied in the early follicular phase of the menstrual cycle (estradiol, 26.7 [2.9] pg/mL; progesterone, 0.6 [0.2] ng/mL).

Inpatient constant routine protocol

All protocol events were scheduled relative to habitual wake time as determined during the 7-day run-in phase for each individual. Participants were admitted to the inpatient CTRC approximately 4 hours prior to their habitual bedtime. Urine pregnancy tests confirmed female participants were not pregnant during the study. Light exposure was maintained at less than 5 lux during scheduled wake and 0 lux during scheduled sleep. Lighting in the room consisted of three main overhead fixtures each containing 3 m × 1.2 m-long, 28-watt fluorescent lamps in the 3,500 k spectrum. Each overhead light fixture was covered with diffusion filter paper and dimmed to its lowest setting to achieve < 5 lux measured from the participants angle of gaze. On night one, participants were provided a baseline 8-hour sleep opportunity. Immediately upon waking the following morning, an indwelling venous catheter was inserted into the antecubital space of the arm. During the constant routine, wakefulness was maintained for 26 hours in a semirecumbent posture (head of the bed raised to 35 degrees) in dim light as described above, and participants were provided hourly isocaloric snacks (matched to the macronutrient content of the run-in diet). The hourly meals consisted of either a custom liquid nutritional supplement or a solid food option (small sandwiches) prepared by the metabolic kitchen. Both options had the same macronutrient composition as the run-in diet. Participants picked one of these options and consumed it exclusively for the duration of the study period. Each snack represented 1/24th of the participants' TDEE. Participants were given ~100 mL water with each hourly snack. Individual energy requirements during the constant routine were calculated as RMR × an activity factor of 1.07 to account for the measured energy cost of 24 hours of waking bedrest (Jung et al., 2011). Wakefulness was confirmed by continuous monitoring by staff. After the completion of the constant routine, participants were given a recovery sleep opportunity (8-10 hours) prior to discharge.

Indirect calorimetry

Minute-by-minute EE and the RQ were measured every 3 hours starting 3 hours after waking using hood calorimetry (ParvoMedics TrueOne 2400 Metabolic Measurement System; Sandy, Utah). Recordings lasted between 10 and 20 minutes, with the first 5 minutes excluded from the analysis (see Supporting Information for additional details). EE was calculated using the Weir equation (Weir, 1949), and RQ was calculated as the ratio of volumes of CO_2 to O_2 (VCO_2/VCO_2). Rates of carbohydrate oxidation and fat oxidation were calculated as milligrams per minute according to the formula of Frayn (Frayn, 1983). Percent contribution of fat and carbohydrate to total oxidation was calculated as follows: percent carbohydrate oxidation = ([RQ − 0.71] / 0.29) × 100; percent fat oxidation = 100 − percent carbohydrate oxidation (Brooks, 2004).

Subjective hunger and satiety

Visual analog scale questionnaires obtained hourly assessed ratings of global hunger and fullness, desire to eat, subjective quantity of food that can be consumed, preoccupation with thoughts of food, desire for meat, desire for fruit, desire for dairy, desire for vegetables, desire for salty foods, and desire for sweet foods.

Blood and saliva sampling

Blood was sampled hourly from an indwelling catheter (~5 mL per draw) starting 1 hour after waking. Plasma was separated from whole blood after centrifugation and stored at −80°C until analysis. Plasma samples were assayed for ghrelin, leptin, and PYY. Saliva was collected every hour beginning immediately upon waking for melatonin. Assay details (kit manufacturer, coefficient of variation, and sensitivity values) are provided in Supporting Information Table S2.1.

Dim light melatonin onset

Salivary melatonin concentrations were assessed hourly beginning immediately after waking. Dim light melatonin onset (DLMO25%) was defined as the linearly interpolated point in

time at which melatonin levels reached 25% of the fitted peak-to-trough amplitude of individual

data, as determined by a three-harmonics least-squares regression analysis (Wright et al.,

2013b; Stothard et al., 2017).

<u>Analysis of circadian rhythmicity</u>

Because of increasing homeostatic sleep pressure over the course of the constant

routine protocol, many variables exhibit a positive linear trend over time (Shea et al., 2005).

Therefore, the linear trend was subtracted from each variable using least-squares regression to

produce a steady baseline without distorting the period or phase of the oscillations prior to any

analyses. Detrended data were then aligned to individual DLMO25% clock times and averaged

into 3-hour bins for EE and substrate oxidation and hourly for hunger ratings and appetitive

hormones. Aligning variables to DLMO25% produced an expected amount of missing data

given individual variability in DLMO25%. For example, a participant with an earlier DLMO25%

will have more available data points after DLMO25% than a participant with a later DLMO25%

time. As such, four participants were missing data from indirect calorimetry occurring + 9 hours

and/or + 12 hours after DLMO25%. To avoid excluding these participants, detrended data from

−12 and −9 hours prior to DLMO25% were used to replace the missing time points. This

assumption was based on visual inspection of the individual raw data, which suggested

rhythmicity in each measured variable (see Supporting Information). To test whether replacing

the missing data created bias for detecting rhythms, statistical analyses were run on the data

set with and without missing time points. Results between data sets were similar.

Data are presented as mean (SEM) unless otherwise indicated. All variables are plotted

in circadian degrees (1 hour = 15°) with DLMO25% set to 0° and a relative clock hour equal to

2000 hours. Variables are double plotted, with the average melatonin curve provided in grey to

improve visualization of rhythms and biological timing of peaks and troughs. However, analyses

were only performed on one circadian cycle during the constant routine. Linear mixed models

were used to evaluate changes in variables over time (SAS Institute Inc., Cary, North Carolina).

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To evaluate rhythmicity, each variable was converted to normalized units (i.e., percent difference from the mean), and statistical analyses were performed using linear harmonic regression (CircWave by R. Hut; The University of Groningen, Groningen, Netherlands). Using ANOVA, CircWave fits one or more sine waves (harmonics) to the data and compares this with a horizontal line through the data. An R^2 value is produced to describe goodness of fit (see Supporting Information for detail).

Results

Wake, sleep, and DLMO25%

Average self-selected habitual wake and bedtime during the run-in period occurred at 0606 hours (56 minutes) and 2152 hours (47 minutes), respectively. These sleep periods were used to calculate the habitual bedtime and wake time for the first inpatient night prior to the start of the constant routine (2201 hours [50 minutes] and 0559 hours [49 minutes], respectively). All study procedures were relative to the individual participant's habitual bed and wake times. During the constant routine, DLMO25% occurred at a clock time of 2003 hours (93 minutes). Average melatonin profiles are presented in Figures 2.1-2.4 as shaded gray areas.

Circadian variation in EE, RQ, and substrate oxidation

EE showed a significant effect of time (P = 0.03) (Table 2.1, Figure 2.1A), but the CircWave curve fit at the group level was not significant (P = 0.17) (Table 2.1, Figure 2.1B), with only one out of seven participants showing rhythmicity in EE (Supporting Information Table S2.2, Supporting Information Figure S2.1A). In contrast, RQ showed both a significant effect of time (P = 0.007) (Table 2.1, Figure 2.1C) and a significant CircWave curve fit at the group level, with a sine wave explaining 29% of the variance in RQ during the constant routine (P < 0.001) (Table 2.1, Figure 2.1D). The fitted peak of the group-level rhythm in the RQ occurred at 177° (~0750 hours), with a peak to nadir amplitude of 4.4%. At the individual level, RQ was rhythmic in three of seven participants (Supporting Information Table S2.2, Supporting Information Figure S2.1B).

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Fat oxidation displayed a significant effect of time (P < 0.001) (Table 2.1, Figure 2.2A) and a significant CircWave curve fit at the group level, with a sine wave explaining 23% of the total variance in fat oxidation during the protocol (P < 0.001) (Table 2.1, Figure2. 2B). The fitted peak in the fat oxidation rhythm occurred at 7° (at a similar time as the DLMO) with an 18.1% peak to nadir amplitude. At the individual level, fat oxidation was rhythmic in three of seven participants (Supporting Information Table S2.2, Supporting Information Figure S2.1C).

Carbohydrate oxidation also demonstrated a significant effect of time (P = 0.006) (Table 2.1, Figure 2.2C) and a significant CircWave group-level fit, explaining 25% of the variance in the data (P < 0.001) (Table 2.1, Figure 2.2D). The fitted peak in the carbohydrate oxidation rhythm occurred at 174° (~0730 hours) with a 28.8% peak to nadir amplitude. Like fat oxidation, three of seven participants showed a significant rhythm in carbohydrate oxidation at the individual level (Supporting Information Table S2.2, Supporting Information Figure S2.1D).

For reference, we also present the percent contribution of fat and carbohydrate oxidation to total substrate oxidation in Figures 2.2D and 2.2E, respectively. On average, fat contributed 58.5% (2.1%) to total oxidation, whereas carbohydrate contributed 41.5% (2.1%) to total oxidation during the constant routine (Table 2.1).

Circadian variation in subjective hunger and fullness ratings

Subjective hunger showed a significant effect of time (P = 0.04) (Table 2.1, Figure 2.3A) and a significant CircWave fit consisting of two harmonics (i.e., two separate peaks; P = 0.001) (Table 2.1, Figure 2.3B). This contrasts with the variables reported above that were best described by a single harmonic. Twelve percent of the variance in the hunger rhythm was explained by the CircWave analysis, with the larger fitted peak in hunger occurring at 257° (~1300 hours) and a smaller peak occurring during the biological night (76°, ~0100 hours). At the individual level, four of the participants demonstrated a rhythm in hunger (Supporting Information Table S2.2, Supporting Information Figure S2.2A). Ratings of fullness did not vary

by time (P = 0.082) (Table 2.1, Figure 2.3C) or rhythmic pattern (P = 0.49) (Table 2.1, Figure 2.3D).

Additional ratings of subjective hunger and desire for certain food groups are presented in Supporting Information Table S2.3 and Supporting Information Figure S2.3. Desire to eat, preoccupation with thoughts of food, desire for meat, desire for fruit, desire for dairy, desire for vegetables, desire for salty foods, and desire for sweet foods all showed a significant CircWave fit (P < 0.006) (Supporting Information Table S2.3). Fitted peaks for ratings of desire to eat and preoccupation with thoughts of food aligned with the peak of overall hunger, whereas the other ratings peaked later in the biological evening (Supporting Information Table S2.3 for statistics; Supporting Information Figures S2.3A-S2.3I for the plots).

Circadian variation in appetite hormones

Ghrelin, the only known orexigenic hormone, demonstrated a significant effect of time (P < 0.001) (Table 2.1, Figure 2.4A) and a significant CircWave fit at the group level explaining 16% of the variance in the data (P < 0.001) (Table 2.1, Figure 2.4B). The fitted peak of the ghrelin rhythm was at 349° (~1910 hours) with an 8.6% peak to nadir amplitude. At the individual level, ghrelin was rhythmic in three of seven participants (Supporting Information Table S2.2, Supporting Information Figure S2.4A). Leptin, an anorexigenic hormone, did not vary by time (P = 0.41) (Table 2.1) or fit a rhythmic profile, although the CirWave fit was a nonsignificant trend (P = 0.06) (Table 2.1, Figure 2.4D). PYY, also an anorexigenic hormone, varied by time (P = 0.01) (Table 2.1, Figure 2.4E) and had a significant rhythm as determined by CircWave (P < 0.002) (Table 2.1, Figure 2.4F). The fitted peak of the PYY rhythms was at 178° (~0750 hours) with a 15.6% peak to nadir amplitude. Five of seven participants demonstrated a rhythm in PYY (Supporting Information Table S2.2, Supporting Information Figure S2.4C).

Discussion

In the present study, we observed circadian variation in RQ, fat and carbohydrate oxidation, hunger ratings, ghrelin, and PYY in a sample of healthy adults under constant routine

conditions. Despite significant rhythms in these variables at the group level, we observed considerable individual variability. Inconsistent with previous reports (Shea et al., 2005; Zitting et al., 2018), we did not observe rhythms in EE or leptin. Our data suggest the possibly that drivers of energy balance operate in anticipation of feed-fasting cycles to optimize metabolic efficiency over 24 hours. Eating or sleeping out of phase with endogenous rhythms in regulators of energy balance may play a role in the link between circadian misalignment and obesity (McHill et al., 2014).

Findings from two previous constant routine studies have identified a rhythm in EE (Krauchi and Wirz-Justice, 1994; Spengler et al., 2000). Spengler et al. (Spengler et al., 2000) studied 10 adult males under constant behavioral and environmental conditions over 41 hours of wakefulness and measured VO2, VCO2, and ventilation via indirect calorimetry every 2 hours. Kräuchi et al. (Krauchi and Wirz-Justice, 1994) performed a 30.5-hour constant routine protocol in seven men with indirect calorimetry measurements occurring hourly. Spengler et al. (Spengler et al., 2000) demonstrated a significant rhythm in EE peaking in the biological morning, whereas Kräuchi et al. (Krauchi and Wirz-Justice, 1994) showed a more complex circadian pattern in EE with two peaks, a small peak slightly preceding the core body temperature minimum and a larger peak in the biological morning. Kräuchi et al. (Krauchi and Wirz-Justice, 1994) also noted significant interindividual variability in EE rhythms but did not present individual-level data.

Findings from our study are also inconsistent with findings from a forced desynchrony study (Zitting et al., 2018) that reported a significant circadian rhythm in fasting EE with a peak in the biological afternoon/evening and a trough near the core body temperature minimum, with a peak-to-trough amplitude of ± 110 kcal. A clear distinction between the forced desynchrony protocol and the constant routine protocol used in the present study (and those of Spengler et al. and Kräuchi et al.) (Krauchi and Wirz-Justice, 1994; Spengler et al., 2000) is the food intake/fasting state. In the current protocol, participants were not fasting; thus, the measure of

EE was composed of both resting EE plus the EE produced by diet-induced thermogenesis induced by the hourly snacks. Diet-induced thermogenesis is the most challenging component of EE to measure independently, and to assess its potential circadian rhythm would require near continuous measurement of EE during a constant routine (i.e., using a whole room calorimeter). More frequent as well as longer duration measures of EE (as was done in the Kräuchi et al. study) (Krauchi and Wirz-Justice, 1994) might be required to capture a rhythm in EE under fed conditions.

In agreement with Zitting et al. (Zitting et al., 2018), but in disagreement with two prior constant routine studies (Krauchi and Wirz-Justice, 1994; Spiegel et al., 2004), we observed a rhythm in the RQ, with higher RQ during the biological morning/day compared with the evening. Our results (and those of Zitting et al.) (Zitting et al., 2018) suggest that carbohydrate oxidation is highest during the biological morning (~12 hours prior to DLMO25%) and lowest during the evening (near the DLMO25%). In contrast, fat oxidation is antiphase (highest during the biological evening and lowest during the biological morning). Our finding of a circadian rhythm in fat oxidation with a peak near the DLMO25% is consistent with the anticipatory nature of the circadian system, which would be primed to oxidize fat during a nocturnal fast under normal sleep/wake and fasting/food intake conditions (Wright et al., 2005; Wright et al., 2013b; Stothard et al., 2017). These data are also consistent with previous reports of higher circulating free fatty acid concentrations in plasma and higher fat oxidation during the biological night compared with the day (Broussard et al., 2015a; Rynders et al., 2018; Rynders et al., 2020). It should be noted that melatonin itself has been implicated in body weight regulation and lipid metabolism in animal models, and it remains possible that rhythms in substrate oxidation are driven by the circadian melatonin rhythm (Rasmussen et al., 1999; Wolden-Hanson et al., 2000; Puchalski et al., 2003; Raskind et al., 2007; Liu et al., 2019).

We observed a rhythm in subjective hunger with a larger peak during the early afternoon and a smaller peak during the biological night (~3 hours after the DLMO). These data are in

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contrast to a forced desynchrony study by Scheer et al. (Scheer et al., 2013), who reported a peak in hunger at 230° (~2200 hours relative time) and a trough at 50° (~0800 hours relative time). Hunger oscillated with a 17% peak-to-trough amplitude in the study by Scheer et al. (Scheer et al., 2013) compared with ~28% in the current study. Results from another forced desynchrony study by McHill et al. (McHill et al., 2018) produced similar results as Scheer et al. (Scheer et al., 2013) and showed a peak in subjective hunger at 240° and a nadir at 60°. One possible explanation for the differences in the phase of subjective hunger between our study and the results of Sheer et al. (Scheer et al., 2013) and McHill et al. (McHill et al., 2018) may be related to differences in food intake and sleep between constant routine and forced desynchrony protocols.

We observed a circadian rhythm in ghrelin during the constant routine with peak levels prior to DLMO25% and a trough ~12 hours later. Results from a prior study in which 33 young adults were fasted for 12 to 48 hours with blood sampling every 3 hours showed a marked diurnal rhythm in serum ghrelin with a nadir in the morning, peak levels in the afternoon, and a gradual decline during the night (Espelund et al., 2005; Natalucci et al., 2005; LeSauter et al., 2009). This pattern in ghrelin secretion was observed despite lack of meal stimuli, suggesting ghrelin plays a role in the anticipation of EI. A circadian rhythm in fasting ghrelin has also been reported under the conditions of forced desynchrony (McHill et al., 2018). Because ghrelin is an orexigenic hormone, we were surprised to find such a large difference between the peak timing of ghrelin and hunger. This is in contrast to findings from forced desynchrony studies in which peaks in hunger ratings and fasting ghrelin colocalized to the biological evening (Scheer et al., 2013; McHill et al., 2018). Again, differences may be due to protocol selection (constant routine vs. forced desynchrony).

Leptin follows a diurnal rhythm under food intake-fasting conditions with a nadir in the mid-afternoon and peak during the evening/sleep (Sinha et al., 1996; Scheer et al., 2009; Nguyen and Wright, 2010; Scheer et al., 2010; Markwald et al., 2013; McHill et al., 2014; Qian

et al., 2019b). However, findings are conflicting as to whether leptin varies with circadian phase (Shea et al., 2005; Scheer et al., 2009; McHill et al., 2018). In the present study, we did not observe a rhythm in leptin, which contrasts with previously published findings using a constant routine protocol (Shea et al., 2005). During a 37-hour constant routine, Shea et al. (Shea et al., 2005) reported a significant rhythm in leptin, with a peak during the biological night (close to the core body temperature minimum) and a peak-to-trough amplitude of 16%. Analysis of data from individual participants from that study revealed the presence of circadian rhythms in leptin in four of the six participants as assessed using cosinor analysis. In the present study, we observed significant circadian rhythms in leptin in three of seven participants (Supporting Information Tables S2.1-S2.2), but the group level fit was not significant. Our results are also at odds with previous forced desynchrony studies that found significant rhythms in fasting concentrations of leptin (Scheer et al., 2009; McHill et al., 2018). While available evidence has suggested a small circadian component to leptin, the hormone appears to be largely driven by sleep/wake and fasting/food intake (Scheer et al., 2009; Nguyen and Wright, 2010; McHill et al., 2014).

PYY is considered a satiety hormone secreted primarily from L cells in the distal gastrointestinal tract and is involved in macronutrient absorption. Circulating PYY concentrations are lower in adults with obesity compared with those who are lean (Pfluger et al., 2007) and are increased in "obesity-resistant" individuals in response to overfeeding (Halliday et al., 2020). Like daily patterns of ghrelin and leptin, PYY predominantly follows the sleep-wakefulness fasting-feeding cycle (McHill et al., 2014); however, our findings also reveal a circadian component. The PYY rhythm peaked in the biological morning (~12 hours prior to DLMO25%). This is in opposition to our hypothesis in which we predicted PYY to peak during the biological night (parallel with the nadir of the hunger rhythm). Given the role of the circadian system in sleep consolidation, we thought that a circadian drive in PYY (as well as leptin) during the biological night would support fasting across the sleep period. While this may be happening

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to some extent (i.e., PYY levels are increasing across the biological night in the current study), we observed the peak in PYY in the biological morning. Although a morning peak in PYY is consistent with Scheer et al. (Scheer et al., 2013), who reported a nadir in hunger at ~0800 hours in forced desynchrony conditions, the apparent dissociation between the phase of PYY and hunger in a constant routine requires further study.

One limitation of the present study is the small sample size. However, findings from previous studies have reported significant circadian rhythms in outcome variables using similar sample sizes. Given the highly controlled nature of the constant routine protocol, circadian rhythms can be detected using these smaller sample sizes. Another limitation is the relatively short duration of our constant routine protocol, which resulted in missing time points when data were aligned to individual DLMO25%. In addition, the current paper did not compare the amplitude of the circadian rhythms to the magnitude of diurnal patterns. This comparison would have allowed us to comment on the strength of the circadian drive versus the influence of behavior and environment. Finally, as we did not measure protein metabolism (e.g., by collecting 24-hour urinary nitrogen), we were not able to adjust our respirometry measures for protein metabolism; thus, observed oscillations in carbohydrate and fat oxidation may be influenced to some extent by oscillations in protein oxidation.

Conclusion

Using a constant routine protocol, we provide evidence of circadian rhythms in fat and carbohydrate oxidation under nonfasted conditions. We also provide evidence of circadian rhythms in the hunger and satiety hormones ghrelin and PYY. Fluctuations in nutrient oxidation, ghrelin, PYY, and appetite are thought to respond primarily to nutrient ingestion. However, the present results suggest the endogenous circadian system also plays an important role, which may have important implications for regulation of body weight and energy metabolism.

Figures and tables
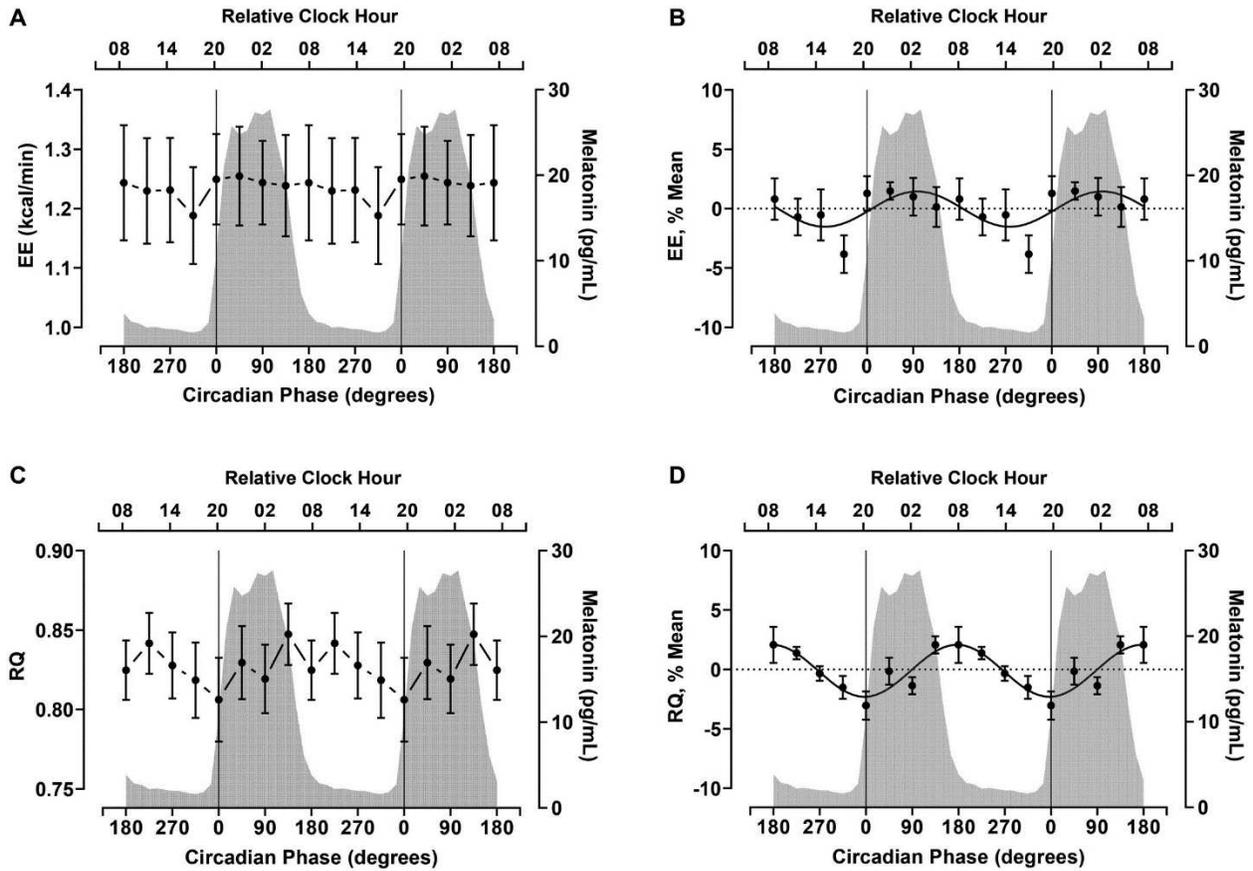
Figure 2.1. Circadian variation of energy expenditure and respiratory quotient during constant routine conditions. Data are double plotted for visualization of circadian rhythms. Circadian phase, 0° = dim light melatonin onset; shaded area indicates average salivary melatonin curve. EE, energy expenditure; RQ, respiratory quotient (ratio of carbon dioxide production to oxygen consumption).

Figure 2.2. Circadian variation in fat and carbohydrate oxidation during constant routine conditions. Data are double plotted for visualization of circadian rhythms. Circadian phase, 0° = dim light melatonin onset; shaded area indicates average salivary melatonin curve. CHO, carbohydrate; Ox, oxidation.

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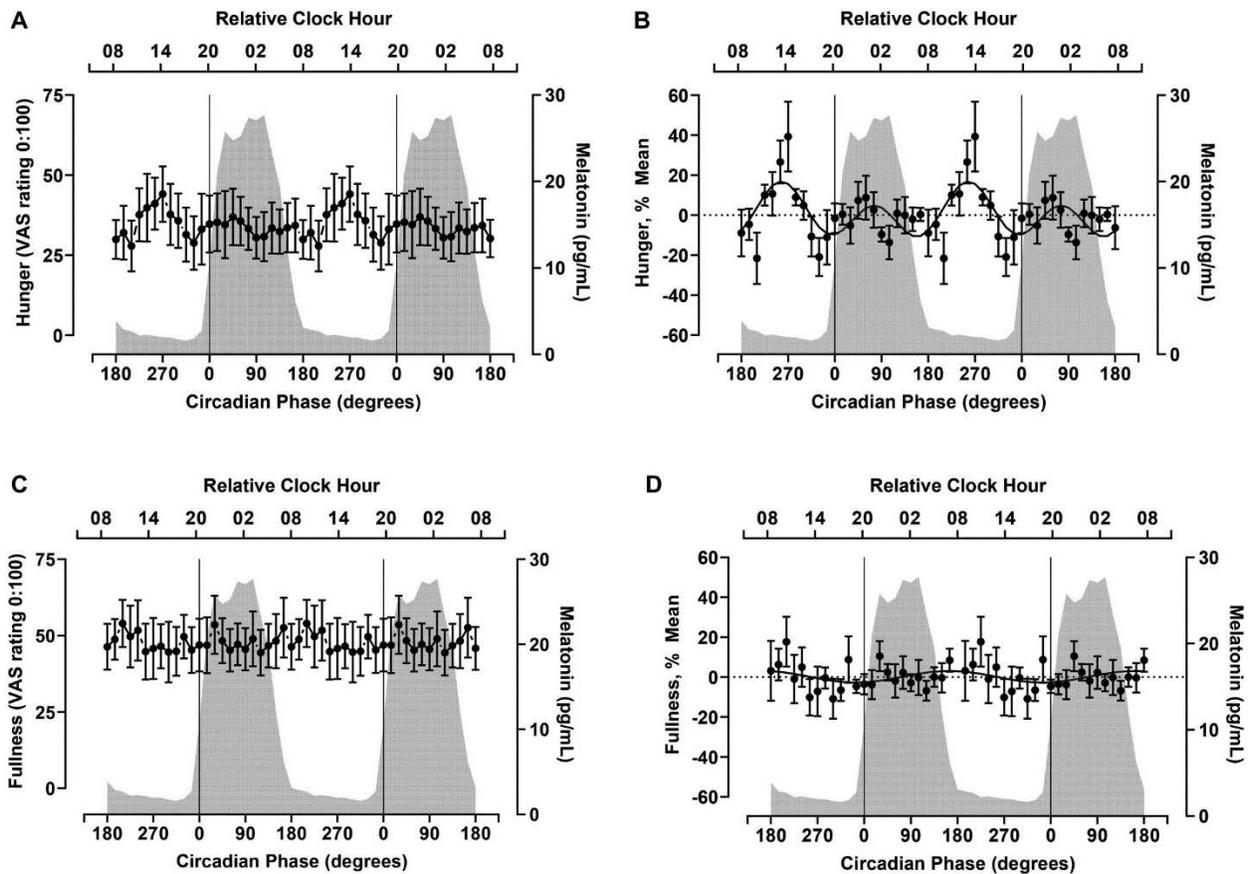
Figure 2.3. Circadian variation in subjective appetite ratings during constant routine conditions. Data are double plotted for visualization of circadian rhythms. Circadian phase, 0° = dim light melatonin onset; shaded area indicates average salivary melatonin curve. VAS, visual analog scale.
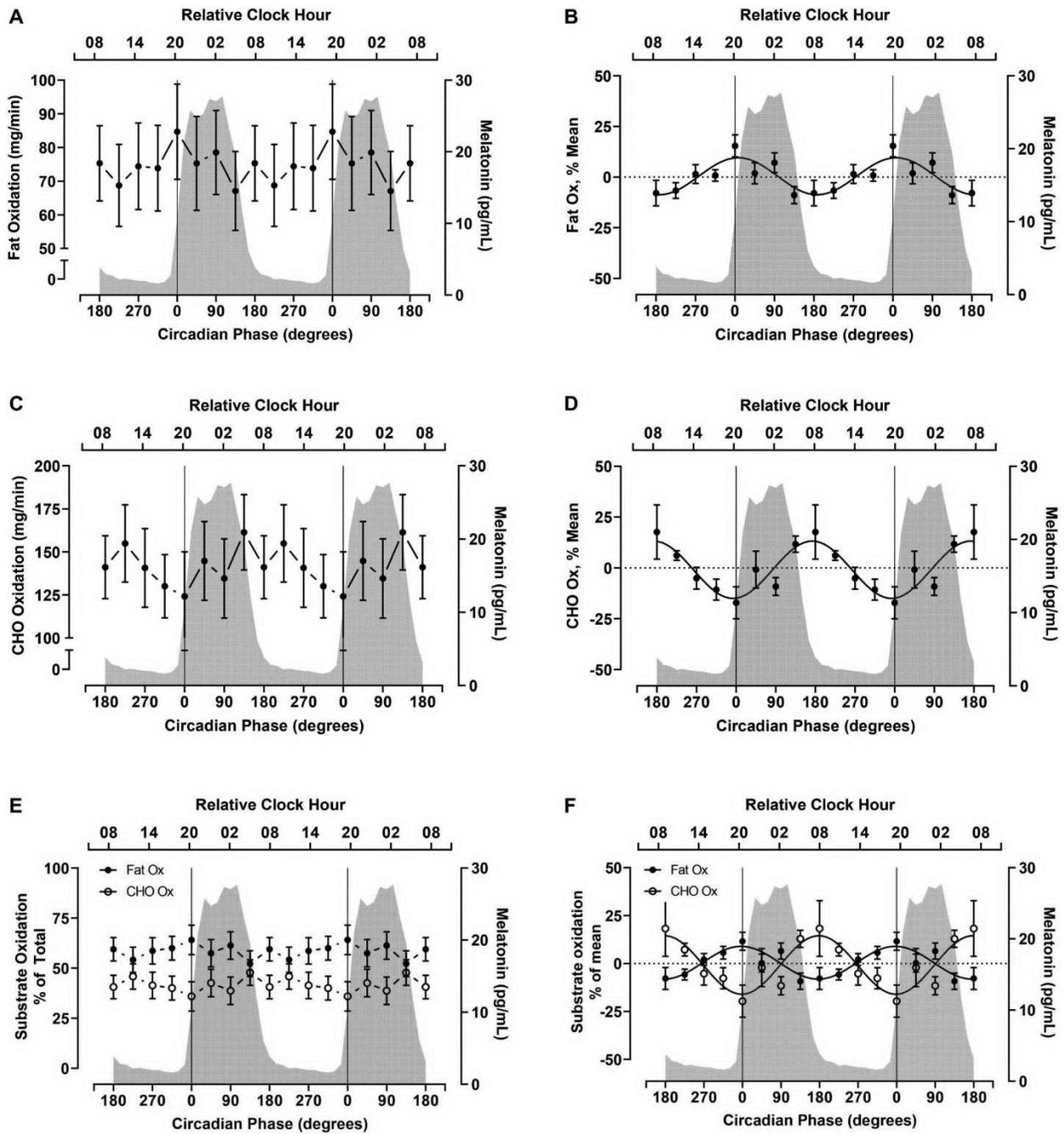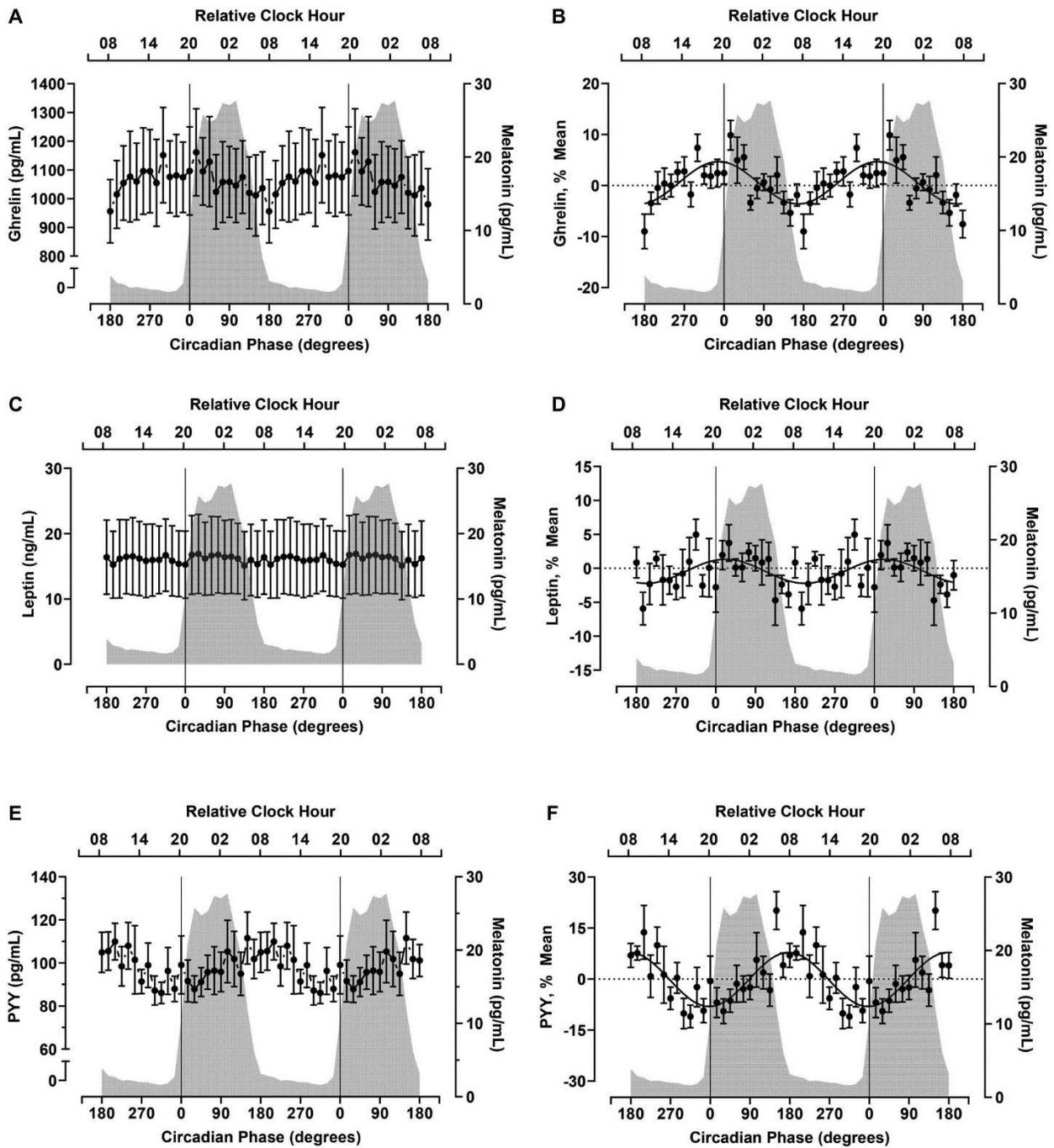
Figure 2.4. Circadian variation in appetite hormones during constant routine conditions. Data are double plotted for visualization of circadian rhythms. Circadian phase, 0° = dim light melatonin onset; shaded area indicates average salivary melatonin curve. PYY, peptide tyrosine (Y) tyrosine (Y).

TABLE 2.1. Circadian rhythmicity evaluated by CircWave

Variable	Data mean (SEM)	LMM (P)	Peak phase degrees (rel. clock hour)	Peak-to nadir-amplitude	ANOVA: F statistic (P)	CircWave: R^2 (P)
Indirect calorimetry						
EE, kcal/min	1.24 (0.03)	0.53	--	--	1.08 (0.40)	0.06 (0.17)
RQ	0.83 (0.007)	0.005	177° (~0750 h)	4.4%	3.54 (<0.001)	0.29 (<0.001)
Fat ox, mg/min	74.8 (4.0)	0.01	7° (~2030 h)	18.1%	2.92 (0.009)	0.23 (<0.001)
CHO ox, mg/min	141.4 (7.1)	0.006	174° (~0730 h)	28.8%	2.89 (0.01)	0.25 (<0.001)
Fat ox, % of total	58.5 (2.1)	0.02	357° (~1950 h)	17.0%	3.12 (0.006)	0.25 (<0.001)
CHO ox, % of total	41.5 (2.1)	0.02	178° (~0750 h)	30.3%	2.76 (0.01)	0.24 (<0.001)
VAS						
Hunger, 0-100 scale[a]	34.0 (1.5)	0.04	257° (~1300 h)	27.6%	2.03 (0.006)	0.12 (0.001)
Fullness, 0-100 scale	48.1 (1.5)	0.82	--	--	0.57 (0.10)	0.01 (0.49)
Hormones						
Ghrelin, pg/mL	1,063.4 (25.6)	<0.001	349° (~1910 h)	8.6%	2.74 (<0.001)	0.16 (<0.001)
Leptin, ng/mL	16.1 (1.0)	0.41	--	--	0.99 (0.48)	0.03 (0.06)
PYY, pg/mL	97.9 (1.9)	0.01	178° (~0750 h)	15.6%	2.20 (0.002)	0.15 (<0.001)

[a]Linear harmonic regression performed by CircWave determined that the profile for hunger was best fit by two sinusoidal waves. All other variables were described by a single sine wave.
"--" indicates that the sine wave fit was not significantly different from a horizontal line.
EE, energy expenditure; RQ, respiratory quotient; fat ox, fat oxidation; CHO ox, carbohydrate oxidation; VAS, visual analog scale; LMM, linear mixed model effect of time; Rel. clock, relative clock time.

INTRODUCTION TO METHODS OF MEASURING WHOLE BODY AND TISSUE SPECIFIC
INSULIN SENSITIVITY

Metabolic physiology

Insulin is the primary hormone responsible for lowering blood glucose concentrations. Fasting insulin levels in a healthy adult are less than 10mU/mL. Insulin moves glucose out of circulation and limits input into circulation. When a meal is consumed, typically comprised of carbohydrates, fats, and protein, glucose is digested from these food products and absorbed into the bloodstream. Constitutively expressed GLUT2 transporters on the pancreas receive a high flow of glucose and respond by releasing insulin. Insulin binds to the alpha subunits of the insulin receptor on the outside of the cell and initiates the insulin signaling cascade. The insulin receptor autophosphorylates at tyrosine residues which allows insulin receptor substrate 1 (IRS-1) to bind to the insulin receptor, and is in turn phosphorylated. This allows for the binding of p85 subunit of phosphoinositide 3-kinase (PI3K) and recruits its catalytic subunit P110 to activate PI3K. PI3K turns phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3, 4, 5- triphosphate (PIP3). This activates phosphoinositide-dependent protein kinase (PDK) and protein kinase B (PKB or AKT). Phosphorylation of AKT subunit 160 (AS160) allows the translocation of GLUT4 vesicles into the membrane allowing more glucose to flow into the cell (Petersen and Shulman, 2018). Overall, insulin binding to its receptor allows for more glucose to be brought into the cell, ultimately lowering blood glucose concentrations. GLUT4 transporters are insulin dependent transporters and are only found in skeletal muscle, white adipose tissue, and cardiomyocytes (Shepherd and Kahn, 1999). Glucose homeostasis is tightly regulated; and while many physiological pathways have built-in back up pathways, there is no back-up secondary to insulin. Thus, when insulin action fails, there are no other pathways or mechanisms to overcome this failure.

Insulin sensitivity

Insulin sensitivity is a measure of how well cells respond to insulin and is primarily a function of these post-receptor signaling events described above. Insulin sensitive tissues are desirable as they require little insulin to complete the job of lowering blood glucose. Tissues with impaired insulin sensitivity may require more insulin to bring in the same amount of glucose. This compensated state which encompasses euglycemia but hyperinsulinemia adds burden to the beta cells of the pancreas. Eventually, if a constantly elevated load of insulin on all cells is sustained, impaired insulin sensitivity may occur.

After eating a meal comprised of glucose, fat, and protein, the pancreas releases insulin, which acts on the three main metabolic tissues: white adipose tissue, liver, and skeletal muscle.

White adipose tissue

The role of insulin on white adipose tissue is to increase fuel storage through glucose uptake and increased lipogenesis and decrease fuel utilization through lipolysis. Insulin binds to the insulin receptor and acts via PI3K-dependent and PI3K-independent pathways to increase GLUT4 vesicle fusion, GLUT4 insertion, and glucose uptake into the cell. PI3K simultaneously decreases lipolysis mainly through perilipin and hormone sensitive lipase. Additionally, AKT can activate SREBP1, which increases lipogenesis (Petersen and Shulman, 2018). Overall, insulin binding to the insulin receptor on white adipose tissue increases glucose uptake, increases lipogenesis, and decreases lipolysis.

Liver

In the liver, insulin acts to increase fuel storage and decrease fuel production. For example, insulin binding to its receptor initiates the same initial cascade described above; however, AKT has a crucial role in subsequent molecular pathways. AKT can inhibit phosphorylase kinase and GSK3α and β, which both independently act on glycogen synthase to increase glycogen synthesis. Glucose transport through GLUT2 receptors can be converted to glucose-6-phosphate through glucokinase, which can also increase glycogen synthase and

therefore glycogen synthesis. Additionally, through mTORC1, SREBP1, and FO9XO1 transcription, gluconeogenesis is inhibited and de novo lipogenesis is increased (Petersen and Shulman, 2018). Overall, insulin action in the liver acts to increases glycogen synthesis, decreases gluconeogenesis, and increases de novo lipogenesis which together increase fuel storage and decrease fuel production.

<u>Skeletal muscle</u>

Activation of the insulin receptor on skeletal myocytes has two main functions: glucose uptake and glycogen synthesis. When insulin binds and initiates insulin signaling, GLUT4 storage vesicles translocate into the plasma membrane which allows glucose to enter the cell. Upon entering the cell, glucose is converted to glucose-6-phosphate (G6P) by hexokinase. Since G6P cannot bind to GLUT proteins, G6P is unable to diffuse out of the cell. G6P ultimately traps glucose in the cell where it is needed. G6P inside the cell can increase the phosphorylation of glycogen synthase which increases glycogen synthesis. G6P also inhibits glycogen phosphorylase which inhibits glycogen synthesis, thereby increasing glycogen synthesis. Additionally, AKT can inhibit GSK3α and β which inhibits the phosphorylation of glycogen synthase thereby increasing glycogen synthesis (Petersen and Shulman, 2018). Ultimately, insulin binding to the skeletal muscle receptors increases glucose uptake and increases glycogen synthesis.

<u>Metabolic assessments</u>

Insulin sensitivity is an important measure in clinical and research settings. However, there are many different methods to measure insulin sensitivity. These various methods allow for metabolic testing in various scenarios, however, there are benefits and limitations to each.

<u>Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)</u>

Fasting measurements can be used to estimate whole body insulin sensitivity. A single fasting blood sample is obtained and glucose and insulin are measured. Some outcomes include fasting plasma glucose, HOMA-IR, and QUICKI, which can all be calculated with one

fasting glucose and insulin value. Fasting plasma glucose values less than 100mg/dL suggest normal blood glucose concentrations, whereas 100-125mg/dL represents a prediabetes range and values 126mg/dl or higher suggest diabetes. While a fasting plasma glucose test only measures glucose, if fasting glucose and fasting insulin are measured, other calculations can be made to estimate insulin sensitivity. One calculation is the homeostatic model assessment of insulin resistance (HOMA-IR) which is calculated using fasting glucose and insulin levels to estimate insulin resistance.

HOMA-IR= (fasting glucose mmol/L x fasting insulin mL/L) / 22.5 (Matthews et al., 1985)

Qualitative insulin sensitivity check index (QUICKI) is derived using the inverse of the sum of the logarithms of the fasting insulin and fasting glucose samples.

QUICKI = 1 / (log(fasting insulin µU/mL) + log(fasting glucose mg/dL)) (Katz et al., 2000)

Using this equation, a lower QUICKI score represents a worse insulin sensitivity. While the QUICKI is a measure of insulin sensitivity, HOMA-IR is an estimate of insulin resistance so a higher HOMA-IR means more insulin resistant. These fasting calculations are beneficial for studies with a large number of subjects as they are simple, inexpensive, and only require a single blood sample. Fasting values and calculations are the easiest to measure; however, fasting values are not dynamic and primarily reflect beta cell function.

Oral Glucose Tolerance Test (OGTT)

An oral glucose tolerance test (OGTT) can be used to estimate whole body insulin sensitivity (Bang, 1913). An intravenous catheter is inserted for blood collection. After baseline blood samples are obtained, a 75g or 100g glucose drink is consumed and blood is collected every ~30 minutes for 2-3 hours. Glucose and insulin concentration will be measured in the

blood samples. Some outcomes include the Matsuda Insulin Sensitivity Index, 2h glucose

level/tolerance, glucose and insulin area under the curve, mean glucose, time to peak glucose,

and outcomes from the oral minimal model. The 2-hour time point is typically the "tolerance"

point with normal vales being below 140 mg/dL, prediabetes or impaired glucose tolerance is

140-199 mg/dL, and diabetes is categorized as over 200mg/dL. Additionally, we can use the

Matsuda Insulin Sensitivity Index as a measure of whole body insulin sensitivity.

Matsuda ISI = 1000 / sqrt [($Insulin_0$ x $Glucose_0$) x ($Insulin_{mean}$ x $Glucose_{mean}$)] (Matsuda

and DeFronzo, 1999)

A Matsuda ISI represents better insulin sensitivity and a value equal to or lower than 2.5

represents insulin resistance. OGTTs have been validated by the gold-standard technique for

measuring insulin sensitivity and are better than simple fasting values because it requires a

challenge to the system (Matsuda and DeFronzo, 1999). OGTTs are relatively simple and

inexpensive, are physiological tests due to the oral glucose consumption, and require rather

simple calculations. However, an OGTT requires trained medical staff for insertion of an

intravenous catheter for frequent blood sampling. Limitations of the OGTT are that neither

glucose nor insulin is constant and changes in absorption may mask changes in glucose

tolerance.

Intravenous Glucose Tolerance Test (IVGTT)

An intravenous glucose tolerance test (IVGTT) is an accurate measure of whole body

insulin sensitivity (Crawford, 1938; Bergman et al., 1979). This test consists of frequent blood

sampling for glucose and insulin during a one-time glucose and subsequent insulin infusion.

Glucose and insulin are infused directly into an intravenous catheter so the gastrointestinal

system is bypassed during this test creating a faster plasma glucose level rise and a smaller

glucose dose needed. However, the insulin dose used is approximately 400mI/L, which is a

supra-physiological level. The IVGTT is highly correlated with the gold standard method of measuring insulin sensitivity and provides additional measures of metabolic function making it a useful clinical and research tool (Beard et al., 1986).

An intravenous catheter is inserted for blood collection and frequent blood samples are collected for 3 hours (T=0, 2, 4, 6, 8, 10, 20, 22, 25, 30, 40, 50, 70, 100, 120, 180 min). A glucose bolus injection is given at T=0 min and an insulin bolus injection is given at T=22 min. The IVGTT can be broken down into four distinct phases. The first phase is immediately after the glucose infusion at time=0min and consists of mixing the injected glucose into the blood. Phase 2 occurs after mixing and is the glucose-mediated glucose disposal due to the increase in endogenous insulin which creates a steady glucose decline. Phase 2 is a measure of beta cell responsiveness. After the insulin injection at T=22 min, phase 3 is the insulin-mediated glucose disposal where there is a faster decline in glucose and is used to measure insulin sensitivity. Finally, Phase 4 is the recovery period when glucose levels trough and then rebound to basal levels based on liver production of glucose (Ader, 1997).

The benefit of the IVGTT is the amount of information the test provides in addition to insulin sensitivity. The primary outcomes obtained from the IVGTT are first-phase insulin release (AIRg), insulin sensitivity (Si), glucose effectiveness (Sg), and Disposition Index (DI). AIRg is the acute insulin response to glucose and is a measure of the beta cell responsiveness to glucose in the first 10 minutes of the test. Therefore, AIRg is the first-phase insulin release in response to the initial glucose bolus and is ultimately a measure of beta cell function. Insulin sensitivity (Si) is a measure of insulin's ability to lower blood glucose concentrations. Glucose effectiveness (Sg) is a measure of glucose effectiveness and is a measure of glucose's ability to stimulate its own uptake independent of insulin. This occurs during Phase 2 of the IVGTT after the glucose injection but before the insulin injection. Finally, DI is calculated as DI = Si x AIRg. DI is a measure of the ability of the pancreatic islet cells to secrete insulin normalized to the

degree of insulin resistance. Using the Bergman Minimal Model of Glucose Kinetics, these parameters of glucose homeostasis can be calculated in addition to insulin sensitivity.

The IVGTT is considered a reference technique for insulin sensitivity and provides an estimate of beta cell function and glucose effectiveness, which are more parameters than can be calculated with any other metabolic test. The IVGTT is relatively short in duration; however, it requires trained staff to perform intensive blood sampling. An IVGTT is non-physiological due to the intravenous injections of glucose and insulin. The IVGTT is more expensive than an OGTT and there are more potential problems with an IVGTT due to the more frequent blood draws. Additionally, the minimal model analyses require specific software which limit the public availability of this technique.

Hyperinsulinemic euglycemic clamp

The gold-standard method for investigating and quantifying insulin sensitivity is the hyperinsulinemic euglycemic clamp because it measures the amount of glucose necessary to compensate for an increased insulin level without causing hypoglycemia (DeFronzo et al., 1979). During a clamp, hyperinsulinemia is induced using a fixed high insulin dose. This would then result in hypoglycemia in healthy participants if the plasma glucose concentration is not replaced with glucose infusion. Thus, glucose is checked every 5 minutes using a standard glucometer or YSI and glucose is infused at a variable rate throughout the assessment in order to maintain euglycemia, usually 90mg/dL. After 2-3 hours steady-state conditions of plasma glucose concentration are obtained. During this steady state, the glucose infused is equivalent to the glucose being removed from the blood and, provided that endogenous glucose production by the liver is completely suppressed, this is an accurate measure of insulin sensitivity. Thus, people who are highly insulin sensitive will require more glucose to be infused and therefore a higher glucose infusion rate represents higher insulin sensitivity. Using only a high dose of insulin and no isotope tracers, the glucose infusion rate, M, from the clamp is a measure of whole body insulin sensitivity. One unique aspect about using a clamp technique is that it can

provide tissue-specific insulin sensitivity given multi-stage variable insulin doses and isotopically labeled tracers.

After eating a meal comprised of glucose, fat, and protein, the pancreas releases insulin which acts on the three main metabolic tissues: white adipose tissue, liver, and skeletal muscle. In healthy human white adipose tissue, insulin will suppress lipolysis. When insulin does not suppress lipolysis as much, this represents impaired white adipose tissue insulin sensitivity as more insulin is required to do the same job. White adipose tissue has GLUT4 receptors which allow for insulin-dependent glucose uptake. White adipose tissue is the most sensitive to insulin requiring the lowest insulin dose to begin to see the effects of insulin on white adipose tissue.

Unlike the white adipose tissue and skeletal muscle, the liver does not depend on insulin for glucose uptake. The liver has constitutively expressed GLUT2 transporters which allow for insulin-independent glucose uptake. Insulin in the liver stimulates hepatic glucose uptake whereas an insulin resistant liver will suppress hepatic glucose uptake. The liver is less insulin sensitive than white adipose tissue and therefore requires a greater insulin dose to see the liver-specific effects of insulin.

Skeletal muscle is responsible for ~80% postprandial glucose uptake in humans (Defronzo et al., 1981). Since skeletal muscle is the largest glucose user, a measure of whole body insulin sensitivity is primarily a measure of skeletal muscle insulin sensitivity. Glucose uptake in skeletal muscle is insulin-dependent as GLUT4s are the primary glucose transporter. In skeletal muscle, insulin stimulates glucose uptake, whereas insulin resistant muscle has suppressed glucose uptake. Skeletal muscle is the least insulin sensitivity which means it requires the highest insulin dose in order to see the effects of insulin.

By harnessing the tissue-specific metabolic physiology outlined above, stable or radioactive isotope tracers can be used to enhance the clamp technique. Using a 3-stage clamp protocol with isotope tracers, an 8-hour test will be conducted with the final 30 minutes of each stage consisting of steady-state sample collection. [6,6-^{2}H^2] Glucose and [1,1,2,3,3-^{2}H$_5$]

Glycerol can be infused for 2 hours before the beginning of the clamp. Then a 4mU/m^2/min insulin dose will be infused for 2 hours to measure white adipose tissue insulin sensitivity. An 8-20mU/m^2/min insulin dose will then be infused for 2 hours to measure liver insulin sensitivity. Finally, a 40mU/m^2/min insulin dose will be infused for 2 hours to measure skeletal muscle insulin sensitivity. Baseline and steady-state insulin-stimulated blood samples will be collected during each stage. Using mass spectrometry, the amount of glucose and/or glycerol being endogenously produced in the system can be measured and compared to the amount infused to calculate isotope enrichment. When using a 3-stage hyperinsulinemic euglycemic clamp with isotope tracers, whole body and tissue-specific insulin sensitivity can be measured.

The clamp technique requires trained staff to perform intensive blood sampling and constant adjustments during the test. Similar to the IVGTT, the clamps is non-physiological due to the intravenous injections of glucose and insulin thereby bypassing the role of the gastrointestinal system and incretins. The clamp can be very long in duration and rather expensive. Moreover, there are many possible methods to the clamp which makes the outcomes difficult to compare between studies. However, the clamp is the only technique in which tissue-specific insulin sensitivity can be distinguished. Using multi-stages consisting of increasing insulin doses and isotopic tracers, white adipose tissue, liver, and skeletal muscle insulin sensitivity can be calculated.

Summary

In conclusion, insulin is a vital hormone required for glucose uptake. While the tissue-specific effects of insulin vary, the overall role of insulin is to increase fuel storage and decrease fuel utilization. Thus, while the three main metabolic tissues, white adipose tissue, liver, and skeletal muscle, have tissue-specific molecular responses to insulin, they work together to achieve the same overall goals. Using the various methods of measuring insulin sensitivity in humans, we can estimate whole body and tissue-specific insulin sensitivity using fasting samples, OGTT, IVGTT, and the hyperinsulinemic euglycemic clamp. While each technique has

benefits and limitations, appropriately utilizing each technique can help us gain a better

understanding how insulin sensitivity and metabolic physiology are impacted by various

pathologies and interventions.

Table 3.1. Metabolic parameters obtained using Bergman's Minimal Model during an
intravenous glucose tolerance test

Metabolic parameter	Description	Calculation
Acute phase insulin release to glucose (AIRg)	First-phase insulin release in response to initial glucose bolus	Bergman's Minimal Model Analysis
Insulin sensitivity (Si)	Measure of insulin's ability to lower blood glucose concentrations	Bergman's Minimal Model Analysis
Glucose effectiveness (Sg)	Measure of glucose's ability to simulate its own uptake independent of insulin	Bergman's Minimal Model Analysis
Disposition Index (DI)	Measure of the ability of pancreatic beta cells to secrete insulin normalized to the degree of insulin resistance	DI = AIRg * Si

Table 3.2. Insulin doses and calculations for measuring whole body and tissue-specific insulin
sensitivity with the hyperinsulinemic euglycemic clamp.

	Insulin dose $(mU/m^2/min)$	Insulin sensitivity calculation
White adipose tissue	4	Ra (glycerol)
Liver	8	Ra (glucose)
Skeletal Muscle	40	Rd (glucose)
Whole body	high	M, Glucose Infusion Rate (GIR)

CHAPTER IV – MANUSCRIPT II
CONSIDERATIONS FOR METABOLIC ESTIMATES IN PEOPLE WITH SLEEP AND
CIRCADIAN DISRUPTION

Summary

To assess the complex interactions between insulin secretion, insulin clearance, and the action of insulin to accelerate glucose disappearance and inhibit endogenous glucose production, a variety of tests using physiologically based models of glucose utilization and insulin kinetics can be implemented. Common examples of such metabolic tests include the oral glucose tolerance test (OGTT), intravenous glucose tolerance test (IVGTT), and hyperinsulinemic euglycemic clamp. Each test is typically performed in the morning in a clinical laboratory setting following a specific set of guidelines given to participants. For example, participants are instructed not to exercise for the preceding 24-hours and to arrive at the laboratory following an overnight fast.

In striking contrast, assessments of sleep and circadian behaviors are rarely conducted, let alone controlled for prior to metabolic testing, even though sleep and circadian disruption can have profound and acute impacts on metabolic variables of interest. For example, sleep and circadian disruption are both significant and independent risk factors for type 2 diabetes. Even a single night of insufficient sleep can impair insulin sensitivity and glucose tolerance. Circadian misalignment is common in people who work non-standard hours, including evening, night, or rotating shifts, and is associated with increased fasting glucose and insulin concentrations, as well as impaired insulin sensitivity. Finally, many sleep disorders, including obstructive sleep apnea, insomnia, periodic limb movement disorders, and narcolepsy, are characterized by short or fragmented sleep, which have been shown in laboratory settings to independently impair insulin sensitivity.

In this article, we present data supporting the relationship between sleep and circadian disruption and impaired metabolic homeostasis. We then discuss considerations for conducting

metabolic investigations in people with sleep and circadian disruption. We conclude by encouraging clinical investigators to collect information on, and control for, sleep and circadian history, sleep disorders, and habitual timing of behaviors, in addition to diet and exercise in participants undergoing metabolic testing.

Introduction

The National Sleep Foundation recommends adults sleep 7-9 hours per day (Hirshkowitz et al., 2015); however, the average sleep duration of American adults has decreased from 8.8 hours to 6.8 hours over the last century with 1 in 3 people report sleeping fewer than 6.5 hours per night during the work week (Terman, 1913b; a; Tune, 1968; 1969; 2000; Hirshkowitz et al., 2015). Further, people who sleep fewer than 6 hours per night have a three-fold higher risk of impaired fasting glucose than those sleeping at least 8 hours per night (Rafalson et al., 2010). Finally, results from a recent meta-analysis reported various forms of insufficient sleep (e.g. sleep duration ≤5 hours, sleep disorders), are considered risk factors for type 2 diabetes (T2D) with similar odds ratios as traditional risk factors, including overweight, family history of diabetes, and physical inactivity (Anothaisintawee et al., 2016).

In addition to insufficient sleep, circadian misalignment, resulting from a mismatch between behavioral and circadian physiology (i.e., being awake and eating during the biological night when the circadian system is anticipating sleep and fasting), is associated with an increased risk for obesity and T2D. For example, people who work non-standard hours, including evening, night, or rotating shifts are more likely to develop T2D (Suwazono et al., 2006; Oberlinner et al., 2009; Kivimaki et al., 2011; Pan et al., 2011; Vyas et al., 2012; Monk and Buysse, 2013; Vetter et al., 2018) and metabolic syndrome (Biggi et al., 2008; Canuto et al., 2013; Leproult et al., 2014; Almoosawi et al., 2019) compared to people who work during the day. Circadian misalignment can also occur as a consequence of insufficient sleep (Markwald et al., 2013). With more than 35% of adults reporting insufficient amounts of sleep, chronic and

acute circadian misalignment are likely even more prevalent than commonly recognized (2011; Centers for Disease and Prevention, 2011).

Sleep disorders are highly prevalent in modern society and are associated with metabolic detriments including impaired insulin sensitivity (Foster et al., 2009; Papanas et al., 2009; Ronksley et al., 2009; Aronsohn et al., 2010; Knutson et al., 2011; Schober et al., 2011). For example, obstructive sleep apnea (OSA) affects approximately 936 million adults globally (Arredondo et al., 2021) and includes complete or partial airway obstruction during sleep, resulting in frequent arousals, reduced sleep quality, daytime sleepiness, and decreased cognitive performance (Maspero et al., 2015). In people with obesity and T2D, the prevalence of OSA has been reported as high as 87% (Foster et al., 2009). Further, OSA is associated with T2D severity, independent of obesity (Papanas et al., 2009; Ronksley et al., 2009; Aronsohn et al., 2010; Schober et al., 2011) and even in young, lean, healthy men, OSA was associated with insulin resistance (Pamidi et al., 2012). Other sleep disorders, including insomnia, narcolepsy, and periodic limb movement disorder, are also associated with insufficient sleep and an increased risk for obesity and T2D (Schuld et al., 2000; Lopes et al., 2005; Merlino et al., 2007; Cuellar and Ratcliffe, 2008; Papanas et al., 2009; Ronksley et al., 2009; Vgontzas et al., 2009; Aronsohn et al., 2010; Knutson et al., 2011; Rizzi et al., 2011; Schober et al., 2011).

Sleep and circadian disruption play an important role in metabolic health, and yet are rarely considered in the context of clinical metabolic testing, either for diagnostic or research purposes. Therefore, we will briefly review the literature regarding the impact of sleep and circadian disruption, including sleep disorders, on insulin sensitivity and metabolic disease risk, and discuss specific sleep and circadian considerations that can be incorporated into metabolic testing protocols to account for the known effects of sleep and circadian disruption on metabolic outcomes.

Impact of insufficient sleep on insulin sensitivity

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) is calculated from fasting glucose and insulin levels, with a higher HOMA-IR indicating worse insulin sensitivity. In highly controlled, inpatient studies of young, healthy, lean adults, insufficient sleep consistently impairs whole-body insulin sensitivity as assessed by HOMA-IR in many studies (Spiegel et al., 2004; Reynolds et al., 2012; Killick et al., 2015; Cedernaes et al., 2016; Cedernaes et al., 2018) but not all (Bosy-Westphal et al., 2008). In contrast, outpatient studies of insufficient sleep in healthy volunteers (Robertson et al., 2013; Wang et al., 2016) as well as older participants (Zielinski et al., 2008) have not observed changes in HOMA-IR. Finally, HOMA-IR was not different between long versus short sleepers (Hart et al., 2015).

Although HOMA-IR calculations are simple and easy to obtain from a single blood sample, major limitations to HOMA-IR exist. HOMA-IR is calculated from fasting glucose and insulin, but insulin resistance often develops in the absence of elevated glycemia (Weir and Bonner-Weir, 2004). Ader et al. reveal serious shortcomings of HOMA-IR to detect confirmed development of insulin resistance when they identified that there were no significant relationships between either clamp- or IVGTT minimal model-based measures of baseline insulin sensitivity and corresponding HOMA-IR (Ader et al., 2014). Additionally, HOMA-IR detected insulin resistance in animals where clamps revealed normal insulin sensitivity; and HOMA-IR accuracy was dependent on the magnitude of the insulin secretory response. When beta cell function was robust, as measured by an acute insulin response to glucose (AIRG) above the mean, HOMA-IR yielded an accurate estimate of insulin sensitivity. However, the limitations of HOMA-IR were exposed in subjects that displayed a lesser insulin secretory response (Ader et al., 2014). HOMA-IR accuracy is weakest under conditions where insulin secretory function is in the normal range, but less robust. The relationship between HOMA-IR may more represent signals for beta cell function, as HOMA-IR may reflect islet cell function or

metabolic clearance of insulin, rather than insulin resistance itself (Bergman et al., 2003). Thus, HOMA-IR should be viewed with skepticism if beta cell function is not known a priori in all individuals.

Oral Glucose Tolerance Test (OGTT)

The Matsuda Insulin Sensitivity Index calculated from the OGTT provides an estimate of whole-body insulin sensitivity in response to an oral glucose load. Insulin sensitivity as estimated by the OGTT is associated with sleep quality. For example, lighter sleep (i.e. less slow wave and more Stage 1 sleep) was associated with lower insulin sensitivity in adolescents (Armitage et al., 2013). Furthermore, insufficient sleep impairs whole body insulin sensitivity, as measured by the Matsuda Insulin Sensitivity Index in tightly controlled inpatient studies of young, healthy, lean adults (Eckel et al., 2015; Cedernaes et al., 2018), as well as in outpatient studies (Killick et al., 2015; Wang et al., 2016; Sweeney et al., 2020). Sleep disruption, achieved by experimental slow wave sleep (SWS) suppression without any reduction in sleep duration, also results in a lower Matsuda Insulin Sensitivity Index (Herzog et al., 2013).

Furthermore, the Oral Minimal Model Method can simultaneously measure insulin sensitivity, beta cell responsivity, and hepatic insulin extraction from an OGTT (Cobelli et al., 2014). While the Oral Minimal Model is less frequently utilized in the sleep and circadian fields, studies reveal impaired insulin sensitivity after sleep and circadian disruption, similar to the results found using the Matsuda Insulin Sensitivity Index. For example, one study demonstrated that circadian misalignment reduced glucose tolerance mainly by lowering insulin sensitivity and not by affecting beta cell function (Qian et al., 2018). While the Oral Minimal Model provides more metabolic parameters in addition to insulin sensitivity (i.e., beta cell responsivity and hepatic insulin extraction), the protocol duration may impact the results and should be considered when designing a study (Bartlette et al., 2021).

Taken together, the OGTT is an ideal method to assess the response to glucose in a physiological manner given its oral administration. Moreover, it provides a systemic challenge

that can provide a functional outcome compared to fasting measures only. However, limitations to the OGTT exist, including the use of a standard glucose load (typically 75g) regardless of body weight, thus the g/kg of glucose delivered is variable between participants. Furthermore, the OGTT does not allow for distinction between tissue-specific contributions. Finally, changes in intestinal absorption may impact glucose tolerance. Investigators must consider the benefits and limitations of using the Matsuda Insulin Sensitivity Index or the Oral Minimal Model when utilizing an OGTT.

Intravenous Glucose Tolerance Test (IVGTT)

The IVGTT is used to assess whole-body insulin sensitivity and other metabolic parameters in response to a venous glucose injection. During an IVGTT, a bolus of glucose is infused in under 2 minutes, followed 20 minutes later by an injection of exogenous insulin. Glucose and insulin are assayed from frequently sampled blood and analyzed using Bergman's Minimal Model of Glucose Regulation (Bergman et al., 1979). In addition to insulin sensitivity, Minimal Model analyses of glucose and insulin results in the following parameters: acute insulin response to glucose (AIRg, the insulin area under the curve from Time 0-19), glucose effectiveness (Sg, the ability of glucose per se to stimulate its own uptake and to suppress its own production independent of insulin concentration), and the Disposition Index (DI, the product of SI and AIRg, often considered an integrated marker of beta cell function for a given insulin sensitivity) (Bergman et al., 1979; Bergman, 2020). Glucose tolerance (kg) can also be derived from the IVGTT and is calculated as the absolute value of the slope of the natural log of glucose from 5-19 minutes, resulting in a measure of glucose clearance.

The majority of investigations on the impact of insufficient sleep on insulin sensitivity have utilized the IVGTT, including the study that launched the field of sleep and metabolism (Spiegel et al., 1999; Leproult and Van Cauter, 2010). In highly controlled inpatient studies that followed, insufficient sleep (4.5-5 hours per night for approximately one week) consistently impaired insulin sensitivity in young, healthy, lean adults (Buxton et al., 2010; Broussard et al.,

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2012; Broussard et al., 2015b; Eckel et al., 2015; Broussard et al., 2016; Ness et al., 2019), as well as in people with pre-existing risk factors for metabolic disease including middle-age and overweight (Nedeltcheva et al., 2009). In one study using self-reported sleep, sleep duration was positively correlated with insulin sensitivity and the highest percentage of individuals with impaired insulin sensitivity reported a sleep duration <6h (Wong et al., 2015).

In addition to insufficient sleep, sleep disruption designed to reduce slow wave sleep, the deepest stage of sleep and often considered to be the most physically restorative, is also associated with reduced insulin sensitivity as assessed by the IVGTT (Tasali et al., 2008a; Stamatakis and Punjabi, 2010).

The chief consideration of the IVGTT is that it does not mimic consumption of a meal because glucose and insulin are injected instead of consumed orally and are provided at supra-physiological doses. Furthermore, the glucose infused during the IVGTT bypasses the gut, thereby eliminating the impact of the incretin system on insulin sensitivity and glucose clearance that would normally be present following a meal or glucose ingestion. Additionally, the IVGTT must be conducted by highly trained staff and analyzed using the Minimal Model (MINMOD Millennium) (Pacini and Bergman, 1986).

Hyperinsulinemic euglycemic clamp

The hyperinsulinemic euglycemic clamp is typically used to distinguish between whole-body and peripheral tissue insulin sensitivity using a fixed single or multi-dose hyperinsulinemic infusion, frequent blood sampling, and a variable glucose infusion designed to maintain euglycemia at 90 mg/dL. Incorporation of various insulin doses and isotopically labeled tracers allows for distinct estimates of tissue-specific insulin sensitivity of adipose, hepatic (liver), and skeletal muscle tissues.

Use of the hyperinsulinemic euglycemic clamp in rigorously controlled, inpatient studies of healthy, lean, young and middle aged adults has revealed that insufficient sleep results in reductions in whole-body (Buxton et al., 2010; Donga et al., 2010b; Rao et al., 2015; Depner et

al., 2019), as well as skeletal muscle-specific insulin sensitivity (Donga et al., 2010b; Rao et al., 2015; Depner et al., 2019), which is consistent with *ex vivo* alterations in skeletal muscle tissue following insufficient sleep (Cedernaes et al., 2015; Cedernaes et al., 2018; Mateus Brandao et al., 2021). The impact of insufficient sleep on hepatic and adipose tissue insulin sensitivity is less clear. Results from one study reported impaired hepatic insulin sensitivity following insufficient sleep (Donga et al., 2010b), whereas two studies reported no change (Rao et al., 2015; Depner et al., 2019), possibly due to differences in insulin doses used between studies. To date, only one study has implemented the hyperinsulinemic euglycemic clamp to investigate the impact of insufficient sleep on adipose tissue insulin sensitivity *in vivo* and found no impairment (Depner et al., 2019). In contrast, *ex vivo* alterations in adipose tissue have been observed following insufficient sleep in humans (Broussard et al., 2012; Cedernaes et al., 2018).

In people with pre-existing metabolic impairments such as type 1 diabetes, insufficient sleep for a single night is associated with impaired skeletal muscle insulin sensitivity compared to a night of sufficient sleep (Donga et al., 2010a; van den Berg et al., 2016). Furthermore, when insufficient sleep is implemented for one week in an outpatient setting, whole-body insulin sensitivity assessed using the clamp is impaired as compared to control participants who maintained habitual sleep duration (Robertson et al., 2013).

Although the hyperinsulinemic euglycemic clamp is considered the gold-standard method for assessing whole-body and tissue-specific insulin sensitivity, it is not without its limitations. Given the constant infusions of glucose, insulin, and isotopes, the assessment of insulin sensitivity is conducted under non-physiological conditions and bypass the gut, similar to an IVGTT. To assess insulin sensitivity across multiple tissues also requires the use of additional infusates (isotopically labeled tracers), which increases the duration, cost and participant burden associated with the study. Finally, successful implementation of the clamp requires highly trained staff to monitor the test and make constant adjustments to glucose infusion rates.

Impact of circadian misalignment on insulin sensitivity

Shift workers account for 20-25% of the total work force in industrialized countries (2010). Compared to day workers, shift workers have a higher risk of developing several chronic medical conditions such as obesity (Di Lorenzo et al., 2003; Ishizaki et al., 2004); diabetes (Kroenke et al., 2007; Pan et al., 2011; Guo et al., 2013; Gan et al., 2015); cardiovascular disease (Knutsson et al., 1986; Knutsson, 2003; Tuchsen et al., 2006; Scheer et al., 2009), metabolic syndrome (Biggi et al., 2008; Spiegel et al., 2009; Cappuccio et al., 2010; Knutson, 2010; Cappuccio et al., 2011; Canuto et al., 2013; Almoosawi et al., 2019), gastro-intestinal diseases (Knutsson, 2003), various types of cancer (Stevens and Rea, 2001; Davis and Mirick, 2006; Schernhammer et al., 2006; Stevens et al., 2007; Straif et al., 2007; Savvidis and Koutsilieris, 2012; Grundy et al., 2013), menstrual irregularities and dysmenorrhea (Labyak et al., 2002), pregnancy problems (Zhu et al., 2004), and psychological disorders (Shields, 2002). Additionally, epidemiologic studies suggest that shift work is an independent risk factor for type 2 diabetes (Suwazono et al., 2006; Oberlinner et al., 2009; Kivimaki et al., 2011; Pan et al., 2011; Vyas et al., 2012; Monk and Buysse, 2013).

Shift workers often attempt to sleep during the daytime when the circadian system is promoting wakefulness, leading to shorter and more fragmented sleep. Indeed, night shift workers report significantly poorer sleep quality, longer sleep latency, shorter sleep duration, sleep disturbances, and daytime dysfunction (Lim et al., 2018). Shift workers also take longer to fall asleep and experience more wakefulness after sleep onset (Chang and Peng, 2021). Stage 2 and rapid eye movement (REM) sleep are both reduced during daytime sleep opportunities following a night shift (Torsvall et al., 1989). It is estimated that 10-38% of shift workers develop Shift Work Disorder (SWD), which is defined as excessive sleepiness or insomnia accompanied by a reduction of total sleep time (Drake et al., 2004; Flo et al., 2012; Wright et al., 2013a). SWD increases the risk for obesity and diabetes (van Amelsvoort et al., 1999; Karlsson et al., 2001; Di Lorenzo et al., 2003; Ishizaki et al., 2004; Guo et al., 2013; Depner et al., 2014). Moreover,

daytime sleep itself is associated with an increase in glucose levels, increase in insulin secretion, and a rise in serum insulin levels (Van Cauter et al., 1991). Thus, it could be argued that insufficient sleep mediates the relationship between shift work and metabolic disease development.

Acute circadian misalignment leads to impaired glucose metabolism in both rodents and humans (Hampton et al., 1996; Lund et al., 2001; Scheer et al., 2009; Fonken et al., 2010; Karatsoreos et al., 2011; Buxton et al., 2012; Qian et al., 2013; Salgado-Delgado et al., 2013; Leproult et al., 2014). For example, circadian misalignment due to simulated shiftwork conducted in controlled laboratory settings leads to an increase in glucose or insulin levels (Scheer et al., 2009; Buxton et al., 2012; Depner et al., 2014). Two in-laboratory studies in which healthy participants were exposed to circadian misalignment indicated reductions in total sleep time and sleep efficiency, increased glucose and insulin concentrations, and postprandial hyperglycemia (many even in the typical range of a prediabetes state), which provides supportive evidence for a deleterious effect on diabetes risk and cardiovascular function (Scheer et al., 2009; Buxton et al., 2012).

A small number of studies have investigated the effects of circadian misalignment on insulin sensitivity using the methods described above. In these tightly controlled, inpatient studies conducted in young, lean, healthy adults, circadian misalignment induced by simulated shiftwork or forced desynchrony protocols impair insulin sensitivity as assessed by OGTT (Qian et al., 2018), IVGTT (Leproult et al., 2014), and the hyperinsulinemic euglycemic clamp (Bescos et al., 2018; Wefers et al., 2018).

Impact of common sleep disorders on metabolic disease risk and severity

Sleep disorders such as Obstructive Sleep Apnea (OSA) and insomnia are fairly common and associated with increased risk for cardiovascular diseases, hypertension, heart failure, type 2 diabetes, and metabolic syndrome (Chen et al., 2015; Senaratna et al., 2016; Javaheri and Redline, 2017; Sokwalla et al., 2017; Larsson and Markus, 2019). These

associations may be due to direct effects of the specific sleep disorder itself, or indirectly via accompanying reductions in sleep duration and/or quality.

Obstructive Sleep Apnea (OSA)

The prevalence of the respiratory sleep disorder OSA in the general population is 22% (9-37%) in men and 17% (4-50%) in women (Franklin and Lindberg, 2015). OSA is defined as an apnea-hypopnea index (AHI) ≥5 (1999). AHI is the mean number of apneas (cessation of breathing) and hypopneas (shallow breathing) and number of respiratory-related arousals that occur per hour of sleep (1999; 2014). Apneas typically last between 10-30 seconds but can extend to minutes (1999) and often result in reduced arterial oxygen saturation and frequent arousals from sleep. Symptoms of OSA include reduced or cessation of airflow during sleep resulting in loud snoring or gasping for air during sleep, daytime sleepiness and fatigue.

Age, male sex, and higher BMI are all associated with OSA disease risk (Senaratna et al., 2017a). Indeed, OSA prevalence has been reported to be as high as 87% in people with obesity and diabetes (Foster et al., 2009), suggesting that a high percentage of participants in metabolic research studies likely present with comorbidities associated with OSA. Furthermore, OSA severity is independently associated with poorer glucose control (Pamidi et al., 2010), as well as higher fasting glucose and insulin, 2h glucose, and HOMA (Tasali et al., 2008b). Additionally, overweight men with OSA have impaired glucose tolerance and insulin sensitivity compared to overweight men without OSA (Kamble et al., 2020). Even in young, lean, healthy men free of cardiometabolic disease, OSA is associated with insulin resistance compared to age and BMI-matched controls (Pamidi et al., 2012). Thus, it is imperative that researchers determine whether potential participants have diagnosed or undiagnosed OSA. Treatment status may also be important, as treatment of OSA with continuous positive airway pressure (CPAP) may lead to improvement in insulin sensitivity, HbA1c, and other components of metabolic syndrome (Morgenstern et al., 2014). For example, CPAP treatment is associated

with improvements in HbA1c in patients who maintain high compliance to CPAP use (Steiropoulos et al., 2009).

Although findings from intervention studies suggest improvements in OSA following weight loss (Peppard et al., 2000; Dixon et al., 2005), it is not clear whether other interventions to improve insulin sensitivity such as exercise or pharmacologic interventions that do not result in weight loss impact OSA. It is also unknown whether pre-existing OSA will limit the therapeutic potential of insulin sensitizing interventions, leading to heterogeneity in outcomes from clinical studies. It is therefore critical to collect information and potentially control for OSA status in potential metabolic research participants.

Insomnia

In the general population, the prevalence of insomnia ranges from 8-40% (Ohayon, 2002; Morin et al., 2006; Vgontzas et al., 2013). Insomnia is defined as a difficulty initiating or maintaining sleep, or earlier awakening than desired, with associated daytime functioning complaints (e.g. fatigue, daytime sleepiness, cognitive impairment, accidents, mood or behavior problems, dissatisfaction with sleep) that occur 3 or more times per week, for 3 or more months (2014). Patients with both insomnia and diabetes display increased fasting glucose, insulin, and HOMA-IR (Knutson et al., 2011). Furthermore, patients with insomnia and sleep durations of ≤5 hours per night have the highest odds ratio of developing type 2 diabetes compared to people without insomnia and a sleep duration ≥6 hours (Vgontzas et al., 2009).

Other less common sleep disorders have been linked to increased risk for diabetes and obesity. For example, patients with narcolepsy have higher BMIs compared to the general population (Schuld et al., 2000), and Periodic Limb Movement Disorder (PLMD) and Restless Leg Syndrome (RLS) are common in patients with diabetes (Lopes et al., 2005; Merlino et al., 2007; Cuellar and Ratcliffe, 2008; Rizzi et al., 2011). Thus, it is important to identify and control for sleep disorders including OSA, insomnia, narcolepsy, PLMD, and RSL prior to metabolic testing in research study participants and clinical assessments.

Implementing sleep and circadian measures in metabolic research

Acute and chronic sleep and circadian disruption impair metabolic homeostasis in a variety of settings including various age groups and BMI ranges. Furthermore, underlying sleep disorders significantly increase the risk for obesity, insulin resistance, and type 2 diabetes. Given such high prevalence of sleep and circadian disruption in the general population and particularly in people with metabolic disease, excluding participants with sleep and circadian disruption is not likely feasible—especially for studies seeking to investigate participants with pre-existing cardiometabolic impairments. Exclusion of participants with sleep and circadian disruption will likely eliminate nearly all potential participants and become a major barrier to research. Alternatively, sleep and circadian history can be collected prior to metabolic testing, similar to diet and exercise. The following presents various methods that can be implemented before metabolic assessments to more fully understand or control for prior sleep and circadian history.

Baseline sleep and circadian assessments:

Sleep disorder screening

Numerous sleep disorder questionnaires exist to help identify sleep disorders. For example, the Sleep Disorder Questionnaire (Douglass et al., 1994) asks a variety of questions related to general sleep habits and characteristics, bed and wake time, and previous sleep history. The Epworth Sleepiness Scale (Johns, 1991) asks about the likeliness of dozing off or falling asleep while doing 8 common daily activities. Scores greater than 11 represent increasing levels of excessive daytime sleepiness, which may suggest an underlying sleep abnormality or insufficient sleep duration or quality. The Morning Eveningness Questionnaire (Horne and Ostberg, 1976) is an assessment of chronotype (phenotype associated with the time one prefers to perform daily behaviors; morning bird vs night owl) and asks questions regarding the time of day one would "feel the best" while performing various activities such as going to bed and waking up, performing physical activity, etc. The Functional Outcomes of Sleep

Questionnaire (FOSQ) (Weaver et al., 1997) evaluates the level of difficulty associated with carrying out certain activities due to sleepiness. For example, the FOSQ includes questions regarding difficulty concentrating, remembering, working on a hobby, and how relationships, chores, and visiting friends and family are impacted by sleep habits. The Berlin Questionnaire (Netzer et al., 1999; Senaratna et al., 2017b) contains various questions related to snoring, daytime fatigue, high blood pressure, and breathing during sleep to assess the risk of sleep apnea. Similarly, the STOPBAG questionnaire (Chung et al., 2008; Chung et al., 2012) asks 4 subjective and 4 clinical yes or no questions regarding risk factors for OSA: snoring, tired, observed stopping breathing, high blood pressure, BMI, age, neck circumference, and male gender.

Questionnaires regarding sleep habits, hygiene, and potential sleep problems are easy to administer in clinical studies. However, polysomnography (PSG) is the gold-standard method for diagnosing sleep disorders. Studies in which participants stay in the laboratory overnight could consider implementing a PSG sleep disorder screen either as exclusionary criteria, reporting purposes, and/or to understand potential confounding effects of sleep disorders on metabolic outcomes. A trained sleep specialist must then score the PSG record for apneic events, leg movements, and other sleep disorders.

Sleep diaries

Self-reported sleep history can be collected from participants before metabolic assessments to better understand habitual bed and wake times during the previous week, month, or year. Additionally, history of shift work prior to a metabolic assessment may have lasting effects on the metabolic outcomes of interest.

Maintenance of a consistent sleep schedule prior to assessment

A consistent sleep schedule for 1-2 weeks is another potential consideration to implement prior to metabolic testing to understand if participants are sleep deprived or have a highly variable sleep schedule when they enter the study. Using sleep diaries and call-ins to a

time-stamped voicemail or online recorder can be used for accurate self-reported bed and wake time collection. A more objective measure of sleep timing is actigraphy, which can provide objective measures of bed and wake times, sleep duration, and light exposure.

Conducting metabolic tests relative to habitual wake time

It is standard practice in clinical research to initiate study protocols at a set clock time for every participant. For example, participants may be asked to arrive to the lab fasted by 7am for a metabolic assessment, for example, a hyperinsulinemic euglycemic clamp, which then begins at 8am. Although this is logistically easier for personnel, such as investigators and nurses, to coordinate study visits, using a set clock time for metabolic procedures likely introduces variability in the circadian timing of events across participants. For example, for someone who habitually wakes up at 6am, 7am may be a normal breakfast time, whereas 7am for someone who habitually wakes up at 8am constitutes the biological night and may impact outcomes such as insulin sensitivity. To schedule testing at similar circadian times for each participant, a procedure may start at 1 hour after habitual wake time, rather than at 7am. This will mean the clamp will begin at a different clock times for each participant, although the circadian time will remain consistent.

Similarly, if repeat testing is involved it should ideally be conducted at the same circadian time for each participant so that within subject comparisons of metabolic outcomes can be made without the confounding effects of different circadian times of assessment.

Conclusions

In summary, considerable evidence suggests that sleep and circadian disruption induce metabolic impairments both acutely and chronically, which can be observed with clinical metabolic tests such as the OGTT, IVGTT, and hyperinsulinemic euglycemic clamp. Furthermore, common sleep disorders such as OSA and insomnia are associated with reduced insulin sensitivity and increases risk for metabolic diseases, including obesity and diabetes. We therefore encourage clinical metabolic investigators to consider methods that can be

implemented to control for sleep and circadian factors, including collection of sleep and circadian history, sleep disorders, and habitual timing of behaviors, in addition to diet and exercise, in participants undergoing metabolic testing. In doing so, we can gain further understanding of the effects of sleep and circadian history on metabolic homeostasis.

CHAPTER V – MANUSCRIPT III
INSUFFICIENT SLEEP INDUCES MUSCLE LIPID ACCUMULATION AND INSULIN
RESISTANCE IN HEALTHY ADULTS

Summary

Insufficient sleep impairs insulin sensitivity; however, mechanisms by which this occurs

are unknown. In other contexts, such as obesity and type 2 diabetes, impaired insulin sensitivity

is associated with ectopic lipid deposition and reduced mitochondrial respiratory capacity, as

well as alterations in tissue-specific expression of genes involved in fuel utilization. It is unknown

whether insufficient sleep elicits these same alterations. We and others have shown elevated

nocturnal free fatty acid levels and altered expression of genes involved in fuel utilization during

insufficient sleep suggesting alterations in lipid metabolism may play a role in reduced insulin

sensitivity. Additionally, changes in mitochondrial respiration have been reported during sleep

and circadian disruption. However, it is not known whether insufficient sleep is associated with

lipid accumulation in skeletal muscle, and whether this is related to altered mitochondrial

function. Therefore, we sought to test the hypothesis that insufficient sleep induces lipid

accumulation and mitochondrial dysfunction, which will be related to impaired insulin sensitivity.

Eighteen young, healthy, lean adults (8F; 24.7±3.5y; 22.7±1.8kg/m^2; mean±SD)

participated in a 6-day in-laboratory protocol with 9h in bed (habitual sleep) followed by 4 nights

of 5h in bed (insufficient sleep). Morning melatonin offset was used to estimate the phase of the

central circadian clock. Insulin sensitivity was assessed using a hyperinsulinemic euglycemic

clamp and muscle biopsies of the vastus lateralis were taken and mitochondrial respiration,

quantitative lipidomic analyses, and RNA sequencing (Novogene Co., Ltd) were performed

before and after insufficient sleep.

Insufficient sleep was associated with a significant reduction in Insulin sensitivity

(9.77±1.24 v 8.77±0.94 mg/kg/min, $p < 0.05$, mean±SEM) in conjunction with increased species

of 1,2-DAGs, including Di-C18:0 ($p < 0.05$). Moreover, insufficient sleep altered skeletal muscle

RNA expression of genes involved in metabolic pathways, including a down-regulation of Lipoprotein Lipase (LPL). Additionally, 17 genes were correlated with impaired insulin sensitivity after 4 nights of 5 hours of sleep. Further, there were no changes in ADP-stimulated respiration (State 3) or maximally uncoupled respiration (State U) in permeabilized *ex vivo* skeletal muscle fibers. Overall, we report that 4 nights of insufficient sleep of 5 hours of sleep per night impaired insulin sensitivity, increased lipid accumulation in skeletal muscle, and altered RNA expression of genes involved in metabolic pathways.

<u>Introduction</u>

Insufficient sleep impairs whole-body and tissue-specific metabolic homeostasis. For example, in adipose tissue, insufficient sleep is associated with impaired insulin sensitivity (Broussard et al., 2012) and up-regulated KEGG pathways such as oxidative phosphorylation (Cedernaes et al., 2018). Furthermore, sleep and circadian disruption are associated with alterations in muscle-specific expression of genes involved in glycolysis (Cedernaes et al., 2018) and in lipid metabolism (Wefers et al., 2018). We and others have previously reported elevations in circulating FFA during insufficient sleep (Broussard et al., 2015a) and circadian disruption (Wefers et al., 2018), suggesting that elevated FFA and alterations in lipid metabolism may play a role in reduced insulin sensitivity in these contexts. Increased circulating FFA levels play a central role in the pathogenesis of insulin resistance and the development of metabolic diseases, and have been associated with muscle lipid accumulation (Roden et al., 1996; Boden et al., 2001; Boden, 2003; DeFronzo, 2004; Belfort et al., 2005; Morino et al., 2005). Furthermore, elevated lipids in muscle are associated with impaired insulin sensitivity (Hulver et al., 2003; Coen et al., 2010; Bergman et al., 2012; Petersen and Shulman, 2018). However, it is not known whether insufficient sleep is associated with lipid accumulation in skeletal muscle.

Elevated lipids in muscle are also associated with decreased mitochondrial respiratory capacity (Chavez et al., 2010). Decreased mitochondrial respiratory capacity in skeletal muscle

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is related to impaired insulin sensitivity and is present in individuals with obesity, type 2 diabetes, and offspring of individuals with type 2 diabetes (Simoneau et al., 1995; Kim et al., 2000; Kelley et al., 2002; Petersen et al., 2004; Phielix et al., 2008; Chomentowski et al., 2011; Fabbri et al., 2017). Furthermore, mitochondrial function is altered during sleep and circadian disruption in people (Wefers et al., 2018; Saner et al., 2021), mice (Zhao et al., 2016) and *Drosophila* (Rodrigues et al., 2018).

Therefore, the purpose of this study was to investigate the effects of 4 nights of insufficient sleep to 5h per night on skeletal muscle lipid accumulation, mitochondrial respiratory capacity, and insulin sensitivity. We hypothesized that insufficient sleep would lead to skeletal muscle lipid accumulation and reduced mitochondrial respiratory capacity, which would be related to impaired insulin sensitivity. To test this hypothesis, we obtained skeletal muscle biopsies from young, lean, healthy adults after habitual sleep and insufficient sleep to assess lipid accumulation and mitochondrial function. We also conducted hyperinsulinemic euglycemic clamps after habitual sleep and insufficient sleep to assess insulin sensitivity.

Methods

Participants

Eighteen healthy, young, sedentary, lean women and men (8F; 24.7±3.5y; 22.7±1.8kg/m^2; mean±SD) participated in the study. Inclusion criteria were healthy adults with no acute chronic diseases; habitual sleep duration <7 or >9.25 hours per night; BMI 18.5-24.9 kg/m^2; not currently participating in another medical research study involving medication or bloodwork; >6 months since participation in night work/rotating shift work; living within 1 time zone of MST for one month prior to the study; lived at altitude for at least 3 months prior to study; no visual or hearing impairment; no medical, sleep, psychological (depression, ADHD, schizophrenia, anxiety, personality disorder) disorders in participants or first-degree family members; BDI>13; BAI>10; ESS>10; no heavy alcohol use (>14 drinks/week or >5 drinks/day); no heavy caffeine (greater than 500 mg/day); no history of drug use or alcohol dependency and

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negative urine toxicology screen for drugs of abuse; not currently on prescription meds (birth control okay); no antidepressants, neuroleptic medications, major tranquilizers; no clinically significant medical or surgical condition in the past year; normal EKG; normal complete blood count and comprehensive metabolic panel (fasting plasma glucose concentration <100 mg/dL, HbA1c ≤5.6%, serum triglyceride concentration <150 mg/dL; and serum HDL-cholesterol concentration ≥40 mg/dL for men and ≥50 mg/dL for women); no history gynecological pathology; at least 1 year post-partum and not breast-feeding; not pregnant or currently thinking about getting pregnant; regular menstrual cycles.

Age criteria was 18-35, with 35 years as the upper age limit to avoid age-related changes in sleep architecture that begin in middle-age (Carrier et al., 2011). Ovulating women were selected on the basis of a history of regular menstrual cycle ranging in length from 25-32 days with a maximum of seven days variation month-to-month. Since insulin sensitivity is recognized to fluctuate over the course of the menstrual cycle, all attempts were made to study women during the early follicular phase as determined by self-reported menstrual cycle logs.

All procedures were approved by the Scientific Advisory Review Committee (SARC), the Colorado Multiple Institutional Review Board (COMIRB), and the Colorado State University Institutional Review Board.

Outpatient monitoring

For one week prior to inpatient admission, participants maintained a 9h sleep schedule based on habitual sleep/wake times in order to dissipate any sleep debt before the study and ensure participants were not coming into the lab already sleep restricted. Sleep timing was confirmed by wrist actigraphy with light-exposure monitoring (Actiwatch-Spectrum; Philips Respironics, Murrysville, Pennsylvania), written sleep logs, and call-ins of bed and wake times to a time-stamped online recorder. No prescription, non-prescription medications, or herbal supplements (except birth control) were permitted for one month prior to the study. Nicotine, marijuana, and illicit drugs were proscribed for one month prior to the study and confirmed by

urine toxicology upon admission to the inpatient laboratory stay. Blood donations and travel beyond one time zone were not permitted for one month prior to the inpatient study. Over-the-counter medications were proscribed for one week prior to the study. For 3 days prior to the inpatient study and throughout the in-laboratory protocol, participants were proscribed caffeine, alcohol, and exercise. For 3 days prior to the inpatient study protocol, participants consumed a provided diet designed to meet daily energy requirements as calculated using the Harris-Benedict formula determined from resting metabolic rate with an activity factor of 1.4. Diets were prepared by the University of Colorado Anschutz Medical Campus Clinical and Translational Research Center metabolic kitchen. Participants were instructed to consume all of the meals provided and nothing else except water to ensure energy balance at the start of the inpatient protocol. Diets provided during the outpatient monitoring as well as during the inpatient visit consisted of 15% protein, 30% fat, and 55% carbohydrate.

Inpatient visit

A protocol schematic of the inpatient laboratory visit is shown in Figure 5.1. Participants arrived at the Clinical and Translational Research Center (CTRC) at the University of Colorado Anschutz Medical Campus in the evening, 6 hours before habitual bedtime. Upon admission to the laboratory, drug- and alcohol-free status were verified with urine toxicology and breath alcohol testing (Lifeloc Technologies; model FC10). On night 1, participants received a 9h sleep opportunity based on habitual bed/wake times calculated from the outpatient monitoring week. Polysomnography (PSG) was recorded every night during the inpatient laboratory visit, with the first night serving as a complete PSG sleep disorder screening. On the morning of day 2, hourly saliva was collected beginning immediately upon awakening. Room lighting was <8lux during the 5h melatonin assessment. Approximately 2.5 hours after awakening, a skeletal muscle biopsy was obtained from the vastus lateralis and a hyperinsulinemic euglycemic clamp procedure immediately followed the biopsy. The exact timing of the biopsy and clamp depended on habitual wake time for each participant, with the biopsy occurring immediately prior to the

start of the clamp. For the next four nights, participants received a 5-hour sleep opportunity achieved by delaying bedtime by 4 hours to maintain wake time. PSG sleep was measured throughout the protocol and consistent monitoring by laboratory staff ensures scheduled wakefulness at all times. On the morning of Day 6, the saliva sampling, skeletal muscle biopsy and hyperinsulinemic euglycemic clamp were repeated. Throughout the inpatient laboratory visit, participants consumed a provided diet designed to meet daily energy requirements as determined from resting metabolic rate with an activity factor of 1.2 plus 5% for sleep deprivation (Markwald et al., 2013). The provided isocaloric diet is designed to maintain stable body weight throughout the study to avoid the confounding effect of overeating typically accompanying sleep restriction studies (McHill et al., 2014; Broussard et al., 2015a). After the clamp concludes on Day 6, participants are provided a 12-hour recovery sleep opportunity prior to discharge on Day 7.

Polysomnography (PSG)

PSG recordings (Siesta, Compumedics, Inc.) were collected every night during the inpatient study using monopolar electroencephalogram (EEG) referenced to contralateral mastoids (C3xA2, C4xA1, O1xA2, and F3xA2), right and left electrooculograms (EOG), chin electromyograms (EMG), and electrocardiograms (EKG). A complete PSG sleep disorder screening was completed on the first night of the in-laboratory stay which included the above measurements as well as abdominal and thoracic respiratory bands, position sensor, nasal thermistor, snore microphone, pulse oximetry, and leg EMGs. Scheduled wakefulness was verified by research staff with continuous monitoring and with wake EEG on study Days 2 and 6 to ensure participants did not fall asleep during the hyperinsulinemic euglycemic clamp. Sleep stages were manually scored from the C3xA2 derivation according to standard criteria (Rechtschaffen, 1968). Slow wave sleep (SWS) includes both Stage 3 and Stage 4 sleep.

Melatonin

Hourly saliva samples were collected beginning immediately upon awakening on inpatient study Days 2 and 6 and continued for 5 hours, resulting in 6 samples per day. Melatonin was measured using a commercially available Direct Saliva Melatonin Radioimmunoassay (RIA) (Bühlmann Laboratories AG, Schönenbuch, Switzerland).

Hyperinsulinemic euglycemic clamp

A standard hyperinsulinemic euglycemic clamp was conducted using established methods (DeFronzo et al., 1979). Participants were fasted overnight and throughout the clamp procedure. Upon habitual waketime, an antecubital vein was cannulated for infusions of insulin and dextrose. On the contralateral side, a dorsal hand vein was catheterized in retrograde fashion for blood sampling using the heated hand technique. A primed continuous infusion of insulin was administered at to 40 mU/m^2/min for 2h to assess whole body insulin sensitivity.

A variable infusion of 20% dextrose was used to maintain blood glucose at about 90 mg/dL throughout each stage of the clamp. Arterialized blood was sampled every 5 minutes for bedside determination of glucose concentration (Analox, Lunenberg, Massachusetts), and the dextrose infusion was adjusted as necessary. During the last 30 minutes of the clamp, arterialized blood was taken for hormone and substrate measurements. Whole body insulin sensitivity was measured as the glucose infusion rate, M, during the last 30 minutes of the clamp.

Skeletal muscle biopsies

A skeletal muscle biopsy was obtained under a local anesthetic (lidocaine) using a percutaneous needle as is commonly done at the University of Colorado Anschutz Medical Campus CTRC as previously described (Sachs et al., 2019; Broussard et al., 2021; Kahn et al., 2021). Briefly, the biopsy was taken from the vastus lateralis muscle midway between the greater trochanter of the femur and patella. This method typically yields 125 mg of tissue per biopsy. A first skeletal muscle biopsy was taken on Day 2 after one week of outpatient sleep of 9h per night and one night of inpatient sleep at the same habitual time. On Day 6, after 4 nights

of 5 hours sleep opportunity per night, another skeletal muscle biopsy was repeated on the same limb. To avoid potential confounding effects from the first biopsy, the second biopsy occurred through a different incision site and 1-2 inches away from the initial biopsy. All biopsies were obtained from the right leg, except for one subject who had the first biopsy on the left leg and second biopsy on the right leg. Approximately 25 mg of tissue was immediately placed in BIOPS (info) for assessment of mitochondrial respiration while the remaining tissue was flash frozen in liquid nitrogen and stored at -80°C.

Quantitative lipidomics using LC/MS/MS

 Frozen skeletal muscle biopsies were homogenized and fortified with internal standards, lipid extracted, and analyzed by an Agilent 1100 HPLC connected to an API 2000 triple quadrupole mass spectrometer (Garcia-Ruiz et al., 1997). The 1,3- and 1,2-DAG isomers were separated chromatographically using a Hilic 2.1 micron, 3x100mm column. Concentration of diacylglycerol (DAG; 1,2- and 1,3- isomers), sphingolipids (ceramide [Cer], dihydroceramide [DHCer], glucosylceramide [GluCer], lactosylceramide [LacCer], galactosylceramide [GalCer], sphingomyelin [SPM]), acylcarnitine (AC), and triacylglycerol (TAG) species were determined by comparing ratios of unknowns to odd chain or deuterated internal standards.

RNA isolation and sequencing

 RNA isolation, sequencing, and gene expression analyses were performed using standard techniques. Briefly, frozen skeletal muscle biopsies were pulverized (Spectrum™ Bessman Tissue Pulverizers, Fisher Scientific) and 20-30 mg tissue were used for RNA isolation. Frozen tissue was lysed in Trizol (ThermoFisher) and rigorously vortexed. RNA was isolated with an RNA-specific spin column kit (Direct-zol™ RNA Miniprep Kit, Zymo Research) and treated with DNase I to remove genomic DNA (Cavalier et al., 2021).

 Nanodrop measurements confirmed concentration and integrity of samples. All samples were sequenced on an Illumina NovaSeq6000 platform (Novogene Co., Ltd., Sacramento, CA). Differential gene expression was analyzed with DESeq2/EdgeR (Robinson et al., 2010; Love et

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al., 2014). Gene Set Enrichment Analysis (GSEA) was carried out using the Bioconductor

package clusterProfiler (Yu et al., 2012) using Gene Ontology (GO) pathways and expert

curated Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al.,

2016).

Mitochondrial respiration

Fresh skeletal muscle biopsies were used to prepare permeabilized fiber bundles to

assess oxygen consumption by high-resolution respirometry Oxygrapgh-2K (OROBOROS

Instruments, Innsbruck, Austria). Tissue was permeabilized in saponin (50 µg/mL) for 20

minutes before starting the measurements as previously described (Kuznetsov et al., 2008).

Measurements were performed at 37°C and respiration rates expressed as $nmolO_2 \cdot min^{-1}$

$\cdot mg \cdot wet \ wt^{-1}$. To estimate the *in vivo* condition, mitochondrial respiration in permeabilized

skeletal muscle tissue was determined with malate + pyruvate + glutamate (MPG) as respiratory

substrates. Respiration is initiated with a saturating concentration of ADP (8 mM), followed by

succinate and the Vmax of maximal ADP-stimulated respiration rate or state 3 respiration rate

was determined. The intactness of mitochondrial membranes were validated by cytochrome c

tests for each experiment. Only samples passing this quality test were used in analyses.

Carbonilcyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) was then titrated to uncouple the

mitochondria and a maximal rate of the electron transport system was determined.

Statistics

Data from our primary outcome measures were analyzed by paired t-tests. Primary

outcomes include (1) change (insufficient sleep – habitual sleep) in insulin sensitivity, (2)

change (insufficient sleep - habitual) in lipid accumulation using a Benjamini-Hochberg

correction for multiple comparisons and (3) change (insufficient sleep – habitual sleep) in

skeletal muscle mitochondrial respiration. Correlations between the change in insulin sensitivity

and change in total lipid species or change in mitochondrial respiration were conducted. Outliers

were determined as data points more than 1.5 interquartile ranges below the first quartile and 1.5 interquartile ranges above the third quartile and were removed from the dataset.

Welch's t-tests for unequal variance were calculated for PSG outcomes. To examine changes in melatonin, a repeated measures ANOVA (R statistical software; version 4.0.3; www.r-project.org) was used with study day and sample number as fixed factors and participant as a random factor. For RNA sequencing analyses, generalized linear model likelihood ratio tests were completed using DESeq2/EdgeR R packages with a false discovery rate (FDR) cut-off of 5% (Robinson et al., 2010; Love et al., 2014). P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method and a corrected p-value of 0.05 and log2 fold-change of 0 were set as the threshold for statistical significance.

Results

Participants

Eighteen young, healthy, sedentary, lean adults (8F; 24.7±3.5 years; mean±SD) participated in the study. Average BMI was 22.7±1.8 kg/m^2. On average, participants slept for 457±6 min during habitual sleep vs 283±1 min during insufficient sleep (Figure 5.2). Additional sleep staging results can be found in Table 5.1. Furthermore, morning melatonin offset was significantly delayed during insufficient sleep indicating morning circadian misalignment (Fig 5.3).

Insufficient sleep impairs insulin sensitivity

Insulin sensitivity, as estimated by the glucose infusion rate (GIR) during the final 30 minutes of the hyperinsulinemic euglycemic clamp was significantly lower during insufficient sleep compared with habitual sleep (insufficient sleep: 8.8±0.9, habitual sleep: 9.8±1.2 mg/kg/min, p<0.05; Fig 5.4).

Insufficient sleep induces skeletal muscle lipid accumulation

We investigated the effects of insufficient sleep on skeletal muscle lipid accumulation. There were no changes in total skeletal muscle concentration or specific species of TAG,

DHCer, GluCer, GalCer, AC, SPM, or TAG (Fig 5.5-5.6). While there were no changes in total Ceramides or total LacCers during insufficient sleep, specific species of ceramides, including C16:1 Cer, C22:0 LacCer, C24:0 LacCer, and C24:1 Lac Cer, were significantly elevated during insufficient sleep (Fig 5.5-5.6). There were no changes in total 1,2-DAGs or total 1,3-DAGs during insufficient sleep; however, specific species of 1,2-DAGs, including 16:0/18:1, 16:0/20:0, 16:0/20:2, 16:0/20:3, 18:0/18:1, di18:0, were significantly elevated after insufficient sleep (Fig 5.5.5-5.6). In contrast, specific species of 1,3-DAGs, including 18:0/18:3 and di14:0, were significantly decreased during insufficient sleep (Fig 5.6). A complete list of specific bioactive lipids and total species concentrations with respective p values are listed in Table 5.2. There were no significant correlations between whole body insulin sensitivity and total lipid species (Fig. 5.7).

Insufficient sleep alters skeletal muscle expression of genes involved in fuel utilization and critical biological pathways

Expression of 23 genes in skeletal muscle were up-regulated and 39 genes were down-regulated following 4 nights of insufficient sleep (Fig 5.8). A complete list of up- and down-regulated genes can be found in Tables 5.3 and 5.4, respectively. Specifically, gene expression of glycerol 3 phosphate dehydrogenase 2 (mitochondrial; GPD2) and frizzled family receptor 7 (FZD7), both associated with diabetes, and coagulation factor XIII A1 polypeptide (F13A1), which enhances glycolysis, were up-regulated during insufficient sleep. Expression of tumor protein p53 inducible nuclear protein 2 (TP53INP2), which is naturally repressed in human insulin resistance, as well as peroxisomal biogenesis factor 7 (PEX7) and lipoprotein lipase (LPL), both involved in lipid metabolism, were down-regulated during insufficient sleep.

To identify biological processes altered by insufficient sleep, pathway analyses using Gene Ontology (GO) annotations and kyoto encyclopedia of genes and genomes (KEGG) pathways were conducted (Fig 5.9). Activated pathways revealed are involved in signaling receptor activity, peptide receptor activity, and Wnt signaling pathways. Suppressed GO and

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KEGG pathways included cotranslational protein targeting to the membrane, translation initiation, and NF-kappa B signaling pathways.

Seventeen genes were significantly correlated with impaired insulin sensitivity, a complete list can be found in Table 5.5. Only one gene, RP11-93209.7, was negatively correlated with impaired insulin sensitivity, whereas 16 genes were positively correlated. Specifically, stomatin (BPB72)-like 2 (STOML2), which is a protein that regulates mitochondrial activity and biogenesis, and acyl-CoA thioesterase 13, which catalyzes the hydrolysis of acyl-CoAs into free fatty acids and coenzyme A, were positively correlated with impaired insulin sensitivity during insufficient sleep.

Insufficient sleep does not alter skeletal muscle mitochondrial respiration

To assess a functional outcome of skeletal muscle lipid accumulation, we measured skeletal muscle mitochondrial respiratory capacity using and Oroboros Oxygraph-2K. There were no changes in ADP-stimulated mitochondrial respiration (State 3) or maximally uncoupled respiration (State U) in permeabilized *ex vivo* skeletal muscle fibers using pyruvate and malate as substrates (Fig 5.10). Additionally, there were no relationships between *in vivo* insulin sensitivity as measured by the glucose infusion rate during the clamp and oxygen consumption during either ADP-stimulated respiration (State 3) (R^2=0.1154; p>0.05) or maximally uncoupled respiration (State U) (R^2=0.0351; p>0.05) (Fig 5.11).

Discussion

We have demonstrated that 4 nights of 5 hours of sleep per night in healthy, young, lean women and men results in an increase in lipid accumulation in skeletal muscle and altered the expression of many genes important in metabolic pathways accompanied by impaired insulin sensitivity. We also found that mitochondrial function in skeletal muscle was unchanged after insufficient sleep. These findings provide novel insights into our understanding of the pathways that may link insufficient sleep to impaired insulin sensitivity and increased diabetes risk.

We found that 4 nights of insufficient sleep of 5 hours per night increased skeletal muscle lipid accumulation, specifically, 1,2-diacylglycerols (1,2-DAGs). 1,2-DAGs are associated with skeletal muscle insulin resistance in contexts such as diabetes and obesity and are known impair insulin sensitivity in muscle cells *in vitro* (Bergman et al., 2012). Therefore, our results suggest that elevated 1,2-DAGs may contribute to impair muscle-specific insulin sensitivity consistently reported during insufficient sleep. In contrast, 1,3-diacylglycerols (1,3-DAGs), which have not previously been implicated in insulin resistance (Petersen and Shulman, 2018), were unchanged or significantly decreased during insufficient sleep. Additionally, we found significant increases in specific ceramide (Cer) and lactosylceramide (LacCer) species during insufficient sleep which supports previous findings of increased ceramides in insulin resistant compared to insulin sensitive participants (Coen et al., 2010). Ceramides and 1,2-DAGs impair insulin sensitivity through different pathways. For example, ceramides inhibit insulin-stimulated glucose uptake via the inhibition of AKT activation. One proposed route is by the activation of protein phosphatase 2A (PP2A) which leads to the dephosphorylation of AKT in response to insulin. Another proposed route is by the activation of PKC zeta which binds, phosphorylates, and translocates AKT (Petersen and Shulman, 2018). Additionally, 1,2-DAGs are proposed to lead to muscle insulin resistance by activating protein kinase C (PKC) theta. PKC may then lead to serine phosphorylation and inhibition of insulin receptor substrate 1 (IRS1) in the insulin signaling cascade (Petersen and Shulman, 2018). Although we did not see any changes in these pathways from RNA sequencing analysis of basal muscle biopsies, we have previously shown that insufficient sleep is associated with impaired insulin-stimulated AKT phosphorylation in adipose tissue (Broussard et al., 2012). Thus, future studies should consider insulin stimulation of muscle—either *in* or *ex vivo*—to elucidate the impact of insufficient sleep and associated lipid accumulation on specific metabolic signaling pathways.

We did not find any change in total triacylglycerols (TAGs) nor specific TAG species in skeletal muscle after insufficient sleep. These results are in contrast to a study found that blood

TAGs were increased after sleep loss (Chua et al., 2015). The differences between the results

of these two studies may be the difference between blood and muscle TAGs after sleep loss.

Additionally, Chua et al., investigated blood lipids after one night of total sleep deprivation

whereas the current study investigated 4 nights of 5 hours of sleep per night (Chua et al., 2015).

Moreover, the total sleep deprivation study included only ethnic-Chinese males whereas the

current study enrolled male and female participants from a variety of race and ethnicity

backgrounds including Asians and Hispanic or Latinos. Indeed, racial and ethnic background of

participants impact the risk for type 2 diabetes and cardiometabolic disease and thus may

influence the differences in TAG results between these studies (Osei et al., 1993; Jensen et al.,

2002; Bajaj and Banerji, 2004).

In addition to lipidomic analyses to investigate lipid accumulation in skeletal muscle, we

performed RNA isolation and sequencing on skeletal muscle biopsies in order to assess gene

expression changes associated with insufficient sleep and impaired insulin sensitivity. Many of

the genes found to be significantly up- and down-regulated during insufficient sleep are involved

in various metabolic pathways. Among the up-regulated genes involved in metabolism include

glycerol 3 phosphate dehydrogenase 2 (mitochondrial) (GPD2) which is associated with

diabetes (Novials et al., 1997; St-Pierre et al., 2001); coagulation factor XIII A1 polypeptide

(F13A1) which is linked to obesity (Dull et al., 2021); DNA damage inducible transcript 4-like

(DDIT4L) which promotes activation of insulin receptors (Corradetti et al., 2005); frizzled family

receptor 7 (FZD7) which are activated in diabetes (von Maltzahn et al., 2011); and AhpC/TSA

antioxidant enzyme domain containing 1 (AAED1) which enhances glycolysis (Zhang et al.,

2018). The up-regulation of these genes during insufficient sleep suggests an increase in

glycolytic pathways and a link to obesity and diabetes.

Some down-regulated genes involved in metabolism include tumor protein p53 inducible

nuclear protein 2 (TP53INP2) which is naturally repressed in human insulin resistance (Sala et

al., 2014); potassium voltage-gated channel Shab related subfamily member 1 (KCNB1) which

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is related to GLUT4 transport and insulin sensitivity (Zhang et al., 2013); protein phosphatase 1 regulatory subunit 3B (PPP1R3B) which is involved in diabetes (Niazi et al., 2019); lipoprotein lipase (LPL) which is involved in triglyceride hydrolase (Mead et al., 2002); and solute carrier family 2 facilitated glucose transporter member 4 (SLC2A4) which is involved in glucose transmembrane transporter activity (Brunetto et al., 2012). The down-regulation of these genes during insufficient sleep suggests a decrease in fatty acid metabolism and impaired glucose transport.

GO and KEGG pathway analyses reveal activation of signal receptor activity, peptide receptor activity, and Wnt signaling pathways. Activation of the Wnt pathway, as we see in these data, have been seen previously in type 2 diabetes (Jin, 2008). GO and KEGG pathway analyses reveal a suppression of cotranslational protein targeting to membrane, translation initiation, and NFκB signaling pathways. NFκB is a mediator of inflammatory responses and induces the expression of pro-inflammatory genes. Deregulated activation of NFκB contributes to pathogenic processes of various inflammatory diseases, including obesity (Baker et al., 2011). We found a suppression of NFκB signaling pathways suggesting fewer pro-inflammatory genes expressed.

Although 17 genes were correlated with impaired insulin sensitivity after 4 nights of 5 hours of sleep, none of these genes was up- or down-regulated with insufficient sleep. However, numerous genes that were correlated with impaired insulin sensitivity are directly related to essential metabolic pathways. For example, stomatin (EPB72-like 2 (STOML2) is a protein that regulates mitochondrial activity and biogenesis (Hajek et al., 2007). Thus, insufficient sleep, per se, may not alter genes to impair insulin sensitivity but it contributes to genes that dysregulated metabolic pathways which may contribute to impaired insulin sensitivity during insufficient sleep.

Elevated lipids in muscle are also associated with decreased mitochondrial respiratory capacity (Phielix et al., 2008; Chavez et al., 2010). Therefore, to assess a functional outcome associated with increased skeletal muscle lipid accumulation, we measured skeletal muscle

mitochondrial respirometry. There were no changes in ADP-stimulated mitochondrial respirations (State 3) or maximally uncoupled respirations (State U). Additionally, there were no correlations between any state of mitochondrial function and insulin sensitivity. This suggests that changes in skeletal muscle mitochondrial function may not be directly altered by insufficient sleep or that more chronic insufficient sleep protocols are required to see changes in mitochondrial function.

One study using a circadian misalignment protocol in men found that circadian misalignment significantly increased ADP-stimulated (State 3) and maximally uncoupled (State U) respiration in the behavioral morning compared with the behavioral night (Wefers et al., 2018). In this circadian misalignment protocol, participants received an 8-hour daytime sleep opportunity. Even though a sufficient duration for the sleep opportunity was provided, we previously reported that during an inpatient study of circadian misalignment, when participants were provided with an 8h daytime sleep opportunity they still only received on average 6h18min of actual sleep (McHill et al., 2014). While sleep characteristics were not reported in this circadian misalignment protocol, it is likely that some sleep restriction occurred. Therefore, it is difficult to compare to the mitochondrial function results from our study to this circadian misalignment study.

A limitation of our mitochondrial function protocol is that we are only included carbohydrate substrates. While it would be interesting to also investigate lipid substrates, such as octanoylcarnitine, another group found changes in oxygen consumption only with carbohydrate substrates and not lipid substrates during a circadian misalignment protocol (Wefers et al., 2018). Another recent study found that using lipid substrates mitochondrial respirations in complex 1 and complex 2 decreased in healthy adult men who received 4 hours of sleep for 5 days (Saner et al., 2021). However, this study conducted an oral glucose tolerance test (OGTT) to estimate insulin sensitivity and performed the skeletal muscle biopsy after the OGTT. In contrast, the current study obtained the skeletal muscle biopsy before the

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measure of insulin sensitivity (hyperinsulinemic euglycemic clamp). Therefore, differences in the mitochondrial function results between these studies may be due to study design in that one study investigated mitochondrial function under insulin-stimulated conditions whereas the other was during fasting conditions. Thus, future studies should continue to investigate mitochondrial function using both carbohydrate and lipid substrates under fasting and insulin-stimulated conditions in the context of sleep and circadian disruption.

Overall, the results of the present study demonstrate that 4 nights of 5 hours of sleep per night alters lipid metabolism resulting in lipid accumulation in skeletal muscle and alters gene expression of genes important in related metabolic pathways. However, we did not see a change in skeletal muscle mitochondrial function after insufficient sleep nor a relationship between skeletal muscle mitochondrial function or lipid accumulation and insulin sensitivity. This suggests that other molecular mechanisms may be contributing to lipid metabolism dysregulation associated with insufficient sleep. While these mechanisms in skeletal muscle have been associated with impaired insulin sensitivity in other contexts, such as obesity and type 2 diabetes, our results suggest that insufficient sleep may impair insulin sensitivity through mechanisms overlapping but not completely similar to these other contexts such as diabetes. Therefore, more research is needed to identify the specificity of mechanisms by which insufficient sleep impairs whole body and tissue specific insulin sensitivity.
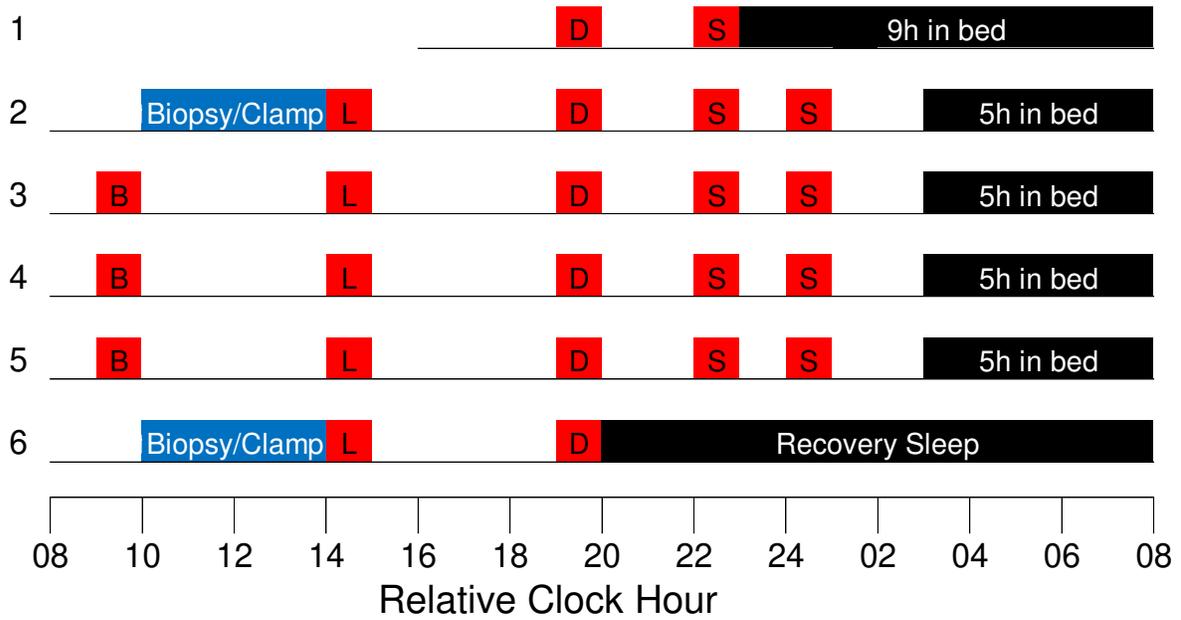
Figure 5.1. Inpatient protocol. Admission on Day 1 permits 9h sleep. Insufficient sleep on Days 2-5 limits to 5h sleep/night. Day 6 includes 12h recovery sleep. Mornings of Days 2 and 6 consist of skeletal muscle biopsies and hyperinsulinemic euglycemic clamp protocols. Day of study on the y-axis. Time of day (h) on x-axis. B=breakfast; L=lunch; D=dinner; S=snack. Black bars represent scheduled sleep.
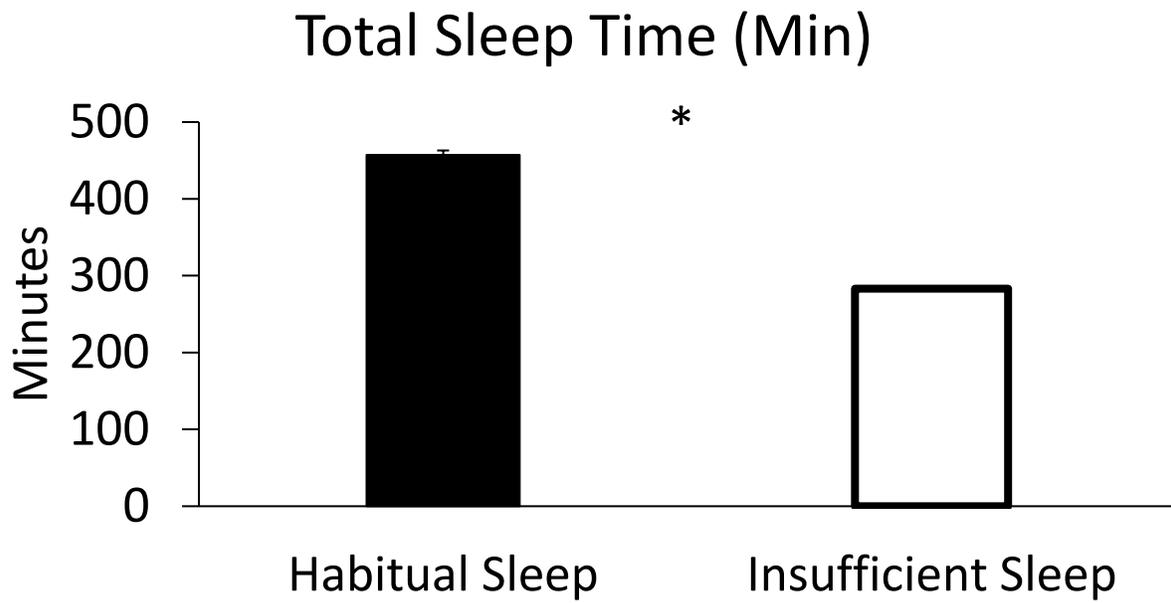
Total Sleep Time (Min)

Figure 5.2. Insufficient sleep reduces total sleep time. Data represent mean ± SEM (n=18). *p<0.05

Table 5.1. Effect of insufficient sleep on sleep stages and total sleep time. Data represents mean ± SEM (n=18). Percentages for each sleep stages were calculated as percentage of total sleep time. REM=rapid eye movement sleep

Table 1 Effects of insufficient sleep on sleep stages			
Sleep variable	**Habitual Sleep**	**Insufficient Sleep**	**P value**
Stage 1, min	22.67±4.02	8.11±0.46	<0.01
Stage 1, %	4.21±0.75	2.71±0.16	0.06
Stage 2, min	275.58±6.57	138.43±3.17	<0.00001
Stage 2, %	51.18±1.23	46.21±1.06	0.67
Slow wave sleep, min	55.5±7.82	73.42±2.59	<0.05
Slow wave sleep, %	10.30±1.44	24.50±0.86	<0.00001
REM, min	103.5±6.12	63.21±2.23	<0.05
REM, %	19.22±1.14	21.10±0.74	0.06
Total Sleep Time, min	457.25±5.80	283.17±1.38	<0.00001
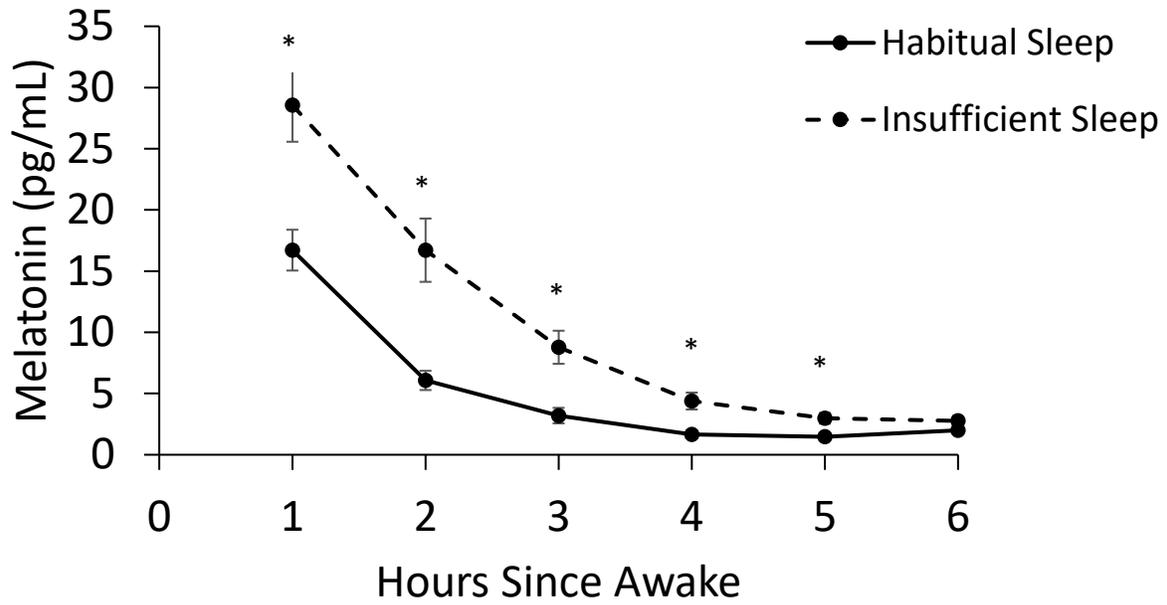
Figure 5.3. Morning melatonin offset curve (mean±SEM) is significantly higher during insufficient sleep (n=18). Melatonin concentration (pg/mL) is on the y-axis and hours since awake is on the x-axis. Habitual sleep is in the solid line. Insufficient sleep is in the dashed line. *p<0.05.
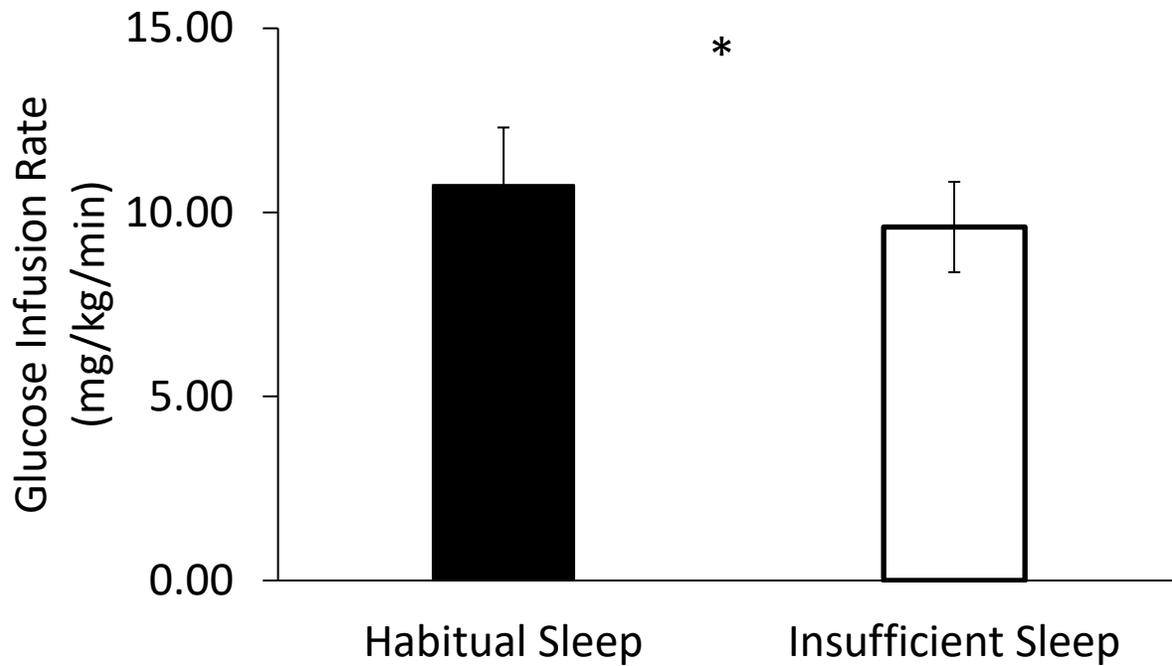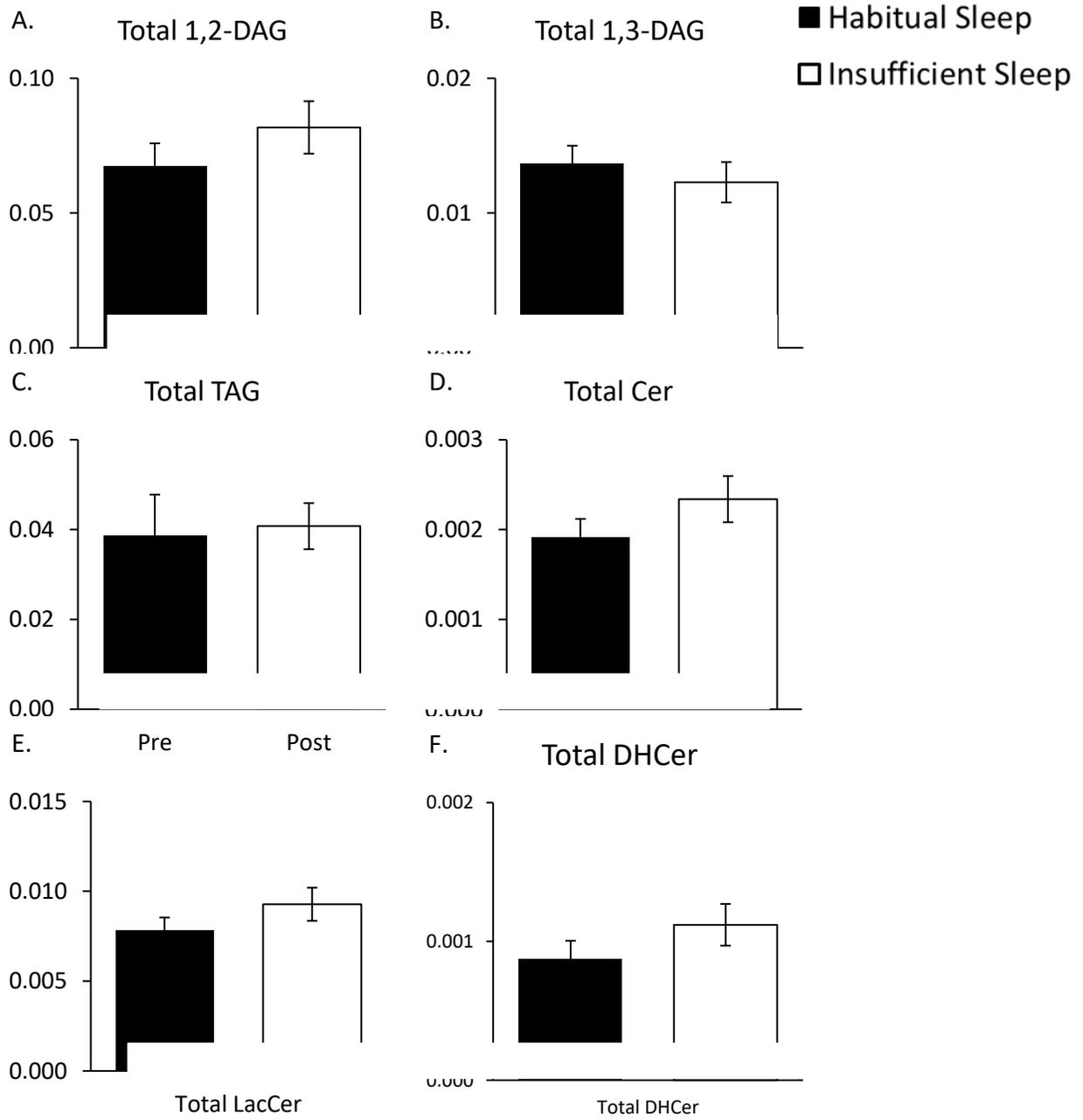
Figure 5.4. Insufficient sleep significantly reduced whole body insulin sensitivity (mean±SEM) as measured by the glucose infusion rate during the hyperinsulinemic euglycemic clamp (n=18). Glucose infusion rate (mg/kg/min) is on the y-axis. Habitual sleep is in the black bar. Insufficient sleep is in the white bar. *p<0.05.
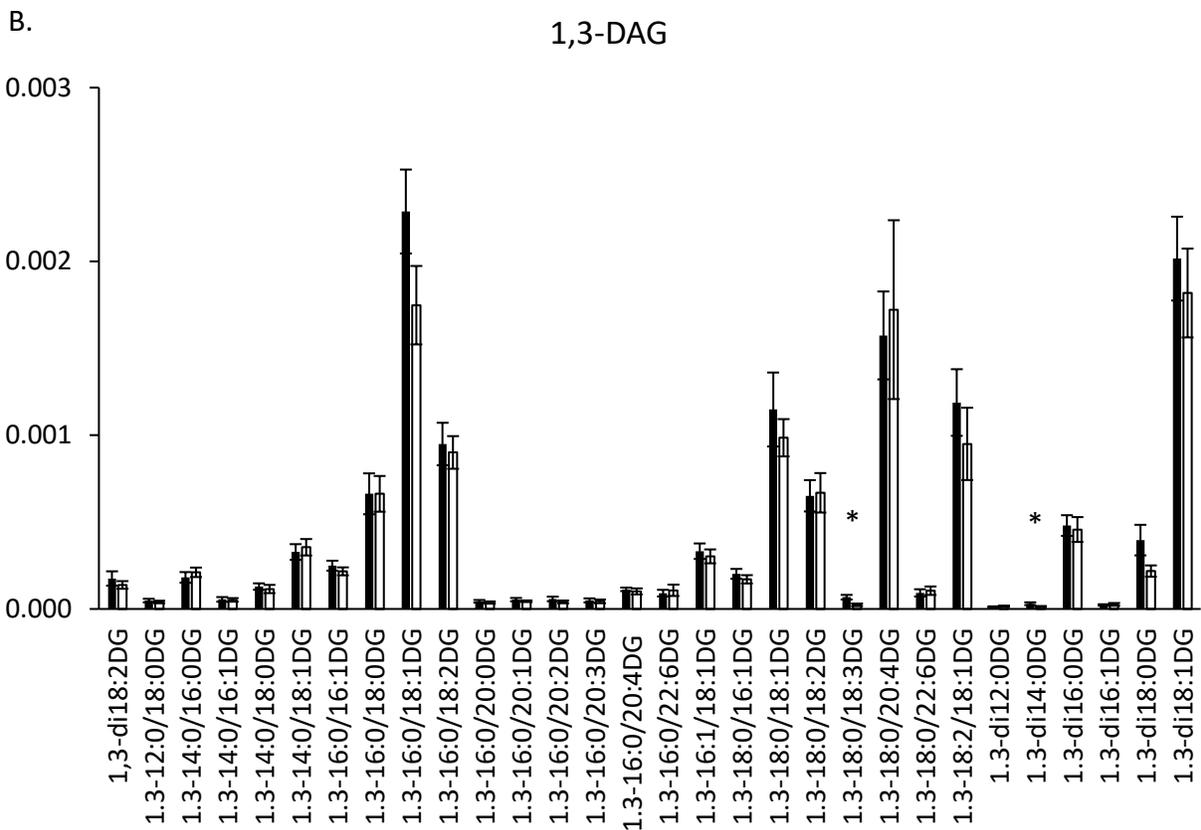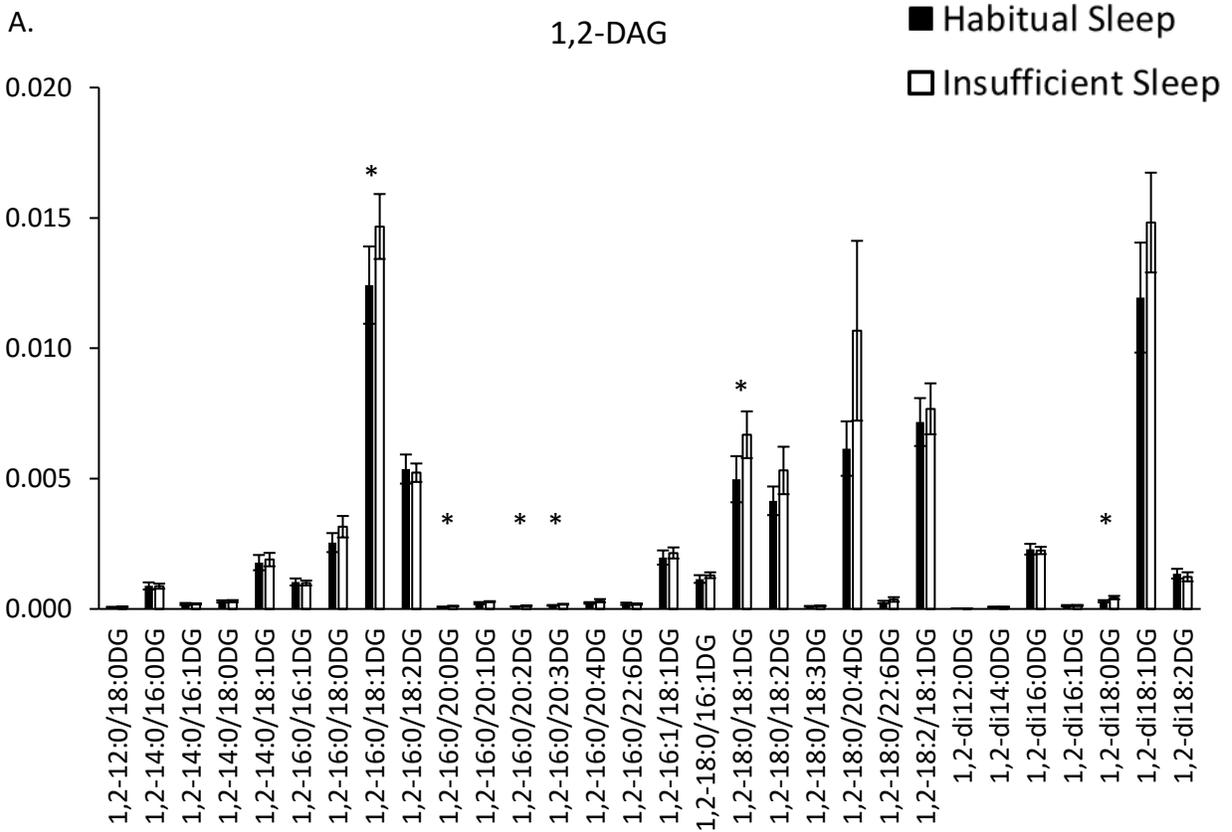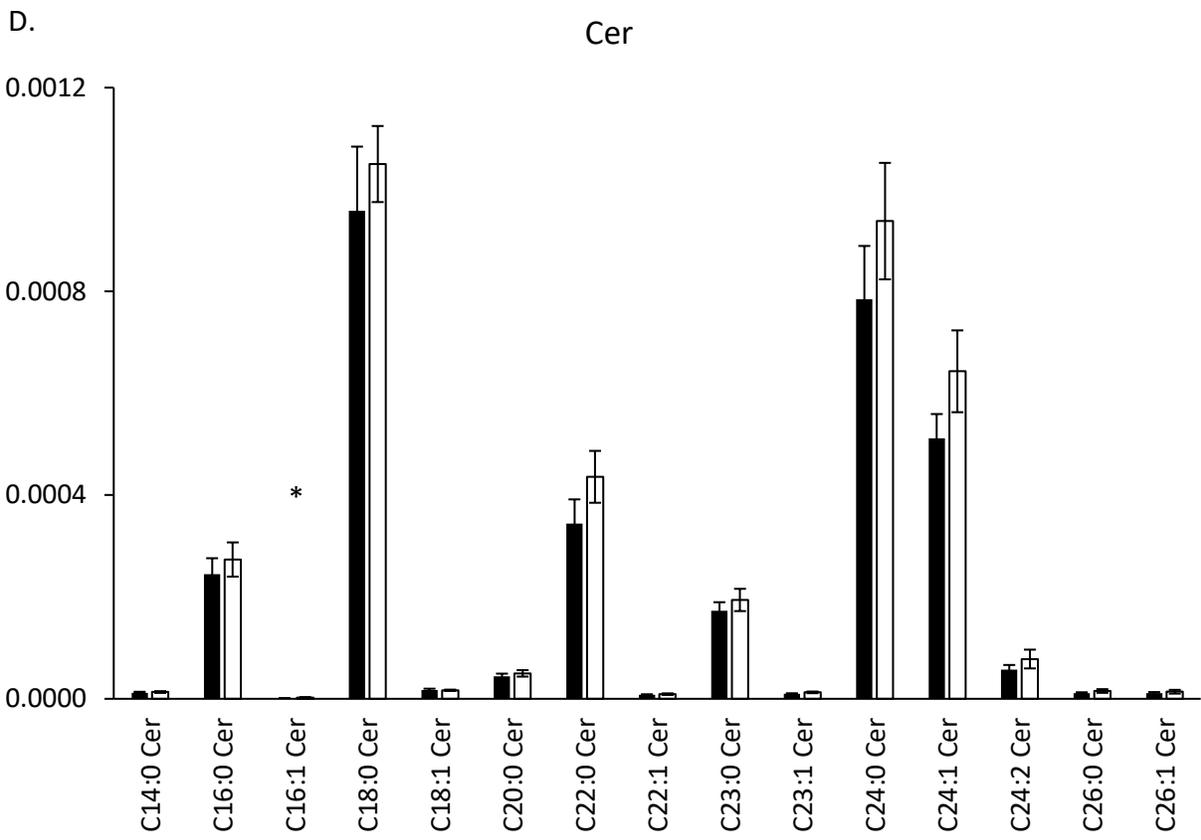
A. Total 1,2-DAG

B. Total 1,3-DAG

C. Total TAG

D. Total Cer

E. Total LacCer

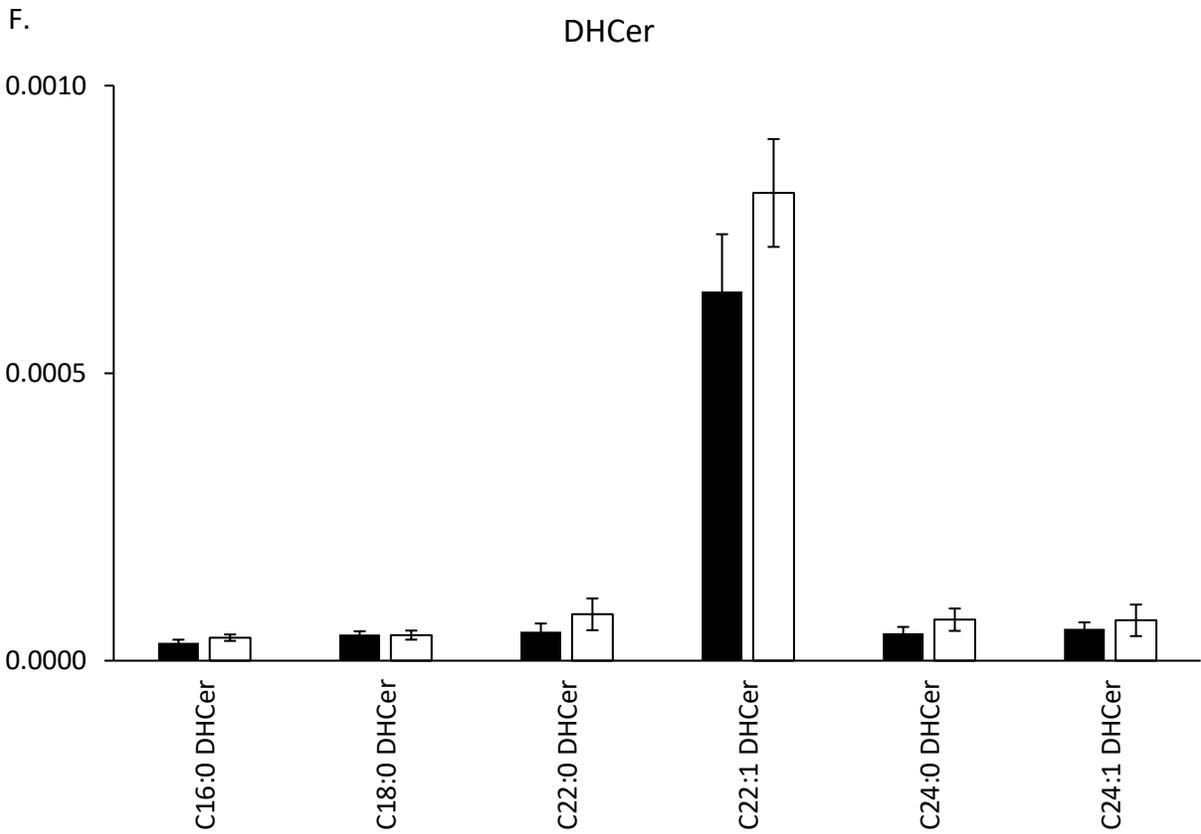F. Total DHCer
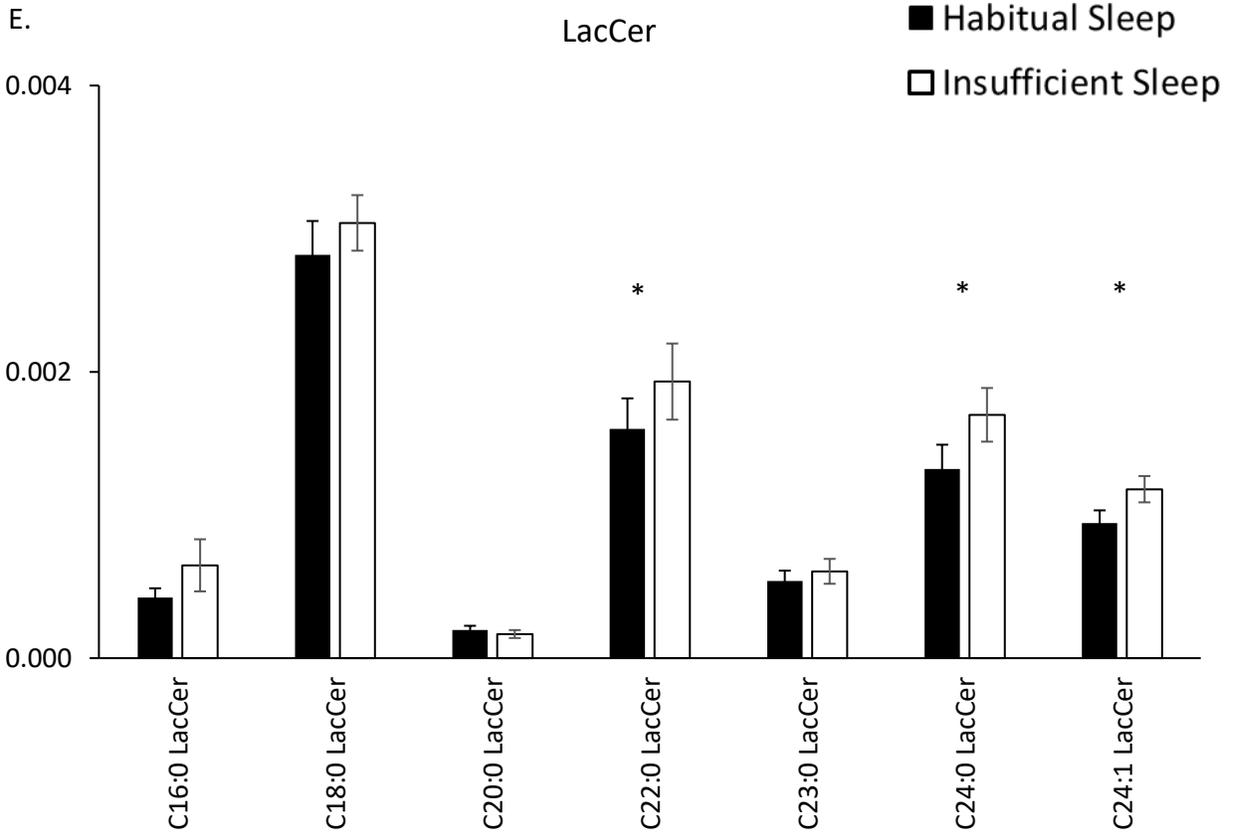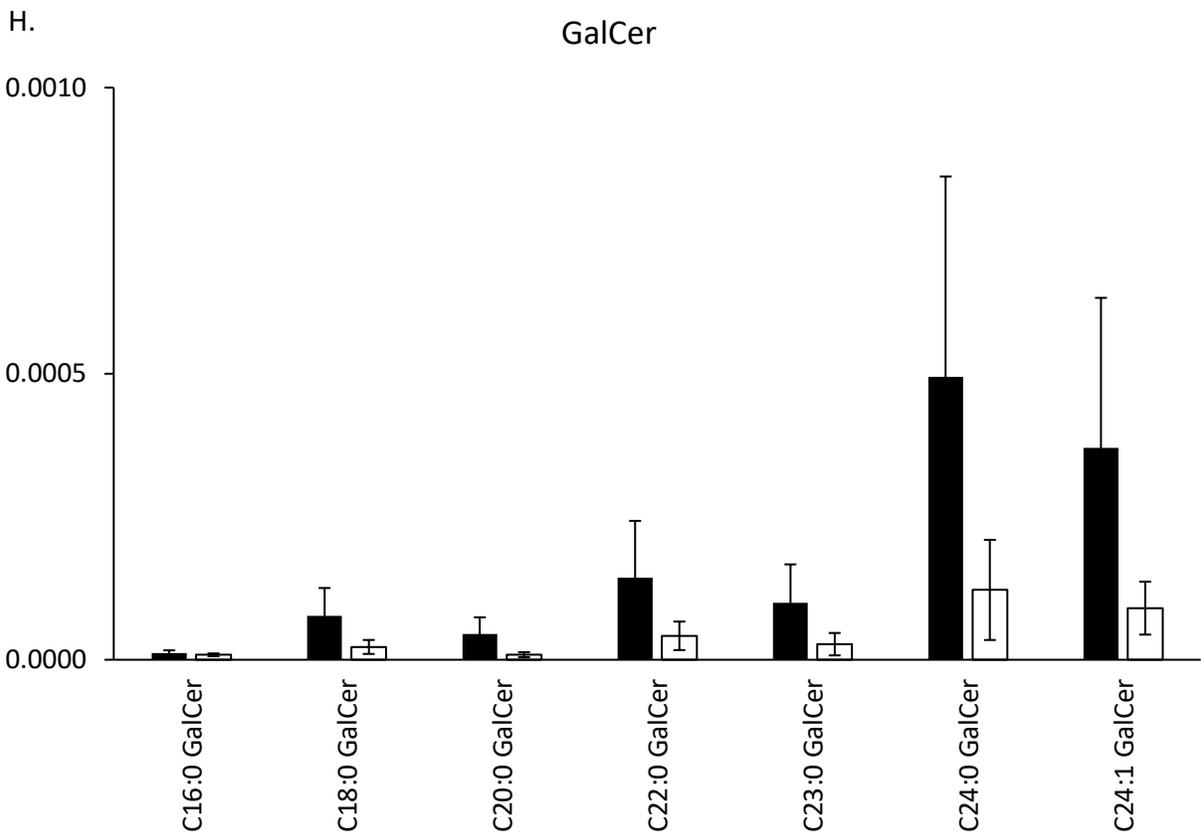
Habitual Sleep
Insufficient Sleep
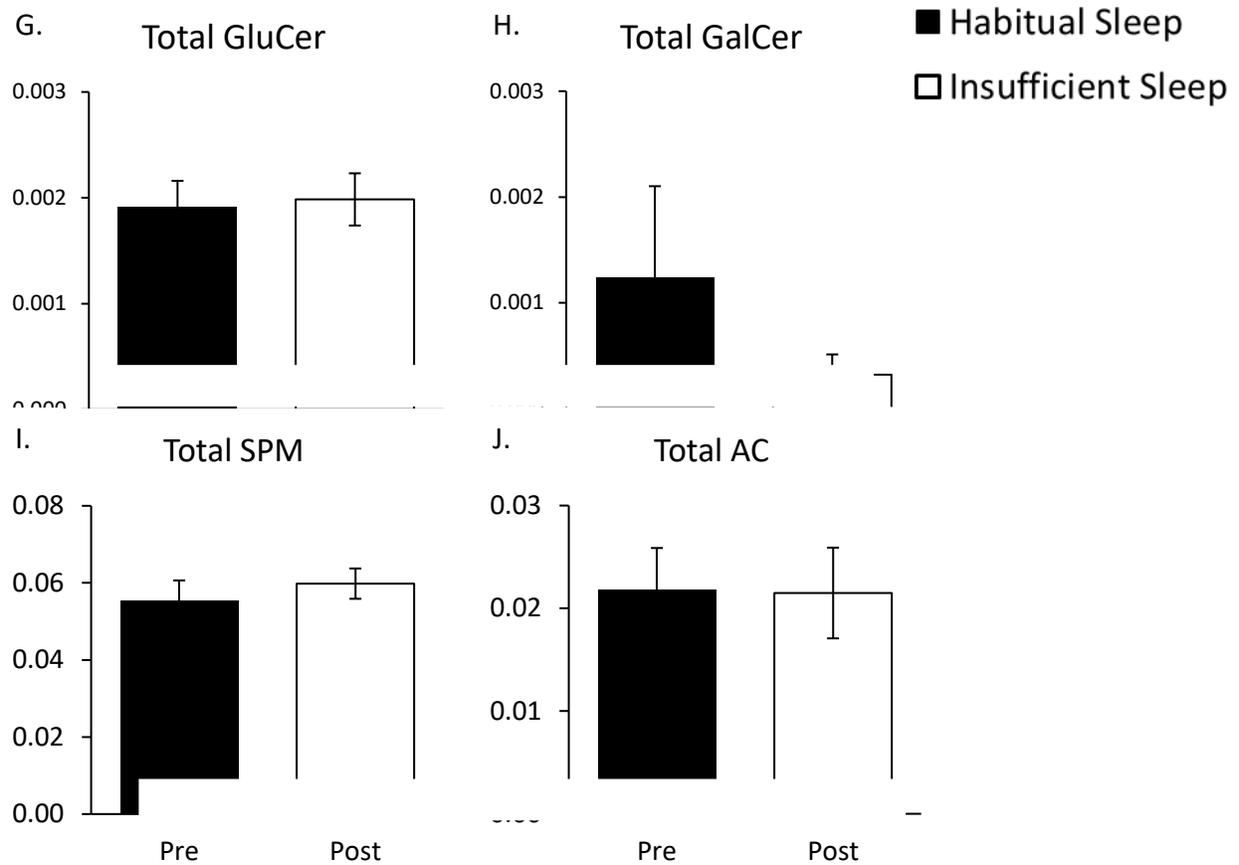
Figure 5.5. Insufficient sleep did not change total lipid levels of (A) 1,2-diacylglycerols, (B) 1,3-diacylglycerols, (C) triacylglycerols, (D) ceramides, (E) lactosylceramides, (F) dihydroceramides, (G) glucosylceramides, (H) galactosylceramides, (I) sphingomyelin, (J) acylcarnitine (n=10). Lipid concentration on the y-axis, Habitual sleep in the black bars, insufficient sleep in the white bars.

A. 1,2-DAG

B. 1,3-DAG

C. TAG

D. Cer

E. LacCer

F. DHCer

G.

GluCer

■ Habitual Sleep
□ Insufficient Sleep

C16:0 GluCer C18:0 GluCer C20:0 GluCer C22:0 GluCer C23:0 GluCer C24:0 GluCer C24:1 GluCer

H.

GalCer

C16:0 GalCer C18:0 GalCer C20:0 GalCer C22:0 GalCer C23:0 GalCer C24:0 GalCer C24:1 GalCer
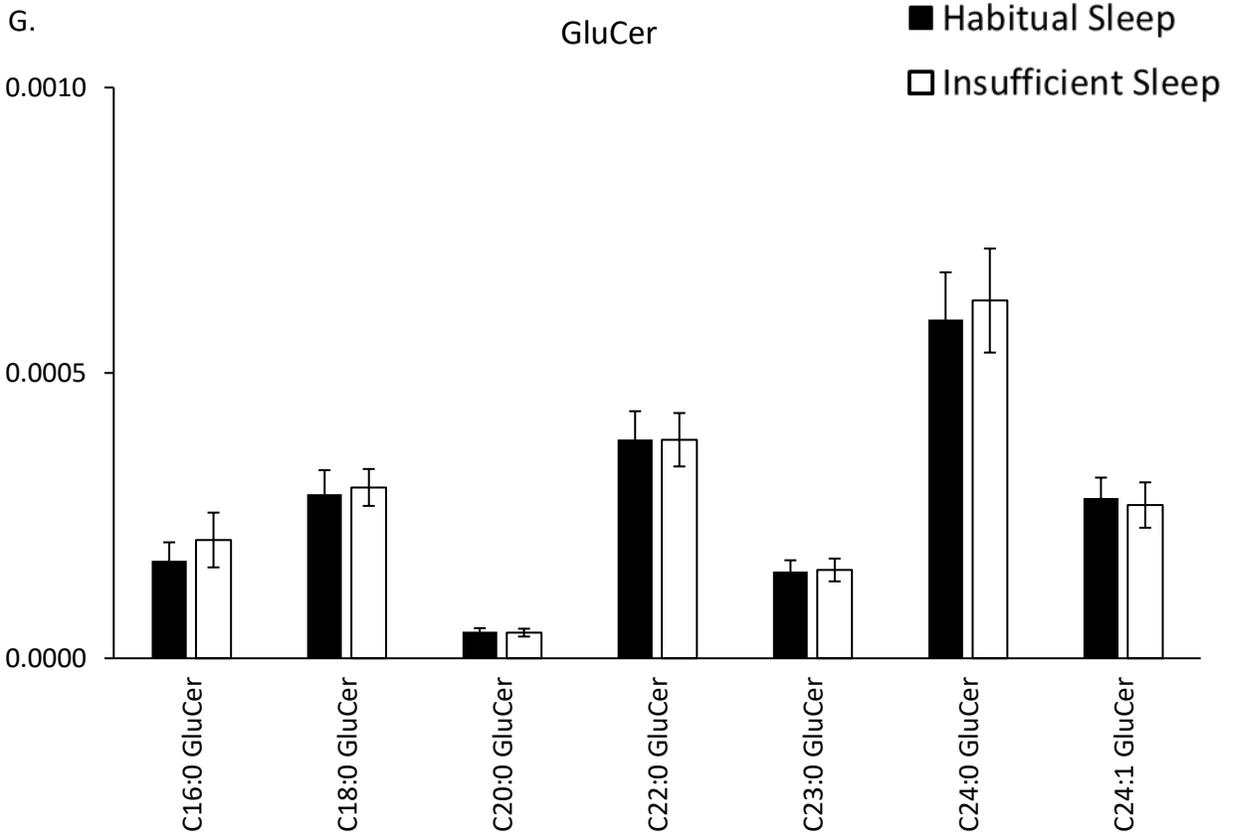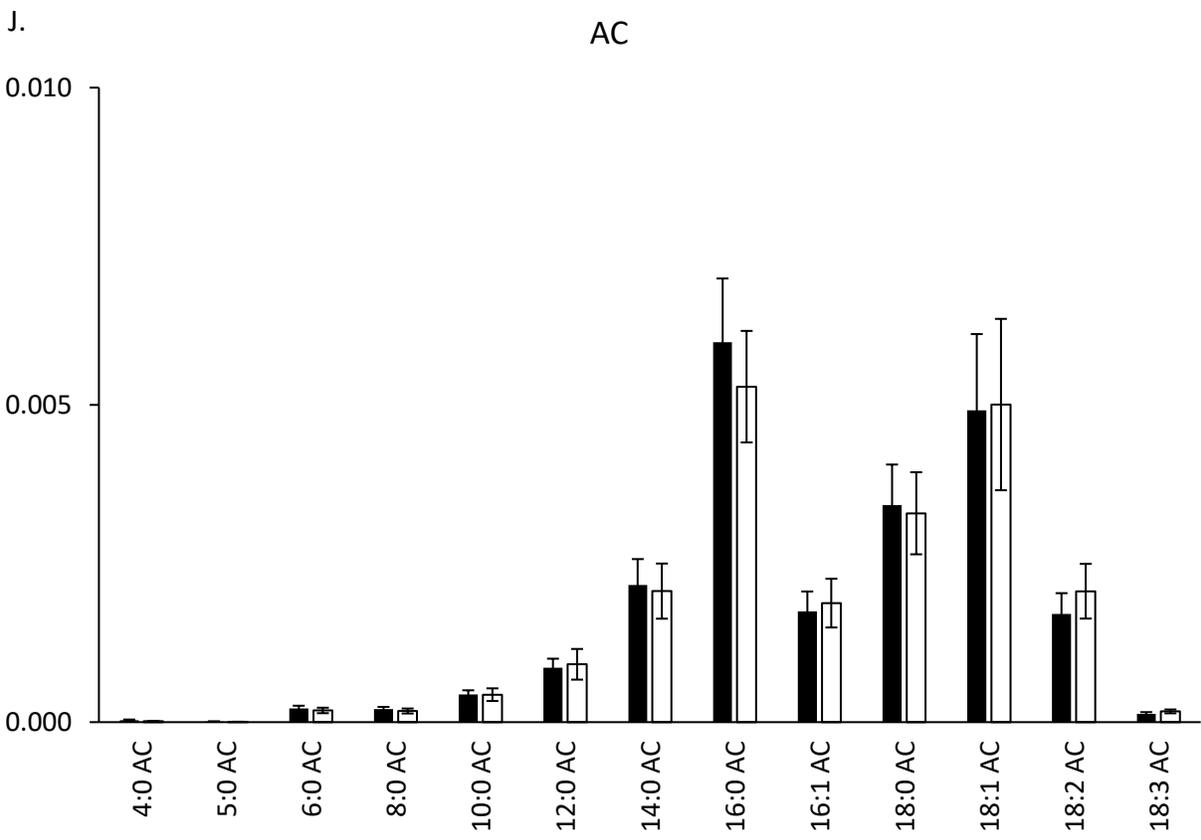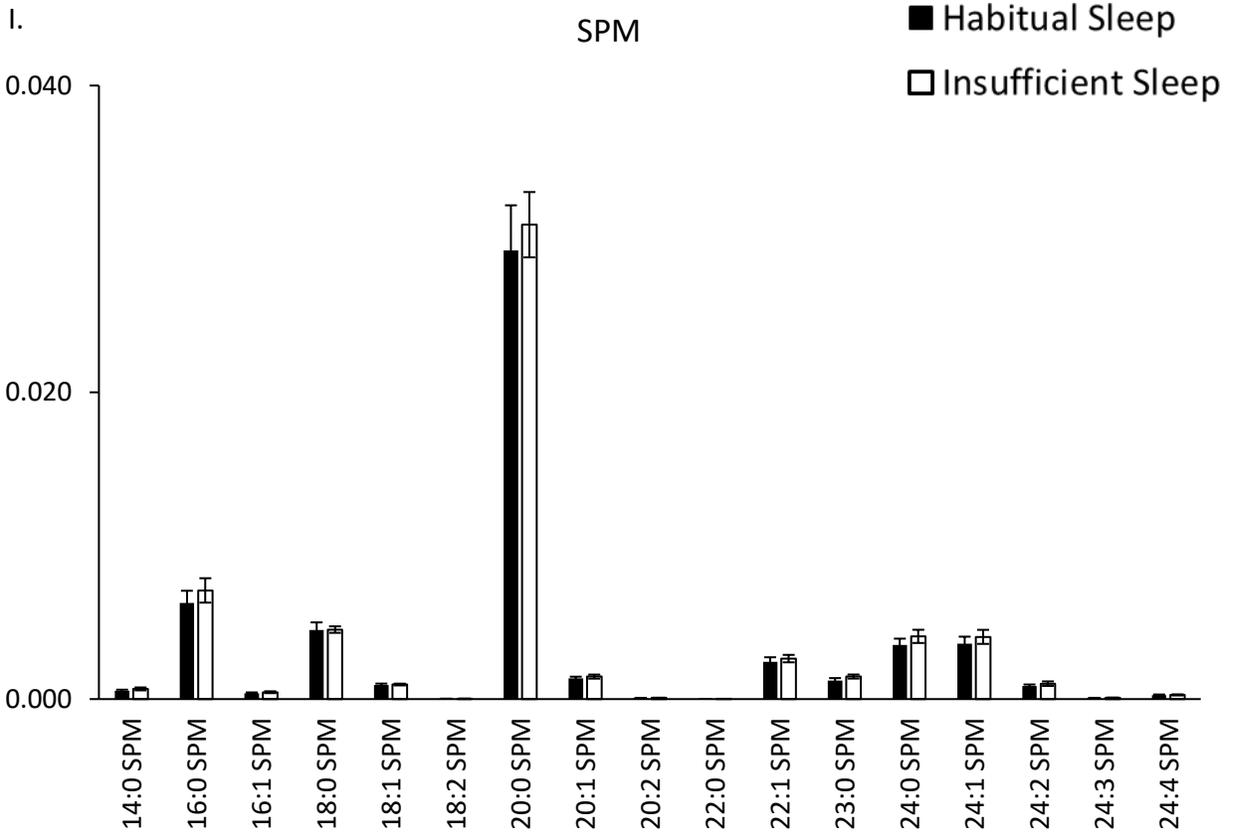
I. SPM

J. AC
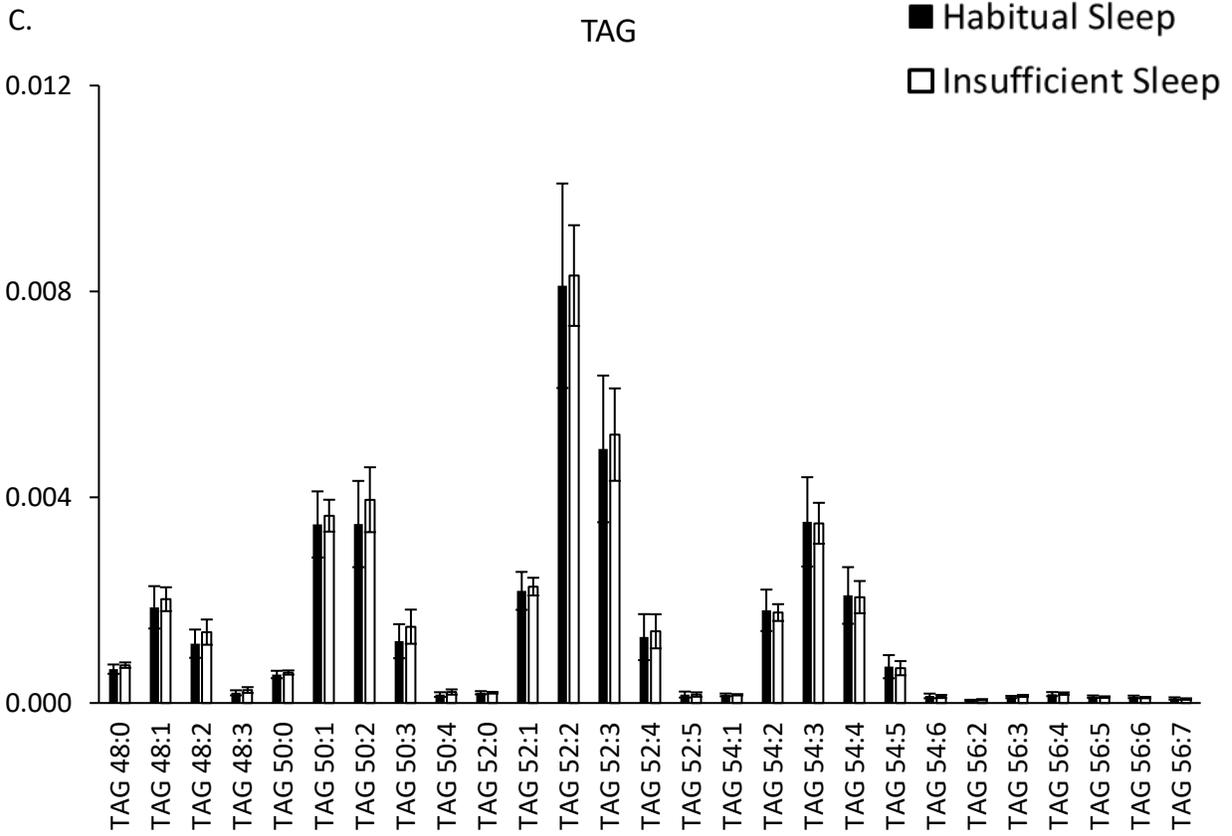
Figure 5.6 Impact of insufficient sleep on specific lipid species of (A) 1,2-diacylglycerols, (B) 1,3-diacylglycerols, (C) triacylglycerols, (D) ceramides, (E) lactosylceramides, (F) dihydroceramides, (G) glucosylceramides, (H) galactosylceramides, (I) sphingomyelin, (J) acylcarnitnes in skeletal muscle (n=10). Lipid concentration is on the x-axis, specific lipid species are on the x-axis. Habitual sleep in the black bars, insufficient sleep in the white bars. *p<0.05

Table 5.2. Change in total lipid concentration as well as each specific lipid species after insufficient sleep (n=10; *p<0.05).
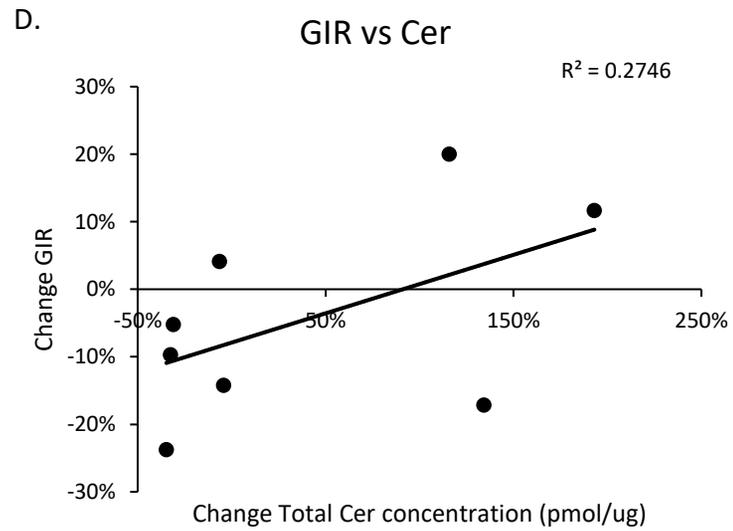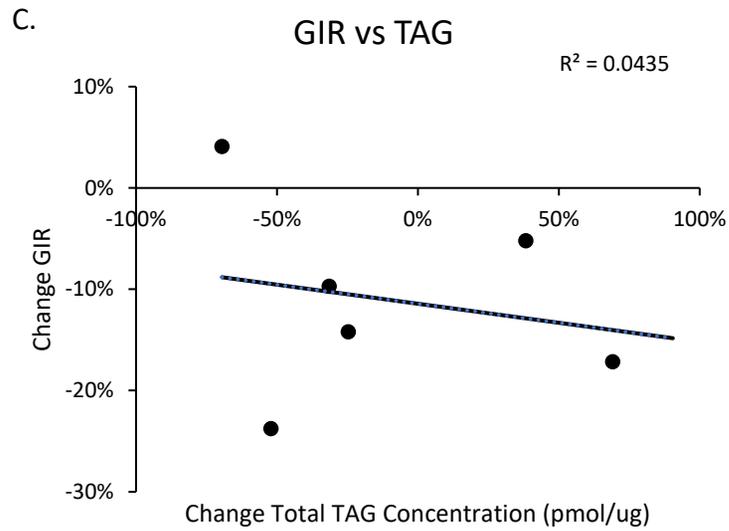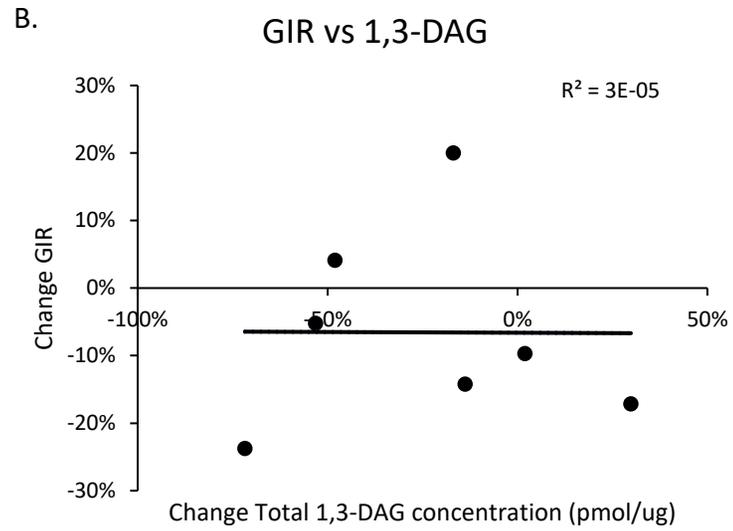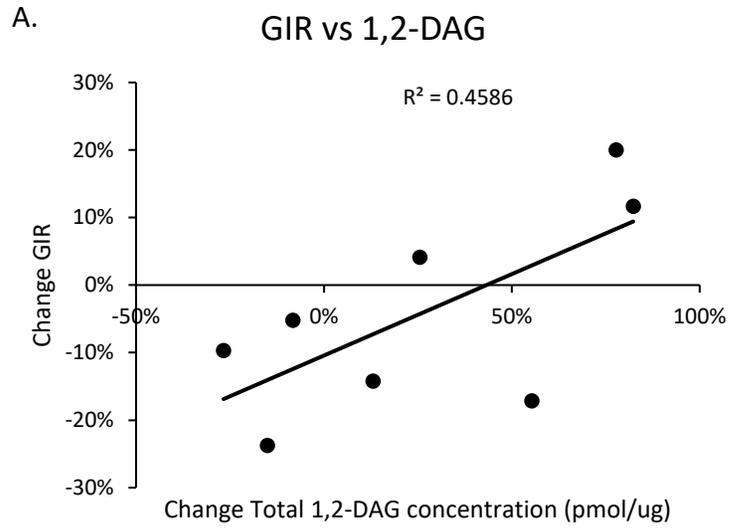
Lipid	P value	Significant
Total 1,2-DAG	0.060999	
Total 1,3-DAG	0.266491	
Total TAG	0.437113	
Total Cer	0.131127	
Total LacCer	0.078659	
Total DHCer	0.093837	
Total GluCer	0.429343	
Total GalCer	0.170937	
Total SPM	0.301959	
Total AC	0.474817	
1,2-12:0/18:0DG	0.070291	
1,2-14:0/16:0DG	0.475339	
1,2-14:0/16:1DG	0.459272	
1,2-14:0/18:0DG	0.389186	
1,2-14:0/18:1DG	0.292522	
1,2-16:0/16:1DG	0.391829	
1,2-16:0/18:0DG	0.051678	
1,2-16:0/18:1DG	0.041259	*
1,2-16:0/18:2DG	0.415942	
1,2-16:0/20:0DG	0.011014	*
1,2-16:0/20:1DG	0.076307	
1,2-16:0/20:2DG	0.045455	*
1,2-16:0/20:3DG	0.047575	*
1,2-16:0/20:4DG	0.082759	
1,2-16:0/22:6DG	0.28904	
1,2-16:1/18:1DG	0.254685	
1,2-18:0/16:1DG	0.121837	
1,2-18:0/18:1DG	0.021353	*
1,2-18:0/18:2DG	0.095606	
1,2-18:0/18:3DG	0.112453	
1,2-18:0/20:4DG	0.070003	
1,2-18:0/22:6DG	0.066576	
1,2-18:2/18:1DG	0.258502	
1,2-di12:0DG	0.180962	
1,2-di14:0DG	0.417234	
1,2-di16:0DG	0.427233	

1,2-di16:1DG	0.402975	
1,2-di18:0DG	0.019889	*
1,2-di18:1DG	0.058769	
1,2-di18:2DG	0.250042	
1.3-12:0/18:0DG	0.249557	
1.3-14:0/16:0DG	0.258171	
1.3-14:0/16:1DG	0.435443	
1.3-14:0/18:0DG	0.233613	
1.3-14:0/18:1DG	0.31604	
1.3-16:0/16:1DG	0.19943	
1.3-16:0/18:0DG	0.499787	
1.3-16:0/18:1DG	0.116971	
1.3-16:0/18:2DG	0.378047	
1.3-16:0/20:0DG	0.292133	
1.3-16:0/20:1DG	0.203013	
1.3-16:0/20:2DG	0.188477	
1.3-16:0/20:3DG	0.290797	
1.3-16:0/20:4DG	0.307061	
1.3-16:0/22:6DG	0.287529	
1.3-16:1/18:1DG	0.300473	
1.3-18:0/16:1DG	0.236144	
1.3-18:0/18:1DG	0.278405	
1.3-18:0/18:2DG	0.446354	
1.3-18:0/18:3DG	0.01101	*
1.3-18:0/20:4DG	0.385069	
1.3-18:0/22:6DG	0.256843	
1.3-18:2/18:1DG	0.229724	
1.3-di12:0DG	0.22654	
1.3-di14:0DG	0.020831	*
1.3-di16:0DG	0.414166	
1.3-di16:1DG	0.357111	
1.3-di18:0DG	0.057508	
1.3-di18:1DG	0.292709	
1,3-di18:2DG	0.223862	
TAG 48:0	0.258094	
TAG 48:1	0.399358	
TAG 48:2	0.328458	
TAG 48:3	0.291737	
TAG 50:0	0.314395	
TAG 50:1	0.422656	

TAG 50:2	0.363341	
TAG 50:3	0.322584	
TAG 50:4	0.291963	
TAG 52:0	0.491582	
TAG 52:1	0.428347	
TAG 52:2	0.470084	
TAG 52:3	0.445931	
TAG 52:4	0.433729	
TAG 52:5	0.497694	
TAG 54:1	0.472574	
TAG 54:2	0.465534	
TAG 54:3	0.490159	
TAG 54:4	0.482184	
TAG 54:5	0.461501	
TAG 54:6	0.430405	
TAG 56:2	0.158393	
TAG 56:3	0.255134	
TAG 56:4	0.427665	
TAG 56:5	0.388587	
TAG 56:6	0.304309	
TAG 56:7	0.342965	
C14:0 Cer	0.279023	
C16:0 Cer	0.298735	
C16:1 Cer	0.005914	*
C18:0 Cer	0.28452	
C18:1 Cer	0.387316	
C20:0 Cer	0.252491	
C22:0 Cer	0.06486	
C22:1 Cer	0.189685	
C23:0 Cer	0.213642	
C23:1 Cer	0.098384	
C24:0 Cer	0.165809	
C24:1 Cer	0.13087	
C24:2 Cer	0.189556	
C26:0 Cer	0.173993	
C26:1 Cer	0.271191	
C16:0 LacCer	0.147748	
C18:0 LacCer	0.227311	
C20:0 LacCer	0.185136	
C22:0 LacCer	0.047657	*

C23:0 LacCer	0.156668	
C24:0 LacCer	0.033631	*
C24:1 LacCer	0.025169	*
C16:0 DHCer	0.130527	
C18:0 DHCer	0.443405	
C22:0 DHCer	0.147913	
C22:1 DHCer	0.059419	
C24:0 DHCer	0.106571	
C24:1 DHCer	0.312007	
C16:0 GluCer	0.289125	
C18:0 GluCer	0.416695	
C20:0 GluCer	0.435479	
C22:0 GluCer	0.495683	
C23:0 GluCer	0.463063	
C24:0 GluCer	0.397945	
C24:1 GluCer	0.417001	
C16:0 GalCer	0.3004	
C18:0 GalCer	0.160327	
C20:0 GalCer	0.134299	
C22:0 GalCer	0.181603	
C23:0 GalCer	0.176894	
C24:0 GalCer	0.174143	
C24:1 GalCer	0.168191	
14:0 SPM	0.169353	
16:0 SPM	0.271094	
16:1 SPM	0.247546	
18:0 SPM	0.478313	
18:1 SPM	0.415783	
18:2 SPM	0.292219	
20:0 SPM	0.363035	
20:1 SPM	0.298749	
20:2 SPM	0.114165	
22:0 SPM	0.174856	
22:1 SPM	0.273246	
23:0 SPM	0.137572	
24:0 SPM	0.19902	
24:1 SPM	0.29028	
24:2 SPM	0.240727	
24:3 SPM	0.245899	
24:4 SPM	0.343009	

4:0 AC	0.054036	
5:0 AC	0.298411	
6:0 AC	0.223081	
8:0 AC	0.232141	
10:0 AC	0.494963	
12:0 AC	0.399333	
14:0 AC	0.427212	
16:0 AC	0.275527	
16:1 AC	0.383637	
18:0 AC	0.427622	
18:1 AC	0.477968	
18:2 AC	0.238011	
18:3 AC	0.177455	

A.

GIR vs 1,2-DAG

R² = 0.4586

Change GIR

Change Total 1,2-DAG concentration (pmol/ug)

B.

GIR vs 1,3-DAG

R² = 3E-05

Change GIR

Change Total 1,3-DAG concentration (pmol/ug)

C.

GIR vs TAG

R² = 0.0435

Change GIR

Change Total TAG Concentration (pmol/ug)

D.

GIR vs Cer

R² = 0.2746

Change GIR

Change Total Cer concentration (pmol/ug)

E. GIR vs LacCer

R² = 0.4463

F. GIR vs DHCer

R² = 0.297

G. GIR vs GluCer

R² = 0.0478

H. GIR vs GalCer

R² = 0.0003

Figure 5.7. There is no significant correlation between insulin sensitivity as measured by the glucose infusion rate (GIR) during the hyperinsulinemic euglycemic clamp and (A) 1,2-diacylglycerols, (B) 1,3-diacylglycerols, (C) triacylglycerols, (D) ceramides, (E) lactosylceramides, (F) dihydroceramides, (G) glucosylceramides, (H) galactosylceramides, (I) sphingomyelin, (J) acylcarnitines.
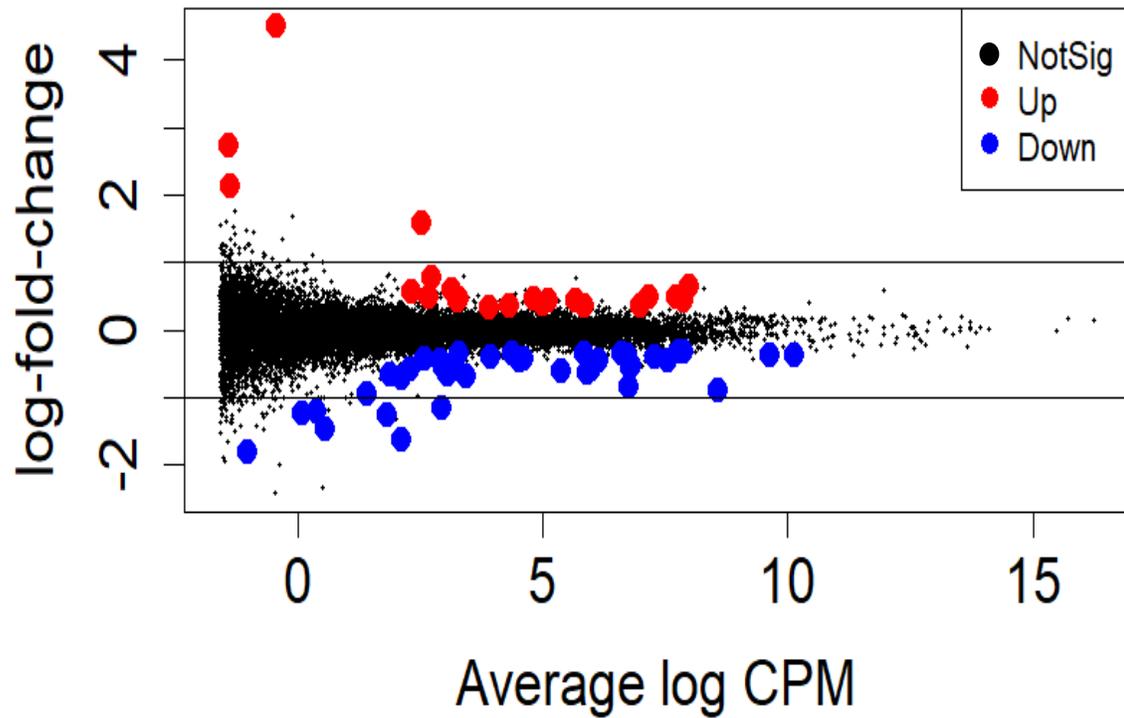
Figure 5.8. Insufficient sleep significantly up-regulated 23 genes (red) and significantly down-regulated 39 genes (blue). (n=12) p<0.05

Table 5.3. Twenty-three genes are significantly up-regulated after insufficient sleep (n=12) p<0.05.

Gene name	Gene type	P value	FDR	Description
CCL18	protein coding	0.000158981	0.045409778	chemokine_(C-C_motif)_ligand_18_(pulmonary_and_activation-regulated)
PIGQ	protein coding	3.18E-05	0.01328385	phosphatidylinositol_glycan_anchor_biosynthesis_class_Q
MYH14	protein coding	5.44E-05	0.018926346	myosin_heavy_chain_14_non-muscle
MPP6	protein coding	1.37E-05	0.007165204	membrane_protein_palmitoylated_6_(MAGUK_p55_subfamily_member_6)
MYL10	protein coding	4.40E-05	0.016708306	myosin_light_chain_10_regulatory
BCL6	protein coding	4.03E-05	0.016027753	B-cell_CLL/lymphoma_6
GPD2	protein coding	2.01E-05	0.009086387	glycerol-3-phosphate_dehydrogenase_2_(mitochondrial)
F13A1	protein coding	2.99E-07	0.000453118	coagulation_factor_XIII_A1_polypeptide
MSTN	protein coding	1.70E-06	0.001489247	myostatin
DDIT4L	protein coding	7.21E-06	0.004540382	DNA-damage-inducible_transcript_4-like
PAK1	protein coding	6.43E-06	0.004292403	p21_protein_(Cdc42/Rac)-activated_kinase_1
FZD7	protein coding	4.34E-06	0.003333747	frizzled_family_receptor_7
AAED1	protein coding	0.000160516	0.04540978	AhpC/TSA_antioxidant_enzyme_domain_containing_1
GDPD5	protein coding	5.42E-05	0.01892635	glycerophosphodiester_phosphodiesterase_domain_containing_5
CMBL	protein coding	4.58E-07	0.000546347	carboxymethylenebutenolidase_homolog_(Pseudomonas)
ZNF219	protein coding	0.000104287	0.03347421	zinc_finger_protein_219
MAPRE2	protein coding	2.27E-06	0.0018935	microtubule-associated_protein_RP/EB_family_member_2
C8orf22	protein coding	2.72E-05	0.01164636	chromosome_8_open_reading_frame_22
GATM	protein coding	1.57E-05	0.007943672	glycine_amidinotransferase_(L-arginine:glycine_amidinotransferase)
BCL2	protein coding	1.54E-06	0.001423647	B-cell_CLL/lymphoma_2
RP11-771D21.2	lincRNA	7.45E-08	0.000155377	-
AC058791.2	processed transcript	3.74E-07	0.000479615	-
RP11-218E20.3	lincRNA	0.000112449	0.03541298	-

Table 5.4. Thirty-nine genes are significantly down-regulated after insufficient sleep (n=12) p<0.05.

Gene name	Gene type	P value	FDR	Description
ST3GAL1	protein coding	1.85559E-09	7.62463E-06	ST3_beta-galactoside_alpha-23-sialyltransferase_1
HDAC4	protein coding	0.000129041	0.039160278	histone_deacetylase_4
WDR62	protein coding	1.14635E-06	0.001125511	WD_repeat_domain_62
TP53INP2	protein coding	3.65534E-07	0.000479615	tumor_protein_p53_inducible_nuclear_protein_2
JAK2	protein coding	0.000177475	0.047777884	Janus_kinase_2
SEL1L2	protein coding	6.58362E-05	0.022425944	sel-1_suppressor_of_lin-12-like_2_(C._elegans)
MYL12A	protein coding	1.27474E-15	2.12767E-11	myosin_light_chain_12A_regulatory_non-sarcomeric
FGF6	protein coding	9.87102E-07	0.001029732	fibroblast_growth_factor_6
PEX7	protein coding	0.000146397	0.043634007	peroxisomal_biogenesis_factor_7
CA14	protein coding	5.07495E-12	4.2353E-08	carbonic_anhydrase_XIV
CASQ2	protein coding	8.27321E-07	0.000920587	calsequestrin_2_(cardiac_muscle)
GOT1	protein coding	0.000125454	0.038776801	glutamic-oxaloacetic_transaminase_1_soluble_(aspartate_aminotransferase_1)
MYOT	protein coding	5.1888E-05	0.018827446	myotilin
COQ10A	protein coding	3.58951E-05	0.014612798	coenzyme_Q10_homolog_A_(S._cerevisiae)
SLCO5A1	protein coding	1.61815E-05	0.007943672	solute_carrier_organic_anion_transporter_family_member_5A1
DNAJA4	protein coding	4.55926E-08	0.000108712	DnaJ_(Hsp40)_homolog_subfamily_A_member_4
SLC16A3	protein coding	2.68986E-07	0.000448964	solute_carrier_family_16_member_3_(monocarboxylic_acid_transporter_4)
MYOM3	protein coding	1.01986E-05	0.005869829	myomesin_3
CREB5	protein coding	4.40456E-05	0.01670831	cAMP_responsive_element_binding_protein_5
KCNB1	protein coding	3.65158E-09	1.01581E-05	potassium_voltage-gated_channel_Shab-related_subfamily_member_1
DNAH3	protein coding	1.74799E-05	0.008335895	dynein_axonemal_heavy_chain_3
LAD1	protein coding	5.59997E-06	0.003894544	ladinin_1
RET	protein coding	7.58946E-05	0.02533513	ret_proto-oncogene
PPP1R3B	protein coding	1.23152E-07	0.000228391	protein_phosphatase_1_regulatory_subunit_3B
HSPB7	protein coding	2.46672E-05	0.01083473	heat_shock_27kDa_protein_family_member_7_(cardiovascular)
LPL	protein coding	7.61672E-06	0.004540382	lipoprotein_lipase
TMEM70	protein coding	0.000159028	0.04540978	transmembrane_protein_70

APOLD1	protein coding	4.39413E-06	0.003333747	apolipoprotein_L_domain_containing_1
SLC2A4	protein coding	1.89691E-05	0.008794802	solute_carrier_family_2_(facilitated_glucose_transporter)_member_4
CCR3	protein coding	1.06344E-05	0.00591665	chemokine_(C-C_motif)_receptor_3
PDE4B	protein coding	1.65285E-09	7.62463E-06	phosphodiesterase_4B_cAMP-specific
KLHL34	protein coding	7.40862E-06	0.004540382	kelch-like_family_member_34
RP11-145A3.1	antisense	2.28405E-09	7.62463E-06	-
LINC00312	lincRNA	4.72965E-06	0.003432285	long_intergenic_non-protein_coding_RNA_312
CTC-454M9.1	processed transcript	4.97874E-05	0.01846668	-
IQCH-AS1	lincRNA	0.000166623	0.04611674	IQCH_antisense_RNA_1
RP4-791K14.2	lincRNA	0.000168541	0.04611674	-
RP11-594N15.3	sense overlapping	0.0000780390	0.02554018	-
MYH4	protein coding	1.25075E-05	0.006734285	myosin_heavy_chain_4_skeletal_muscle

A.
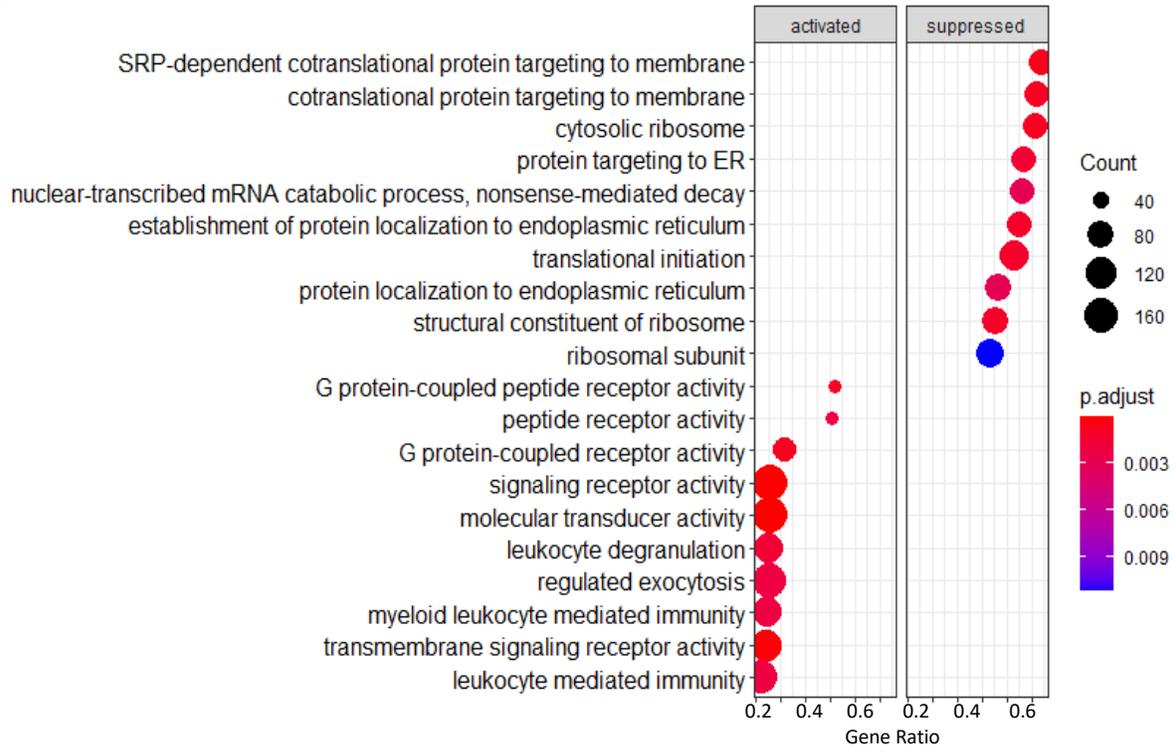
B.
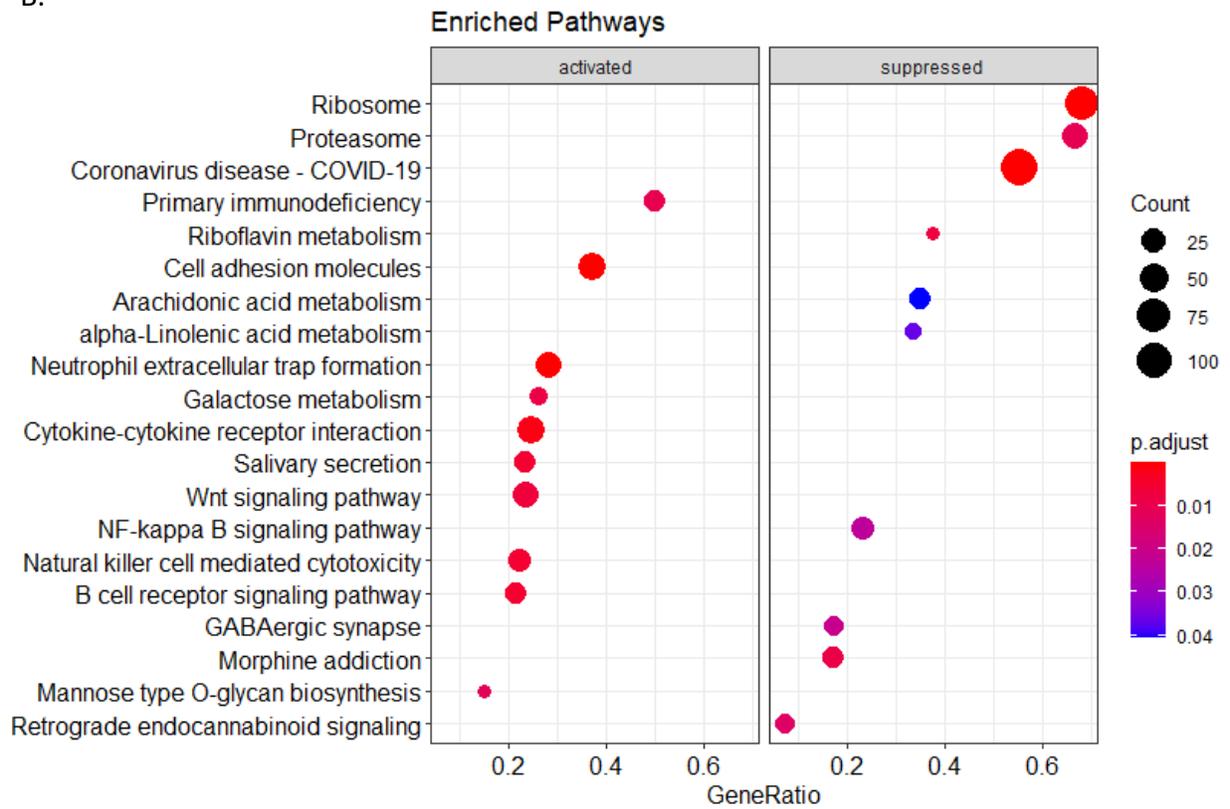
Figure 5.9. Significant (A) gene ontology (GO) and (B) Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses reveals activation and suppression of pathways increasing the risk for obesity and diabetes (n=12).

Table 5.5. Seventeen genes are significantly correlated with impaired insulin sensitivity after insufficient sleep (n=12) p<0.05.

Gene name	Gene type	Correlation	P value	P adjust	Description
RP11-932O9.7	antisense	-0.976	0	0	-
SLC28A1	protein coding	0.945	0	0	solute_carrier_family_28_(sodium-coupled_nucleoside_transporter)_member_1
RP11-37C7.3	antisense	0.941	0	0	-
C12orf76	protein coding	0.936	0	0	chromosome_12_open_reading_frame_76
STOML2	protein coding	0.918	0	0	stomatin_(EPB72)-like_2
HEXIM2	protein coding	0.909	0	0	hexamethylene_bis-acetamide_inducible_2
MCOLN1	protein coding	0.9	0	0	mucolipin_1
HMOX1	protein coding	0.9	0	0	heme_oxygenase_(decycling)_1
DUSP14	protein coding	0.9	0	0	dual_specificity_phosphatase_14
GALNT11	protein coding	0.9	0	0	UDP-N-acetyl-alpha-D-galactosamine:polypeptide_N-acetylgalactosaminyltransferase_11_(GalNAc-T11)
B9D1	protein coding	0.897	0	0	B9_protein_domain_1
TNNI3	protein coding	0.895	0	0	troponin_I_type_3_(cardiac)
ACOT13	protein coding	0.891	0	0	acyl-CoA_thioesterase_13
TUSC2	protein coding	0.891	0	0	tumor_suppressor_candidate_2
LYSMD2	protein coding	0.891	0	0	LysM_putative_peptidoglycan-binding_domain_containing_2
NHP2	protein coding	0.891	0	0	NHP2_ribonucleoprotein
CSNK2B	protein coding	0.891	0	0	casein_kinase_2_beta_polypeptide
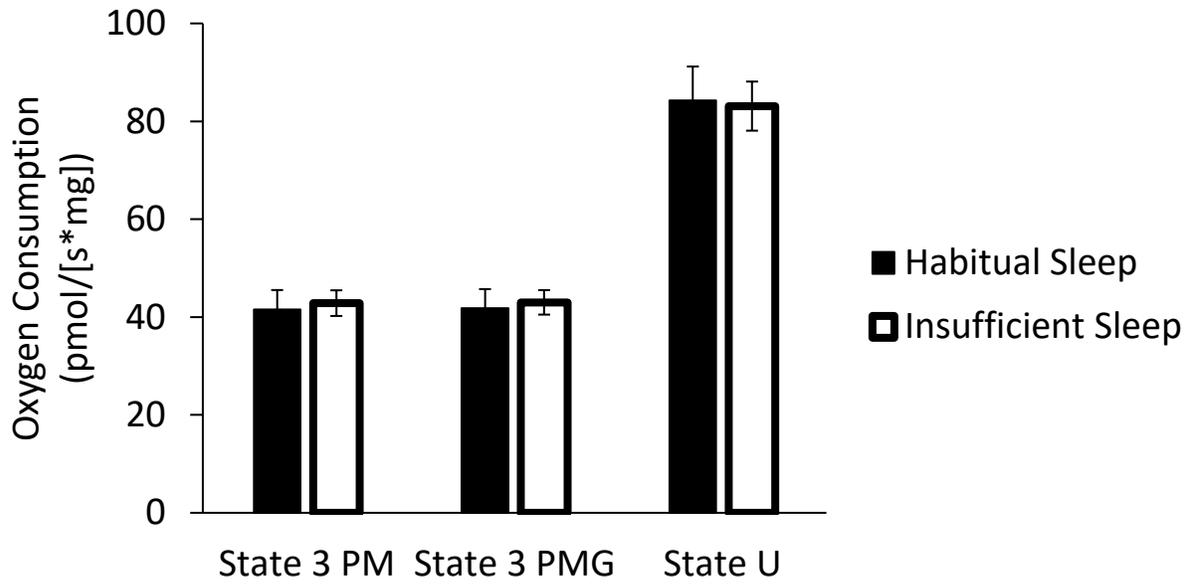
Figure 5.10. Mitochondrial oxidative capacity (mean±SEM) is not different in ADP-stimulated (State 3) or maximally uncoupled (State U) respirations between habitual sleep and insufficient sleep (n=17). Oxygen consumption (pmol/[s*mg]) is on the y-axis. P=pyruvate; M=malate; G=glutamate.

A.

State 3 PM

R² = 0.1128

Change GIR

Change O2 Consumption

B.

State 3 PMG

R² = 0.1154

Change GIR

Change O2 Consumption

C.

State U

R² = 0.0351

Change GIR

Change O2 Consuption
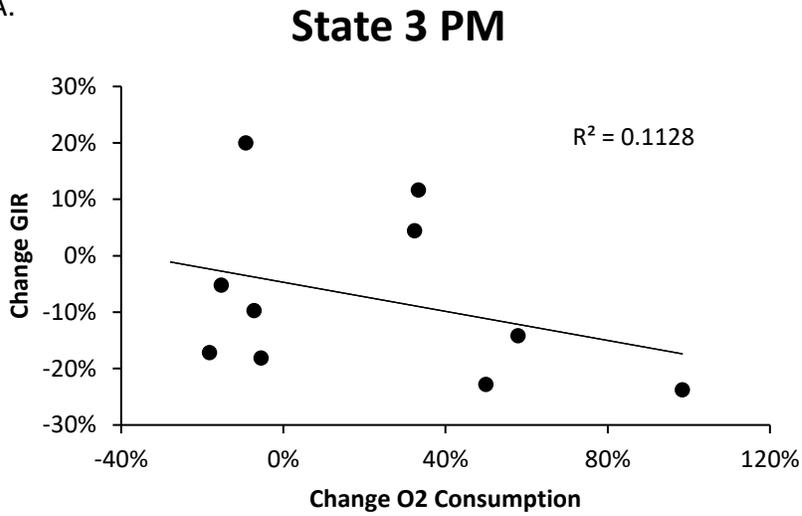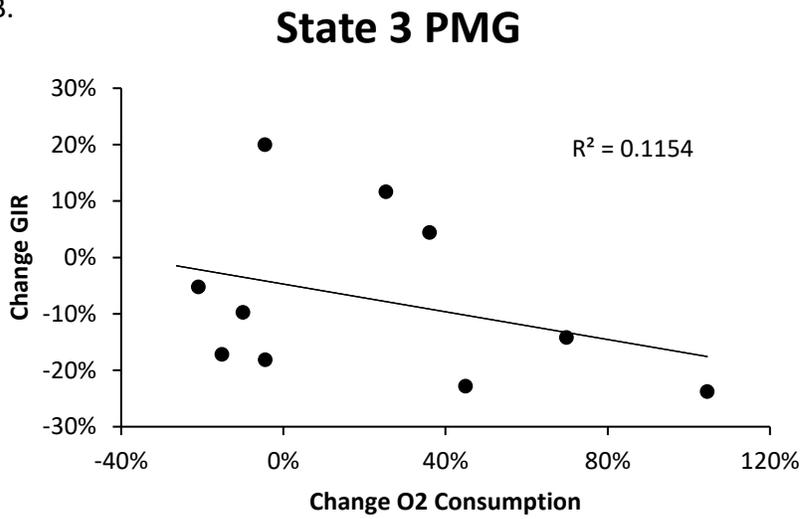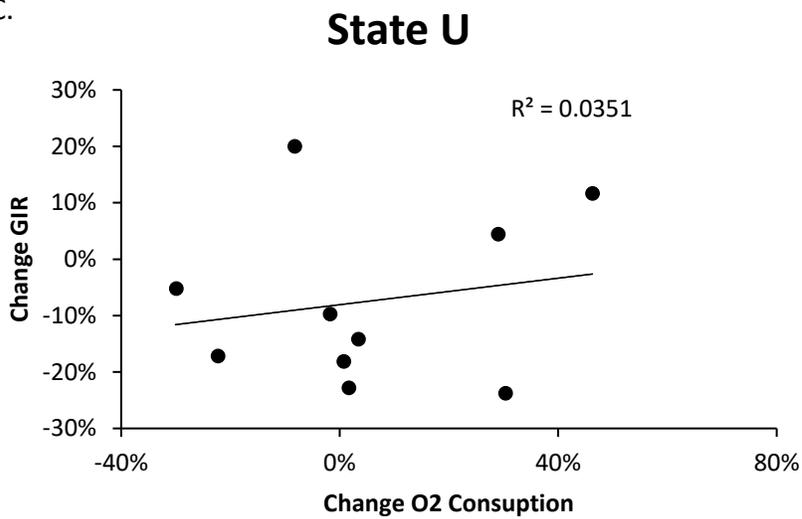
Figure 5.11. There are no correlations between ADP-stimulated (State 3) or maximally uncoupled (State U) mitochondrial respirations and insulin sensitivity as measured by the glucose infusion rate (GIR) during the hyperinsulinemic euglycemic clamp. Change in GIR is on the y-axis. Change in oxygen consumption is on the x-axis.

CHAPTER VI – DISCUSSION AND CONCLUSIONS

Sleep and circadian rhythms have been studied for thousands of years. In ancient times, it was thought that the soul left the body during sleep and that the brain turned off so there were no experiences during sleep. The 2017 finding that jellyfish show sleep-like behavior was the first study to show that an organism without a central nervous system had sleep-like behaviors suggesting the evolutionary benefit and ubiquitous nature of sleep (Nath et al., 2017). Additionally, the first studies of the circadian system found diurnal leaf movements independent of external factors, including sunlight (de Mairan, 1729; Bretzl, 1903). More recently, it has been found that circadian rhythms are ubiquitous among most cells as well as numerous organisms (Zhang et al., 2014; Andreani et al., 2015; Takahashi, 2017). With the 2017 Nobel Prize in Physiology or Medicine awarded to Hall, Rosbash, and Young, for the discoveries of the molecular mechanisms controlling circadian rhythms, research in circadian rhythms is booming. However, now, we have evidence that sleep and circadian disruption is linked to numerous diseases and disorders including metabolic diseases, cardiovascular diseases, various types of cancer, and reproductive and mental health (Knutsson et al., 1986; Stevens and Rea, 2001; Labyak et al., 2002; Di Lorenzo et al., 2003; Knutsson, 2003; Ishizaki et al., 2004; Davis and Mirick, 2006; Schernhammer et al., 2006; Tuchsen et al., 2006; Kroenke et al., 2007; Stevens et al., 2007; Biggi et al., 2008; Scheer et al., 2009; Spiegel et al., 2009; Cappuccio et al., 2010; Knutson, 2010; Cappuccio et al., 2011; Pan et al., 2011; Canuto et al., 2013; Guo et al., 2013; Gan et al., 2015; Almoosawi et al., 2019). A meta-analysis found that sleep and circadian disruption (i.e., sleep duration ≤5 hours, shift work) were as significant risk factors for type 2 diabetes as traditional risk factors of overweight, family history, and physical inactivity (Anothaisintawee et al., 2016). Therefore, the overall purpose of this dissertation is to identify aspects of metabolic homeostasis influenced by circadian regulation and sleep disruption.

The first study of this dissertation (Chapter 2, Study 1) utilized the constant routine protocol to identify circadian rhythms in substrate oxidation and appetite hormones. Eight healthy, young, lean adults participated in a 26-hour inpatient constant routine protocol. The constant routine is a circadian protocol that removes, equally distributes, or makes constant all factors that may influence the circadian system in order to identify circadian rhythms. We found a circadian rhythm in substrate oxidation such that fat oxidation was highest in the biological evening and carbohydrate oxidation was highest in the biological morning. Additionally, circadian rhythms were revealed in ghrelin with a peak in the biological evening and peptide YY with a peak in the biological morning. This study suggests the circadian system influences factors important for substrate oxidation and hormonal regulators of energy balance (Rynders et al., 2020).

While this study was the first to investigate substrate oxidation and appetite hormones simultaneously during a constant routine protocol, it is not without its limitations. First, we did not measure protein metabolism and thus were unable to adjust our respirometry outcomes for protein metabolism. Therefore, our measures of carbohydrate and fat oxidation may be influenced by the unmeasured protein metabolism. In addition, this study used a short duration constant routine protocol; however, the 26-hour protocol utilized for this study was longer than the average 24h rhythm. Thus, even with this short protocol duration, we were still able to collect a full circadian cycle. Finally, this study utilized a small sample size; however, similar studies utilizing circadian protocols have reported significant outcomes using a similar sample size. Moreover, with the highly controlled nature of the constant routine protocol, circadian rhythms can be detected using these smaller sample sizes.

The next chapter (Chapter 3) reviewed the methods of measuring insulin sensitivity in humans. Here, we review metabolic physiology and insulin sensitivity in the whole body as well as at the level of each main metabolic tissue: white adipose tissue, liver, and skeletal muscle. In general, insulin acts on each of these tissues to increase fuel storage and decrease fuel

production; however, the mechanisms by which this occurs is tissue-specific. For example, in white adipose tissue, insulin increases glucose uptake, increases lipogenesis, and decreases lipolysis. In the liver, insulin increases glycogen synthesis, decreases gluconeogenesis, and increases de novo lipogenesis. Finally, in the skeletal muscle, insulin increases glucose uptake and increases glycogen synthesis.

We then review the methods of measuring whole body and tissue-specific insulin sensitivity in humans. Homeostatic Assessment of Insulin Resistance (HOMA-IR) uses fasting glucose and insulin concentrations to estimate whole body insulin resistance (Matthews et al., 1985). While this method is simple and inexpensive, severe limitations exist including the inability of HOMA-IR to detect changes in insulin resistance confirmed by the gold-standard technique the hyperinsulinemic euglycemic clamp. The Oral Glucose Tolerance Test (OGTT) measures whole body insulin sensitivity using glucose and insulin levels at baseline and for 2-3 hours after oral consumption of a glucose drink (Bang, 1913; Matsuda and DeFronzo, 1999). The OGTT is the only method that assess the response to glucose in a physiological manner given its oral administration. While the OGTT does measure a systemic challenge, the standard glucose load (typically 75 g) regardless of bodyweight delivers a variable amount of glucose (g/kg) between participants. The Intravenous Glucose Tolerance Test (IVGTT) measures whole body insulin sensitivity as well as various other metabolic parameters (Crawford, 1938; Bergman et al., 1979). Blood glucose and insulin levels are measured at baseline, after a glucose bolus, and after an insulin bolus to calculate insulin sensitivity (Si), Disposition Index (DI), acute insulin response to glucose (AIRg), and glucose effectiveness (Sg) (Bergman et al., 1979; Bergman, 2020). The advantage of the IVGTT includes these other metabolic parameters that can be measured as part of the assessment, in addition to insulin sensitivity. However, glucose and insulin are injected intravenously at supra-physiological doses thereby not representing a physiological condition. And finally, the hyperinsulinemic euglycemic clamp is the gold standard method of measuring insulin sensitivity (DeFronzo et al., 1979). The clamp

method requires a constant hyperinsulinemic infusion, frequent blood sampling, and variable glucose infusion to maintain euglycemia at 90mg/dL. The clamp also uses supra-physiological intravenous infusions of glucose and insulin, thereby bypassing the gut and incretin system. However, using multi-dose insulin infusions and isotopically labeled tracers, distinct measurements of whole body, white adipose tissue, liver, and skeletal muscle specific insulin sensitivity can be measured. Thus, Chapter 3 reviews methods of measuring whole body and tissue-specific insulin resistance in humans.

In Chapter 4, we revisit the above methods of measuring insulin sensitivity in view of special considerations for people with sleep and circadian disruption. Using the HOMA-IR, many studies show impaired insulin sensitivity after insufficient sleep in well controlled, inpatient studies of young, healthy, lean adults (Spiegel et al., 2004; Reynolds et al., 2012; Killick et al., 2015; Cedernaes et al., 2016; Cedernaes et al., 2018). In contrast, outpatient studies and older participants with insufficient sleep have not observed changes in HOMA-IR (Zielinski et al., 2008; Robertson et al., 2013; Wang et al., 2016). Using the OGTT, tightly controlled inpatient studies of young, healthy, lean adults and in outpatient studies reported insufficient sleep impairs whole body insulin sensitivity (Eckel et al., 2015; Killick et al., 2015; Wang et al., 2016; Cedernaes et al., 2018; Sweeney et al., 2020). The IVGTT was the method used by the first study that launched the intersect of the fields of sleep, circadian rhythms, and metabolic physiology (Spiegel et al., 1999). Since that first 1999 study, highly controlled inpatient studies of insufficient sleep in healthy, young, lean adults as well as adults with pre-existing risk factors for metabolic disease, including middle-age and overweight, consistently reported impaired insulin sensitivity using the IVGTT (Spiegel et al., 1999; Nedeltcheva et al., 2009; Buxton et al., 2010; Leproult and Van Cauter, 2010; Broussard et al., 2012; Broussard et al., 2015b; Eckel et al., 2015; Broussard et al., 2016; Ness et al., 2019). Finally, using the gold standard hyperinsulinemic euglycemic clamp, rigorously controlled inpatient studies of healthy, lean, young and middle aged adults revealed that insufficient sleep impairs whole body as well as

skeletal muscle-specific insulin sensitivity (Buxton et al., 2010; Donga et al., 2010b; Rao et al., 2015; Depner et al., 2019). However, one study reported impaired hepatic insulin sensitivity following insufficient sleep whereas two others reported no change (Donga et al., 2010b; Rao et al., 2015; Depner et al., 2019). Only one study has investigated the impact of insufficient sleep on adipose tissue insulin sensitivity using the hyperinsulinemic euglycemic clamp and found no impairments (Depner et al., 2019).

A small number of studies have investigated the effects of circadian misalignment on insulin sensitivity using tightly controlled inpatient protocols in healthy, young, lean adults and report impaired insulin sensitivity as assessed by OGTT, IVGTT, and the hyperinsulinemic euglycemic clamp (Leproult et al., 2014; Bescos et al., 2018; Qian et al., 2018; Wefers et al., 2018). Sleep disorders such as Obstructive Sleep Apnea (OSA) and insomnia are common and are associated with increased risk for cardiovascular diseases, hypertension, heart failure, type 2 diabetes, and metabolic syndrome (Chen et al., 2015; Senaratna et al., 2016; Javaheri and Redline, 2017; Sokwalla et al., 2017; Larsson and Markus, 2019). However, it is unknown whether these associations are due to the direct effects of the specific sleep disorder or indirectly via accompanying reductions in sleep duration and/or quality. Therefore, prior sleep and circadian history cannot be ignored prior to metabolic testing. Thus, we provide various methods that can be implemented, including sleep disorder screening, sleep diaries, consistent sleep schedules, and habitual bed/wake times, before metabolic assessments to more fully understand and control for prior sleep and circadian history.

Insufficient sleep impairs insulin sensitivity; however, the mechanisms by which this occurs are unknown. Thus, Study 2 (Chapter 5) measured skeletal muscle lipid accumulation, gene expression, and mitochondrial function in 18 healthy, young, lean participants and their relationship to insulin sensitivity before and after insufficient sleep. We found that insulin sensitivity was significantly impaired following insufficient sleep. We report increased levels of specific 1,2-DAG species, previously implicated in impaired insulin resistance. However, there

were no correlations between any total lipid concentration and whole body insulin sensitivity. Moreover, we found alterations in gene expression of genes involved in lipid metabolism, such as lipoprotein lipase. However, we found no functional outcome changes in mitochondrial respiration in skeletal muscle after insufficient sleep. Overall, the results of this study suggest that insufficient sleep impairs skeletal muscle lipid metabolism, which is related to impaired insulin sensitivity in healthy adults.

One limitation to this study was the use of only carbohydrate substrates for mitochondrial respirations. Given the results of this study, lipid substrates should be used in future studies. Additionally, this study was powered for changes in insulin sensitivity. While we did see a significant decrease in insulin sensitivity after insufficient sleep, it is possible that our other outcomes of interest (i.e., lipid accumulation and mitochondrial function) were underpowered. Especially with the missing data and outliers for the correlation analyses with change in insulin sensitivity and change in total lipids or changes in mitochondrial function, it is possible that if we had more participants then significant correlations would have emerged.

The studies included in this dissertation move the field forward with the identification of circadian rhythms in substrate oxidation and appetite hormones as well as the observation that insufficient sleep induces skeletal muscle lipid accumulation and insulin resistance in healthy adults. Future studies are needed to continue progressing the field. For example, mitochondrial function using lipid substrates should be investigated after insufficient sleep. In addition, circadian rhythms in protein metabolism should be measured to complete the full equation of rhythms in substrate metabolism. We identified skeletal muscle lipid accumulation after insufficient sleep; however, more research is needed to continue investigating other mechanisms by which insufficient sleep impairs metabolic physiology. Unfortunately, sleep and circadian disruptions will always be a major part of industrialized society. Thus, countermeasures must be identified to limit metabolic impairments after sleep and circadian disruption. Some countermeasures are beginning to be investigated, including light therapy and

time restricted eating. More research is needed to clarify the relationship between circadian interventions (i.e., light exposure, timing of behaviors) and health and diseases.

In conclusion, this dissertation introduced sleep and circadian physiology (Chapter 1) and investigated the circadian regulation of substrate oxidation and appetite hormones (Chapter 2, Study 1). Then, methods of measuring insulin resistance were reviewed (Chapter 3) followed by special considerations for metabolic assessments for people with sleep and circadian disruption (Chapter 4). Moreover, we investigate lipid accumulation after insufficient sleep and its relation to insulin sensitivity (Chapter 5, Study 2). Finally, we provide a summary of these studies, limitations, and future directions for the field, including possible countermeasures (Chapter 6).

REFERENCES

(1999). Sleep-related breathing disorders in adults: recommendations for syndrome definition and measurement techniques in clinical research. The Report of an American Academy of Sleep Medicine Task Force. *Sleep* 22(5)**,** 667-689.

(2000). National Sleep Foundation Executive Summary of the 2000 Omnibus Sleep in America Poll.

(2010). Painting, firefighting, and shiftwork. *IARC Monogr Eval Carcinog Risks Hum* 98**,** 9-764.

(2011). National Sleep Foundation Annual Sleep in America Poll.

(2014). *International Classification of Sleep Disorders.* American Academy of Sleep Medicine.

Adam, E.K., Quinn, M.E., Tavernier, R., McQuillan, M.T., Dahlke, K.A., and Gilbert, K.E. (2017). Diurnal cortisol slopes and mental and physical health outcomes: A systematic review and meta-analysis. *Psychoneuroendocrinology* 83**,** 25-41. doi: 10.1016/j.psyneuen.2017.05.018.

Ader, M. (1997). Physiologic principles underlying glucose effectiveness. *The Minimal Model Approach and Determinants of Glucose Tolerance. Pennington Center Nutrition Series* 7**,** 159-186.

Ader, M., Stefanovski, D., Richey, J.M., Kim, S.P., Kolka, C.M., Ionut, V., et al. (2014). Failure of homeostatic model assessment of insulin resistance to detect marked diet-induced insulin resistance in dogs. *Diabetes* 63(6)**,** 1914-1919. doi: 10.2337/db13-1215.

Alberti, C. (1958). [Melatonin: the first hormone isolated from the pineal body]. *Farmaco Sci* 13(8)**,** 604-605.

Allada, R., and Chung, B.Y. (2010). Circadian organization of behavior and physiology in Drosophila. *Annu Rev Physiol* 72**,** 605-624. doi: 10.1146/annurev-physiol-021909-135815.

Allada, R., and Siegel, J.M. (2008). Unearthing the phylogenetic roots of sleep. *Curr Biol* 18(15)**,** R670-R679. doi: 10.1016/j.cub.2008.06.033.

Almoosawi, S., Vingeliene, S., Gachon, F., Voortman, T., Palla, L., Johnston, J.D., et al. (2019). Chronotype: Implications for Epidemiologic Studies on Chrono-Nutrition and Cardiometabolic Health. *Adv Nutr* 10(1)**,** 30-42. doi: 10.1093/advances/nmy070.

Alvarez, J.D., and Sehgal, A. (2005). The thymus is similar to the testis in its pattern of circadian clock gene expression. *J Biol Rhythms* 20(2)**,** 111-121. doi: 10.1177/0748730404274078.

Andreani, T.S., Itoh, T.Q., Yildirim, E., Hwangbo, D.S., and Allada, R. (2015). Genetics of Circadian Rhythms. *Sleep Med Clin* 10(4)**,** 413-421. doi: 10.1016/j.jsmc.2015.08.007.

Anothaisintawee, T., Reutrakul, S., Van Cauter, E., and Thakkinstian, A. (2016). Sleep disturbances compared to traditional risk factors for diabetes development: Systematic review and meta-analysis. *Sleep Med Rev* 30**,** 11-24. doi: 10.1016/j.smrv.2015.10.002.

Armitage, R., Lee, J., Bertram, H., and Hoffmann, R. (2013). A preliminary study of slow-wave EEG activity and insulin sensitivity in adolescents. *Sleep Med* 14(3)**,** 257-260. doi: 10.1016/j.sleep.2012.11.012.

Aronsohn, R.S., Whitmore, H., Van Cauter, E., and Tasali, E. (2010). Impact of untreated obstructive sleep apnea on glucose control in type 2 diabetes. *Am J Respir Crit Care Med* 181(5)**,** 507-513. doi: 10.1164/rccm.200909-1423OC.

Arredondo, E., Udeani, G., Panahi, L., Taweesedt, P.T., and Surani, S. (2021). Obstructive Sleep Apnea in Adults: What Primary Care Physicians Need to Know. *Cureus* 13(9), e17843. doi: 10.7759/cureus.17843.

Aserinsky, E., and Kleitman, N. (1953). Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. *Science* 118(3062), 273-274. doi: 10.1126/science.118.3062.273.

Aserinsky, E., and Kleitman, N. (1955). Two types of ocular motility occurring in sleep. *J Appl Physiol* 8(1), 1-10. doi: 10.1152/jappl.1955.8.1.1.

Bajaj, M., and Banerji, M.A. (2004). Type 2 diabetes in South Asians: a pathophysiologic focus on the Asian-Indian epidemic. *Curr Diab Rep* 4(3), 213-218. doi: 10.1007/s11892-004-0026-4.

Baker, R.G., Hayden, M.S., and Ghosh, S. (2011). NF-kappaB, inflammation, and metabolic disease. *Cell Metab* 13(1), 11-22. doi: 10.1016/j.cmet.2010.12.008.

Bang, I.C. (1913). *Der Blutzucker.* Bergmann.

Barger, L.K., Wright, K.P., Jr., Hughes, R.J., and Czeisler, C.A. (2004). Daily exercise facilitates phase delays of circadian melatonin rhythm in very dim light. *Am J Physiol Regul Integr Comp Physiol* 286(6), R1077-1084. doi: 10.1152/ajpregu.00397.2003.

Bargiello, T.A., Jackson, F.R., and Young, M.W. (1984). Restoration of circadian behavioural rhythms by gene transfer in Drosophila. *Nature* 312(5996), 752-754. doi: 10.1038/312752a0.

Bartlette, K., Carreau, A.M., Xie, D., Garcia-Reyes, Y., Rahat, H., Pyle, L., et al. (2021). Oral minimal model-based estimates of insulin sensitivity in obese youth depend on oral glucose tolerance test protocol duration. *Metabol Open* 9, 100078. doi: 10.1016/j.metop.2021.100078.

Beard, J.C., Bergman, R.N., Ward, W.K., and Porte, D., Jr. (1986). The insulin sensitivity index in nondiabetic man. Correlation between clamp-derived and IVGTT-derived values. *Diabetes* 35(3), 362-369. doi: 10.2337/diab.35.3.362.

Belfort, R., Mandarino, L., Kashyap, S., Wirfel, K., Pratipanawatr, T., Berria, R., et al. (2005). Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* 54(6), 1640-1648. doi: 10.2337/diabetes.54.6.1640.

Benloucif, S., Guico, M.J., Reid, K.J., Wolfe, L.F., L'Hermite-Baleriaux, M., and Zee, P.C. (2005). Stability of melatonin and temperature as circadian phase markers and their relation to sleep times in humans. *J Biol Rhythms* 20(2), 178-188. doi: 10.1177/0748730404273983.

Bergman, B.C., Hunerdosse, D.M., Kerege, A., Playdon, M.C., and Perreault, L. (2012). Localisation and composition of skeletal muscle diacylglycerol predicts insulin resistance in humans. *Diabetologia* 55(4), 1140-1150. doi: 10.1007/s00125-011-2419-7.

Bergman, R.N. (2020). Origins and History of the Minimal Model of Glucose Regulation. *Front Endocrinol (Lausanne)* 11, 583016. doi: 10.3389/fendo.2020.583016.

Bergman, R.N., Ider, Y.Z., Bowden, C.R., and Cobelli, C. (1979). Quantitative estimation of insulin sensitivity. *Am J Physiol* 236(6), E667-677. doi: 10.1152/ajpendo.1979.236.6.E667.

Bergman, R.N., Zaccaro, D.J., Watanabe, R.M., Haffner, S.M., Saad, M.F., Norris, J.M., et al. (2003). Minimal model-based insulin sensitivity has greater heritability and a different genetic basis than homeostasis model assessment or fasting insulin. *Diabetes* 52(8), 2168-2174. doi: 10.2337/diabetes.52.8.2168.

Bescos, R., Boden, M.J., Jackson, M.L., Trewin, A.J., Marin, E.C., Levinger, I., et al. (2018). Four days of simulated shift work reduces insulin sensitivity in humans. *Acta Physiol (Oxf)* 223(2)**,** e13039. doi: 10.1111/apha.13039.

Biggi, N., Consonni, D., Galluzzo, V., Sogliani, M., and Costa, G. (2008). Metabolic syndrome in permanent night workers. *Chronobiol Int* 25(2)**,** 443-454. doi: 10.1080/07420520802114193.

Boden, G. (2003). Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes. *Exp Clin Endocrinol Diabetes* 111(3)**,** 121-124. doi: 10.1055/s-2003-39781.

Boden, G., Lebed, B., Schatz, M., Homko, C., and Lemieux, S. (2001). Effects of acute changes of plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects. *Diabetes* 50(7)**,** 1612-1617. doi: 10.2337/diabetes.50.7.1612.

Borbely, A.A. (1982). A two process model of sleep regulation. *Hum Neurobiol* 1(3)**,** 195-204.

Bosy-Westphal, A., Hinrichs, S., Jauch-Chara, K., Hitze, B., Later, W., Wilms, B., et al. (2008). Influence of partial sleep deprivation on energy balance and insulin sensitivity in healthy women. *Obes Facts* 1(5)**,** 266-273. doi: 10.1159/000158874.

Bretzl, H. (1903). Botanische Forschungen des Alexanderzuges. *Nature* 68**,** 292-293. doi: https://doi.org/10.1038/068292a0.

Brooks, G., Fahey TD, Baldwin KM (2004). *Exercise Physiology: Human Bioenergetics and its Applications, 4th edn.*: McGraw-Hill Education.

Broussard, J.L., Chapotot, F., Abraham, V., Day, A., Delebecque, F., Whitmore, H.R., et al. (2015a). Sleep restriction increases free fatty acids in healthy men. *Diabetologia* 58(4)**,** 791-798. doi: 10.1007/s00125-015-3500-4.

Broussard, J.L., Ehrmann, D.A., Van Cauter, E., Tasali, E., and Brady, M.J. (2012). Impaired insulin signaling in human adipocytes after experimental sleep restriction: a randomized, crossover study. *Ann Intern Med* 157(8)**,** 549-557. doi: 10.7326/0003-4819-157-8-201210160-00005.

Broussard, J.L., Kolka, C.M., Castro, A.V., Asare Bediako, I., Paszkiewicz, R.L., Szczepaniak, E.W., et al. (2015b). Elevated nocturnal NEFA are an early signal for hyperinsulinaemic compensation during diet-induced insulin resistance in dogs. *Diabetologia* 58(11)**,** 2663-2670. doi: 10.1007/s00125-015-3721-6.

Broussard, J.L., Perreault, L., Macias, E., Newsom, S.A., Harrison, K., Bui, H.H., et al. (2021). Sex Differences in Insulin Sensitivity are Related to Muscle Tissue Acylcarnitine But Not Subcellular Lipid Distribution. *Obesity (Silver Spring)* 29(3)**,** 550-561. doi: 10.1002/oby.23106.

Broussard, J.L., Reynolds, A.C., Depner C.M., Ferguson S.A., Dawson D., Wright K.P. Jr. (2017). "Circadian Rhythms Versus Daily Patterns in Human Physiology and Behavior," in *Biological Timekeeping: Clocks, Rhythms and Behaviour,* ed. V. Kumar. 1 ed (India: Springer), 279-295.

Broussard, J.L., Wroblewski, K., Kilkus, J.M., and Tasali, E. (2016). Two Nights of Recovery Sleep Reverses the Effects of Short-term Sleep Restriction on Diabetes Risk. *Diabetes Care* 39(3)**,** e40-41. doi: 10.2337/dc15-2214.

Brunetto, E.L., Teixeira Sda, S., Giannocco, G., Machado, U.F., and Nunes, M.T. (2012). T3 rapidly increases SLC2A4 gene expression and GLUT4 trafficking to the plasma membrane in

skeletal muscle of rat and improves glucose homeostasis. *Thyroid* 22(1), 70-79. doi: 10.1089/thy.2010.0409.

Buxton, O.M., Cain, S.W., O'Connor, S.P., Porter, J.H., Duffy, J.F., Wang, W., et al. (2012). Adverse metabolic consequences in humans of prolonged sleep restriction combined with circadian disruption. *Sci Transl Med* 4(129), 129ra143. doi: 10.1126/scitranslmed.3003200.

Buxton, O.M., Lee, C.W., L'Hermite-Baleriaux, M., Turek, F.W., and Van Cauter, E. (2003). Exercise elicits phase shifts and acute alterations of melatonin that vary with circadian phase. *Am J Physiol Regul Integr Comp Physiol* 284(3), R714-724. doi: 10.1152/ajpregu.00355.2002.

Buxton, O.M., Pavlova, M., Reid, E.W., Wang, W., Simonson, D.C., and Adler, G.K. (2010). Sleep restriction for 1 week reduces insulin sensitivity in healthy men. *Diabetes* 59(9), 2126-2133. doi: 10.2337/db09-0699.

Campbell, S.S., and Tobler, I. (1984). Animal sleep: a review of sleep duration across phylogeny. *Neurosci Biobehav Rev* 8(3), 269-300. doi: 10.1016/0149-7634(84)90054-x.

Canuto, R., Garcez, A.S., and Olinto, M.T. (2013). Metabolic syndrome and shift work: a systematic review. *Sleep Med Rev* 17(6), 425-431. doi: 10.1016/j.smrv.2012.10.004.

Cappuccio, F.P., Cooper, D., D'Elia, L., Strazzullo, P., and Miller, M.A. (2011). Sleep duration predicts cardiovascular outcomes: a systematic review and meta-analysis of prospective studies. *Eur Heart J* 32(12), 1484-1492. doi: 10.1093/eurheartj/ehr007.

Cappuccio, F.P., D'Elia, L., Strazzullo, P., and Miller, M.A. (2010). Quantity and quality of sleep and incidence of type 2 diabetes: a systematic review and meta-analysis. *Diabetes Care* 33(2), 414-420. doi: 10.2337/dc09-1124.

Carrier, J., Viens, I., Poirier, G., Robillard, R., Lafortune, M., Vandewalle, G., et al. (2011). Sleep slow wave changes during the middle years of life. *Eur J Neurosci* 33(4), 758-766. doi: 10.1111/j.1460-9568.2010.07543.x.

Cavalier, A.N., Clayton, Z.S., Hutton, D.A., Wahl, D., Lark, D.S., Reisz, J.A., et al. (2021). Accelerated aging of the brain transcriptome by the common chemotherapeutic doxorubicin. *Exp Gerontol* 152, 111451. doi: 10.1016/j.exger.2021.111451.

Cedernaes, J., Lampola, L., Axelsson, E.K., Liethof, L., Hassanzadeh, S., Yeganeh, A., et al. (2016). A single night of partial sleep loss impairs fasting insulin sensitivity but does not affect cephalic phase insulin release in young men. *J Sleep Res* 25(1), 5-10. doi: 10.1111/jsr.12340.

Cedernaes, J., Osler, M.E., Voisin, S., Broman, J.E., Vogel, H., Dickson, S.L., et al. (2015). Acute Sleep Loss Induces Tissue-Specific Epigenetic and Transcriptional Alterations to Circadian Clock Genes in Men. *J Clin Endocrinol Metab* 100(9), E1255-1261. doi: 10.1210/JC.2015-2284.

Cedernaes, J., Schonke, M., Westholm, J.O., Mi, J., Chibalin, A., Voisin, S., et al. (2018). Acute sleep loss results in tissue-specific alterations in genome-wide DNA methylation state and metabolic fuel utilization in humans. *Sci Adv* 4(8), eaar8590. doi: 10.1126/sciadv.aar8590.

Centers for Disease, C., and Prevention (2011). Effect of short sleep duration on daily activities--United States, 2005-2008. *MMWR Morb Mortal Wkly Rep* 60(8), 239-242.

Challet, E., Solberg, L.C., and Turek, F.W. (1998). Entrainment in calorie-restricted mice: conflicting zeitgebers and free-running conditions. *Am J Physiol* 274(6), R1751-1761. doi: 10.1152/ajpregu.1998.274.6.R1751.

Chang, W.P., and Peng, Y.X. (2021). Meta-analysis of differences in sleep quality based on actigraphs between day and night shift workers and the moderating effect of age. *J Occup Health* 63(1)**,** e12262. doi: 10.1002/1348-9585.12262.

Chavez, A.O., Kamath, S., Jani, R., Sharma, L.K., Monroy, A., Abdul-Ghani, M.A., et al. (2010). Effect of short-term free Fatty acids elevation on mitochondrial function in skeletal muscle of healthy individuals. *J Clin Endocrinol Metab* 95(1)**,** 422-429. doi: 10.1210/jc.2009-1387.

Chen, L.J., Lai, Y.J., Sun, W.J., Fox, K.R., Chu, D., and Ku, P.W. (2015). Associations of exercise, sedentary time and insomnia with metabolic syndrome in Taiwanese older adults: A 1-year follow-up study. *Endocr Res* 40(4)**,** 220-226. doi: 10.3109/07435800.2015.1020547.

Chomentowski, P., Coen, P.M., Radikova, Z., Goodpaster, B.H., and Toledo, F.G. (2011). Skeletal muscle mitochondria in insulin resistance: differences in intermyofibrillar versus subsarcolemmal subpopulations and relationship to metabolic flexibility. *J Clin Endocrinol Metab* 96(2)**,** 494-503. doi: 10.1210/jc.2010-0822.

Chua, E.C., Shui, G., Cazenave-Gassiot, A., Wenk, M.R., and Gooley, J.J. (2015). Changes in Plasma Lipids during Exposure to Total Sleep Deprivation. *Sleep* 38(11)**,** 1683-1691. doi: 10.5665/sleep.5142.

Chung, F., Subramanyam, R., Liao, P., Sasaki, E., Shapiro, C., and Sun, Y. (2012). High STOP-Bang score indicates a high probability of obstructive sleep apnoea. *Br J Anaesth* 108(5)**,** 768-775. doi: 10.1093/bja/aes022.

Chung, F., Yegneswaran, B., Liao, P., Chung, S.A., Vairavanathan, S., Islam, S., et al. (2008). STOP questionnaire: a tool to screen patients for obstructive sleep apnea. *Anesthesiology* 108(5)**,** 812-821. doi: 10.1097/ALN.0b013e31816d83e4.

Cobelli, C., Dalla Man, C., Toffolo, G., Basu, R., Vella, A., and Rizza, R. (2014). The oral minimal model method. *Diabetes* 63(4)**,** 1203-1213. doi: 10.2337/db13-1198.

Coen, P.M., Dube, J.J., Amati, F., Stefanovic-Racic, M., Ferrell, R.E., Toledo, F.G., et al. (2010). Insulin resistance is associated with higher intramyocellular triglycerides in type I but not type II myocytes concomitant with higher ceramide content. *Diabetes* 59(1)**,** 80-88. doi: 10.2337/db09-0988.

Corradetti, M.N., Inoki, K., and Guan, K.L. (2005). The stress-inducted proteins RTP801 and RTP801L are negative regulators of the mammalian target of rapamycin pathway. *J Biol Chem* 280(11)**,** 9769-9772. doi: 10.1074/jbc.C400557200.

Crawford, T. (1938). A standard intravenous glucose tolerance test. *Arch Dis Child* 13(73)**,** 69-78. doi: 10.1136/adc.13.73.69.

Cuellar, N.G., and Ratcliffe, S.J. (2008). A comparison of glycemic control, sleep, fatigue, and depression in type 2 diabetes with and without restless legs syndrome. *J Clin Sleep Med* 4(1)**,** 50-56.

Czeisler, C.A., Duffy, J.F., Shanahan, T.L., Brown, E.N., Mitchell, J.F., Rimmer, D.W., et al. (1999a). Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science* 284(5423)**,** 2177-2181. doi: 10.1126/science.284.5423.2177.

Czeisler, C.A., Wright, K., Turek, F., and Zee, P. (1999b). Influence of light on circadian rhythmicity in humans. *Lung biology in health and disease* 133**,** 149-149.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., and Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central

pacemaker in the suprachiasmatic nucleus. *Genes Dev* 14(23)**,** 2950-2961. doi: 10.1101/gad.183500.

Davis, H., Davis, P.A., Loomis, A.L., Harvey, E.N., and Hobart, G. (1938). HUMAN BRAIN POTENTIALS DURING THE ONSET OF SLEEP. *Journal of Neurophysiology* 1(1)**,** 24-38. doi: 10.1152/jn.1938.1.1.24.

Davis, S., and Mirick, D.K. (2006). Circadian disruption, shift work and the risk of cancer: a summary of the evidence and studies in Seattle. *Cancer Causes Control* 17(4)**,** 539-545. doi: 10.1007/s10552-005-9010-9.

de Mairan, J. (1729). Pbservation Botinique. *Histoire de l'Académie Royale des Sciences***,** 35-36.

de Manacéïne, M. (1894). Quelques observations experimentales sur l'influence de l'insomnie absolue. *Arch Ital. Biol.* 21**,** 322-325.

DeFronzo, R.A. (2004). Dysfunctional fat cells, lipotoxicity and type 2 diabetes. *Int J Clin Pract Suppl* (143)**,** 9-21. doi: 10.1111/j.1368-504x.2004.00389.x.

Defronzo, R.A., Simonson, D., Ferrannini, E., and Barrett, E. (1981). Insulin resistance: a universal finding in diabetic states. *Bull Schweiz Akad Med Wiss***,** 223-238.

DeFronzo, R.A., Tobin, J.D., and Andres, R. (1979). Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237(3)**,** E214-223. doi: 10.1152/ajpendo.1979.237.3.E214.

Dement, W., and Kleitman, N. (1957a). Cyclic variations in EEG during sleep and their relation to eye movements, body motility, and dreaming. *Electroencephalogr Clin Neurophysiol* 9(4)**,** 673-690. doi: 10.1016/0013-4694(57)90088-3.

Dement, W., and Kleitman, N. (1957b). The relation of eye movements during sleep to dream activity: an objective method for the study of dreaming. *J Exp Psychol* 53(5)**,** 339-346. doi: 10.1037/h0048189.

Depner, C.M., Melanson, E.L., Eckel, R.H., Snell-Bergeon, J.K., Perreault, L., Bergman, B.C., et al. (2019). Ad libitum Weekend Recovery Sleep Fails to Prevent Metabolic Dysregulation during a Repeating Pattern of Insufficient Sleep and Weekend Recovery Sleep. *Curr Biol* 29(6)**,** 957-967 e954. doi: 10.1016/j.cub.2019.01.069.

Depner, C.M., Stothard, E.R., and Wright, K.P., Jr. (2014). Metabolic consequences of sleep and circadian disorders. *Curr Diab Rep* 14(7)**,** 507. doi: 10.1007/s11892-014-0507-z.

Di Lorenzo, L., De Pergola, G., Zocchetti, C., L'Abbate, N., Basso, A., Pannacciulli, N., et al. (2003). Effect of shift work on body mass index: results of a study performed in 319 glucose-tolerant men working in a Southern Italian industry. *Int J Obes Relat Metab Disord* 27(11)**,** 1353-1358. doi: 10.1038/sj.ijo.0802419.

Dixon, J.B., Schachter, L.M., and O'Brien, P.E. (2005). Polysomnography before and after weight loss in obese patients with severe sleep apnea. *Int J Obes (Lond)* 29(9)**,** 1048-1054. doi: 10.1038/sj.ijo.0802960.

Donga, E., van Dijk, M., van Dijk, J.G., Biermasz, N.R., Lammers, G.J., van Kralingen, K., et al. (2010a). Partial sleep restriction decreases insulin sensitivity in type 1 diabetes. *Diabetes Care* 33(7)**,** 1573-1577. doi: 10.2337/dc09-2317.

Donga, E., van Dijk, M., van Dijk, J.G., Biermasz, N.R., Lammers, G.J., van Kralingen, K.W., et al. (2010b). A single night of partial sleep deprivation induces insulin resistance in multiple metabolic pathways in healthy subjects. *J Clin Endocrinol Metab* 95(6)**,** 2963-2968. doi: 10.1210/jc.2009-2430.

Douglass, A.B., Bornstein, R., Nino-Murcia, G., Keenan, S., Miles, L., Zarcone, V.P., Jr., et al. (1994). The Sleep Disorders Questionnaire. I: Creation and multivariate structure of SDQ. *Sleep* 17(2)**,** 160-167. doi: 10.1093/sleep/17.2.160.

Drake, C.L., Roehrs, T., Richardson, G., Walsh, J.K., and Roth, T. (2004). Shift work sleep disorder: prevalence and consequences beyond that of symptomatic day workers. *Sleep* 27(8)**,** 1453-1462. doi: 10.1093/sleep/27.8.1453.

Duffy, J.F., Cain, S.W., Chang, A.M., Phillips, A.J., Munch, M.Y., Gronfier, C., et al. (2011). Sex difference in the near-24-hour intrinsic period of the human circadian timing system. *Proc Natl Acad Sci U S A* 108 Suppl 3**,** 15602-15608. doi: 10.1073/pnas.1010666108.

Duffy, J.F., and Dijk, D.J. (2002). Getting through to circadian oscillators: why use constant routines? *J Biol Rhythms* 17(1)**,** 4-13. doi: 10.1177/074873002129002294.

Duffy, J.F., Kronauer, R.E., and Czeisler, C.A. (1996). Phase-shifting human circadian rhythms: influence of sleep timing, social contact and light exposure. *J Physiol* 495 (Pt 1)**,** 289-297. doi: 10.1113/jphysiol.1996.sp021593.

Dull, K., Fazekas, F., and Torocsik, D. (2021). Factor XIII-A in Diseases: Role Beyond Blood Coagulation. *Int J Mol Sci* 22(3). doi: 10.3390/ijms22031459.

Dunlap, J.C. (1999). Molecular bases for circadian clocks. *Cell* 96(2)**,** 271-290. doi: 10.1016/s0092-8674(00)80566-8.

Eckel, R.H., Depner, C.M., Perreault, L., Markwald, R.R., Smith, M.R., McHill, A.W., et al. (2015). Morning Circadian Misalignment during Short Sleep Duration Impacts Insulin Sensitivity. *Curr Biol* 25(22)**,** 3004-3010. doi: 10.1016/j.cub.2015.10.011.

Espelund, U., Hansen, T.K., Hojlund, K., Beck-Nielsen, H., Clausen, J.T., Hansen, B.S., et al. (2005). Fasting unmasks a strong inverse association between ghrelin and cortisol in serum: studies in obese and normal-weight subjects. *J Clin Endocrinol Metab* 90(2)**,** 741-746. doi: 10.1210/jc.2004-0604.

Everson, C.A., Bergmann, B.M., and Rechtschaffen, A. (1989a). Sleep deprivation in the rat: III. Total sleep deprivation. *Sleep* 12(1)**,** 13-21. doi: 10.1093/sleep/12.1.13.

Everson, C.A., Gilliland, M.A., Kushida, C.A., Pilcher, J.J., Fang, V.S., Refetoff, S., et al. (1989b). Sleep deprivation in the rat: IX. Recovery. *Sleep* 12(1)**,** 60-67.

Fabbri, E., Chia, C.W., Spencer, R.G., Fishbein, K.W., Reiter, D.A., Cameron, D., et al. (2017). Insulin Resistance Is Associated With Reduced Mitochondrial Oxidative Capacity Measured by 31P-Magnetic Resonance Spectroscopy in Participants Without Diabetes From the Baltimore Longitudinal Study of Aging. *Diabetes* 66(1)**,** 170-176. doi: 10.2337/db16-0754.

Flo, E., Pallesen, S., Mageroy, N., Moen, B.E., Gronli, J., Hilde Nordhus, I., et al. (2012). Shift work disorder in nurses--assessment, prevalence and related health problems. *PLoS One* 7(4)**,** e33981. doi: 10.1371/journal.pone.0033981.

Fonken, L.K., Workman, J.L., Walton, J.C., Weil, Z.M., Morris, J.S., Haim, A., et al. (2010). Light at night increases body mass by shifting the time of food intake. *Proc Natl Acad Sci U S A* 107(43)**,** 18664-18669. doi: 10.1073/pnas.1008734107.

Foster, G.D., Sanders, M.H., Millman, R., Zammit, G., Borradaile, K.E., Newman, A.B., et al. (2009). Obstructive sleep apnea among obese patients with type 2 diabetes. *Diabetes Care* 32(6)**,** 1017-1019. doi: 10.2337/dc08-1776.

Franklin, K.A., and Lindberg, E. (2015). Obstructive sleep apnea is a common disorder in the population-a review on the epidemiology of sleep apnea. *J Thorac Dis* 7(8)**,** 1311-1322. doi: 10.3978/j.issn.2072-1439.2015.06.11.

Frayn, K.N. (1983). Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol Respir Environ Exerc Physiol* 55(2)**,** 628-634. doi: 10.1152/jappl.1983.55.2.628.

Gan, Y., Yang, C., Tong, X., Sun, H., Cong, Y., Yin, X., et al. (2015). Shift work and diabetes mellitus: a meta-analysis of observational studies. *Occup Environ Med* 72(1)**,** 72-78. doi: 10.1136/oemed-2014-102150.

Garcia-Ruiz, C., Colell, A., Mari, M., Morales, A., and Fernandez-Checa, J.C. (1997). Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J Biol Chem* 272(17)**,** 11369-11377. doi: 10.1074/jbc.272.17.11369.

Goel, N. (2005). Late-night presentation of an auditory stimulus phase delays human circadian rhythms. *Am J Physiol Regul Integr Comp Physiol* 289(1)**,** R209-216. doi: 10.1152/ajpregu.00754.2004.

Gronfier, C., Wright, K.P., Jr., Kronauer, R.E., and Czeisler, C.A. (2007). Entrainment of the human circadian pacemaker to longer-than-24-h days. *Proc Natl Acad Sci U S A* 104(21)**,** 9081-9086. doi: 10.1073/pnas.0702835104.

Grundy, A., Richardson, H., Burstyn, I., Lohrisch, C., SenGupta, S.K., Lai, A.S., et al. (2013). Increased risk of breast cancer associated with long-term shift work in Canada. *Occup Environ Med* 70(12)**,** 831-838. doi: 10.1136/oemed-2013-101482.

Gulevich, G., Dement, W., and Johnson, L. (1966). Psychiatric and EEG observations on a case of prolonged (264 hours) wakefulness. *Arch Gen Psychiatry* 15(1)**,** 29-35. doi: 10.1001/archpsyc.1966.01730130031005.

Guo, Y., Liu, Y., Huang, X., Rong, Y., He, M., Wang, Y., et al. (2013). The effects of shift work on sleeping quality, hypertension and diabetes in retired workers. *PLoS One* 8(8)**,** e71107. doi: 10.1371/journal.pone.0071107.

Hajek, P., Chomyn, A., and Attardi, G. (2007). Identification of a novel mitochondrial complex containing mitofusin 2 and stomatin-like protein 2. *J Biol Chem* 282(8)**,** 5670-5681. doi: 10.1074/jbc.M608168200.

Halliday, T.M., Rynders, C.A., Thomas, E., Bergouignan, A., Pan, Z., Kealey, E.H., et al. (2020). Appetite-Related Responses to Overfeeding and Longitudinal Weight Change in Obesity-Prone and Obesity-Resistant Adults. *Obesity (Silver Spring)* 28(2)**,** 259-267. doi: 10.1002/oby.22687.

Hampton, S.M., Morgan, L.M., Lawrence, N., Anastasiadou, T., Norris, F., Deacon, S., et al. (1996). Postprandial hormone and metabolic responses in simulated shift work. *J Endocrinol* 151(2)**,** 259-267. doi: 10.1677/joe.0.1510259.

Hart, C.N., Carskadon, M.A., Demos, K.E., Van Reen, E., Sharkey, K.M., Raynor, H.A., et al. (2015). Acute Changes in Sleep Duration on Eating Behaviors and Appetite-Regulating Hormones in Overweight/Obese Adults. *Behav Sleep Med* 13(5)**,** 424-436. doi: 10.1080/15402002.2014.940105.

Hastings, M.H., Reddy, A.B., Garabette, M., King, V.M., Chahad-Ehlers, S., O'Brien, J., et al. (2003). Expression of clock gene products in the suprachiasmatic nucleus in relation to circadian

behaviour. *Novartis Found Symp* 253**,** 203-217; discussion 102-209, 218-222, 281-204. doi: 10.1002/0470090839.ch15.

Herzog, N., Jauch-Chara, K., Hyzy, F., Richter, A., Friedrich, A., Benedict, C., et al. (2013). Selective slow wave sleep but not rapid eye movement sleep suppression impairs morning glucose tolerance in healthy men. *Psychoneuroendocrinology* 38(10)**,** 2075-2082. doi: 10.1016/j.psyneuen.2013.03.018.

Hill, B.R., De Souza, M.J., and Williams, N.I. (2011). Characterization of the diurnal rhythm of peptide YY and its association with energy balance parameters in normal-weight premenopausal women. *Am J Physiol Endocrinol Metab* 301(2)**,** E409-415. doi: 10.1152/ajpendo.00171.2011.

Hirshkowitz, M., Whiton, K., Albert, S.M., Alessi, C., Bruni, O., DonCarlos, L., et al. (2015). National Sleep Foundation's updated sleep duration recommendations: final report. *Sleep Health* 1(4)**,** 233-243. doi: 10.1016/j.sleh.2015.10.004.

Hoffmann, K. (1968). Temperaturzyklen als Zeitgeber der circadianen Periodik. *Verh Dtsch Zool Ges (Innsbruck)* 62**,** 265-274.

Horne, J.A., and Ostberg, O. (1976). A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* 4(2)**,** 97-110.

Hulver, M.W., Berggren, J.R., Cortright, R.N., Dudek, R.W., Thompson, R.P., Pories, W.J., et al. (2003). Skeletal muscle lipid metabolism with obesity. *Am J Physiol Endocrinol Metab* 284(4)**,** E741-747. doi: 10.1152/ajpendo.00514.2002.

Ishimori, K. (1909). True cause of sleep: a hypnogenic substance as evidenced in the brain of sleep-deprived animals. *Tokyo Igakkai Zasshi* 23(429).

Ishizaki, M., Morikawa, Y., Nakagawa, H., Honda, R., Kawakami, N., Haratani, T., et al. (2004). The influence of work characteristics on body mass index and waist to hip ratio in Japanese employees. *Ind Health* 42(1)**,** 41-49. doi: 10.2486/indhealth.42.41.

Javaheri, S., and Redline, S. (2017). Insomnia and Risk of Cardiovascular Disease. *Chest* 152(2)**,** 435-444. doi: 10.1016/j.chest.2017.01.026.

Jensen, C.C., Cnop, M., Hull, R.L., Fujimoto, W.Y., Kahn, S.E., and American Diabetes Association, G.S.G. (2002). Beta-cell function is a major contributor to oral glucose tolerance in high-risk relatives of four ethnic groups in the U.S. *Diabetes* 51(7)**,** 2170-2178. doi: 10.2337/diabetes.51.7.2170.

Jin, T. (2008). The WNT signalling pathway and diabetes mellitus. *Diabetologia* 51(10)**,** 1771-1780. doi: 10.1007/s00125-008-1084-y.

Johns, M.W. (1991). A new method for measuring daytime sleepiness: the Epworth sleepiness scale. *Sleep* 14(6)**,** 540-545. doi: 10.1093/sleep/14.6.540.

Joiner, W.J. (2016). Unraveling the Evolutionary Determinants of Sleep. *Curr Biol* 26(20)**,** R1073-R1087. doi: 10.1016/j.cub.2016.08.068.

Jung, C.M., Melanson, E.L., Frydendall, E.J., Perreault, L., Eckel, R.H., and Wright, K.P. (2011). Energy expenditure during sleep, sleep deprivation and sleep following sleep deprivation in adult humans. *J Physiol* 589(Pt 1)**,** 235-244. doi: 10.1113/jphysiol.2010.197517.

Kahn, D., Perreault, L., Macias, E., Zarini, S., Newsom, S.A., Strauss, A., et al. (2021). Subcellular localisation and composition of intramuscular triacylglycerol influence insulin sensitivity in humans. *Diabetologia* 64(1)**,** 168-180. doi: 10.1007/s00125-020-05315-0.

Kamble, P.G., Theorell-Haglow, J., Wiklund, U., Franklin, K.A., Hammar, U., Lindberg, E., et al. (2020). Sleep apnea in men is associated with altered lipid metabolism, glucose tolerance, insulin sensitivity, and body fat percentage. *Endocrine* 70(1)**,** 48-57. doi: 10.1007/s12020-020-02369-3.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* 44(D1)**,** D457-462. doi: 10.1093/nar/gkv1070.

Karatsoreos, I.N., Bhagat, S., Bloss, E.B., Morrison, J.H., and McEwen, B.S. (2011). Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. *Proc Natl Acad Sci U S A* 108(4)**,** 1657-1662. doi: 10.1073/pnas.1018375108.

Karlsson, B., Knutsson, A., and Lindahl, B. (2001). Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people. *Occup Environ Med* 58(11)**,** 747-752. doi: 10.1136/oem.58.11.747.

Katz, A., Nambi, S.S., Mather, K., Baron, A.D., Follmann, D.A., Sullivan, G., et al. (2000). Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 85(7)**,** 2402-2410. doi: 10.1210/jcem.85.7.6661.

Kelley, D.E., He, J., Menshikova, E.V., and Ritov, V.B. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51(10)**,** 2944-2950. doi: 10.2337/diabetes.51.10.2944.

Killick, R., Hoyos, C.M., Melehan, K.L., Dungan, G.C., 2nd, Poh, J., and Liu, P.Y. (2015). Metabolic and hormonal effects of 'catch-up' sleep in men with chronic, repetitive, lifestyle-driven sleep restriction. *Clin Endocrinol (Oxf)* 83(4)**,** 498-507. doi: 10.1111/cen.12747.

Kim, J.Y., Hickner, R.C., Cortright, R.L., Dohm, G.L., and Houmard, J.A. (2000). Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab* 279(5)**,** E1039-1044. doi: 10.1152/ajpendo.2000.279.5.E1039.

Kivimaki, M., Batty, G.D., and Hublin, C. (2011). Shift work as a risk factor for future type 2 diabetes: evidence, mechanisms, implications, and future research directions. *PLoS Med* 8(12)**,** e1001138. doi: 10.1371/journal.pmed.1001138.

Kleitman, N. (1939). *Sleep and wakefulness.* Chicago: The University of Chicago Press.

Klerman, E.B., Gershengorn, H.B., Duffy, J.F., and Kronauer, R.E. (2002). Comparisons of the variability of three markers of the human circadian pacemaker. *J Biol Rhythms* 17(2)**,** 181-193. doi: 10.1177/074873002129002474.

Klerman, E.B., Rimmer, D.W., Dijk, D.J., Kronauer, R.E., Rizzo, J.F., 3rd, and Czeisler, C.A. (1998). Nonphotic entrainment of the human circadian pacemaker. *Am J Physiol* 274(4 Pt 2)**,** R991-996. doi: 10.1152/ajpregu.1998.274.4.r991.

Knutson, K.L. (2010). Sleep duration and cardiometabolic risk: a review of the epidemiologic evidence. *Best Pract Res Clin Endocrinol Metab* 24(5)**,** 731-743. doi: 10.1016/j.beem.2010.07.001.

Knutson, K.L., Van Cauter, E., Zee, P., Liu, K., and Lauderdale, D.S. (2011). Cross-sectional associations between measures of sleep and markers of glucose metabolism among subjects with and without diabetes: the Coronary Artery Risk Development in Young Adults (CARDIA) Sleep Study. *Diabetes Care* 34(5)**,** 1171-1176. doi: 10.2337/dc10-1962.

Knutsson, A. (2003). Health disorders of shift workers. *Occup Med (Lond)* 53(2)**,** 103-108. doi: 10.1093/occmed/kqg048.

Knutsson, A., Akerstedt, T., Jonsson, B.G., and Orth-Gomer, K. (1986). Increased risk of ischaemic heart disease in shift workers. *Lancet* 2(8498)**,** 89-92. doi: 10.1016/s0140-6736(86)91619-3.

Ko, C.H., and Takahashi, J.S. (2006). Molecular components of the mammalian circadian clock. *Hum Mol Genet* 15 Spec No 2**,** R271-277. doi: 10.1093/hmg/ddl207.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proc Natl Acad Sci U S A* 68(9)**,** 2112-2116. doi: 10.1073/pnas.68.9.2112.

Krauchi, K., and Wirz-Justice, A. (1994). Circadian rhythm of heat production, heart rate, and skin and core temperature under unmasking conditions in men. *Am J Physiol* 267(3 Pt 2)**,** R819-829. doi: 10.1152/ajpregu.1994.267.3.R819.

Kroenke, C.H., Spiegelman, D., Manson, J., Schernhammer, E.S., Colditz, G.A., and Kawachi, I. (2007). Work characteristics and incidence of type 2 diabetes in women. *Am J Epidemiol* 165(2)**,** 175-183. doi: 10.1093/aje/kwj355.

Kuznetsov, A.V., Veksler, V., Gellerich, F.N., Saks, V., Margreiter, R., and Kunz, W.S. (2008). Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat Protoc* 3(6)**,** 965-976. doi: 10.1038/nprot.2008.61.

Labyak, S., Lava, S., Turek, F., and Zee, P. (2002). Effects of shiftwork on sleep and menstrual function in nurses. *Health Care Women Int* 23(6-7)**,** 703-714. doi: 10.1080/07399330290107449.

Larsson, S.C., and Markus, H.S. (2019). Genetic Liability to Insomnia and Cardiovascular Disease Risk. *Circulation* 140(9)**,** 796-798. doi: 10.1161/CIRCULATIONAHA.119.041830.

Legendre, R., and Piéron, H. (1910). *Le problème des facteurs du sommeil: résultats d'injections vasculaires et intra-cérébrales de liquides insomniques.* Masson et Cie, éditeurs.

Legendre, R., and Piéron, H. (1913). Recherches sur le besoin de sommeil consécutif à une veille prolongée. *Z allg Physiol* 14**,** 235-262.

Leproult, R., Holmback, U., and Van Cauter, E. (2014). Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss. *Diabetes* 63(6)**,** 1860-1869. doi: 10.2337/db13-1546.

Leproult, R., and Van Cauter, E. (2010). Role of sleep and sleep loss in hormonal release and metabolism. *Endocr Dev* 17**,** 11-21. doi: 10.1159/000262524.

LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D.W., and Silver, R. (2009). Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proc Natl Acad Sci U S A* 106(32)**,** 13582-13587. doi: 10.1073/pnas.0906426106.

Levine, J.D., Funes, P., Dowse, H.B., and Hall, J.C. (2002). Resetting the circadian clock by social experience in Drosophila melanogaster. *Science* 298(5600)**,** 2010-2012. doi: 10.1126/science.1076008.

Lim, Y.C., Hoe, V.C.W., Darus, A., and Bhoo-Pathy, N. (2018). Association between night-shift work, sleep quality and metabolic syndrome. *Occup Environ Med* 75(10)**,** 716-723. doi: 10.1136/oemed-2018-105104.

Liu, K., Yu, W., Wei, W., Zhang, X., Tian, Y., Sherif, M., et al. (2019). Melatonin reduces intramuscular fat deposition by promoting lipolysis and increasing mitochondrial function. *J Lipid Res* 60(4)**,** 767-782. doi: 10.1194/jlr.M087619.

Liu, Y., Merrow, M., Loros, J.J., and Dunlap, J.C. (1998). How temperature changes reset a circadian oscillator. *Science* 281(5378)**,** 825-829. doi: 10.1126/science.281.5378.825.

Lopes, L.A., Lins Cde, M., Adeodato, V.G., Quental, D.P., de Bruin, P.F., Montenegro, R.M., Jr., et al. (2005). Restless legs syndrome and quality of sleep in type 2 diabetes. *Diabetes Care* 28(11)**,** 2633-2636. doi: 10.2337/diacare.28.11.2633.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15(12)**,** 550. doi: 10.1186/s13059-014-0550-8.

Lowrey, P.L., and Takahashi, J.S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu Rev Genomics Hum Genet* 5**,** 407-441. doi: 10.1146/annurev.genom.5.061903.175925.

Lu, G. (2002). *Celestial Lancets.* Psychology Press.

Lund, J., Arendt, J., Hampton, S.M., English, J., and Morgan, L.M. (2001). Postprandial hormone and metabolic responses amongst shift workers in Antarctica. *J Endocrinol* 171(3)**,** 557-564. doi: 10.1677/joe.0.1710557.

Manella, G., Aviram, R., Bolshette, N., Muvkadi, S., Golik, M., Smith, D.F., et al. (2020). Hypoxia induces a time- and tissue-specific response that elicits intertissue circadian clock misalignment. *Proc Natl Acad Sci U S A* 117(1)**,** 779-786. doi: 10.1073/pnas.1914112117.

Manella, G., Sabath, E., Aviram, R., Dandavate, V., Ezagouri, S., Golik, M., et al. (2021). The liver-clock coordinates rhythmicity of peripheral tissues in response to feeding. *Nat Metab* 3(6)**,** 829-842. doi: 10.1038/s42255-021-00395-7.

Markwald, R.R., Melanson, E.L., Smith, M.R., Higgins, J., Perreault, L., Eckel, R.H., et al. (2013). Impact of insufficient sleep on total daily energy expenditure, food intake, and weight gain. *Proc Natl Acad Sci U S A* 110(14)**,** 5695-5700. doi: 10.1073/pnas.1216951110.

Maspero, C., Giannini, L., Galbiati, G., Rosso, G., and Farronato, G. (2015). Obstructive sleep apnea syndrome: a literature review. *Minerva Stomatol* 64(2)**,** 97-109.

Mateus Brandao, L.E., Espes, D., Westholm, J.O., Martikainen, T., Westerlund, N., Lampola, L., et al. (2021). Acute sleep loss alters circulating fibroblast growth factor 21 levels in humans: A randomised crossover trial. *J Sleep Res**,*** e13472. doi: 10.1111/jsr.13472.

Matsuda, M., and DeFronzo, R.A. (1999). Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 22(9)**,** 1462-1470. doi: 10.2337/diacare.22.9.1462.

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., and Turner, R.C. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28(7)**,** 412-419. doi: 10.1007/bf00280883.

McClung, C.R. (2006). Plant circadian rhythms. *Plant Cell* 18(4)**,** 792-803. doi: 10.1105/tpc.106.040980.

McHill, A.W., Hull, J.T., McMullan, C.J., and Klerman, E.B. (2018). Chronic Insufficient Sleep Has a Limited Impact on Circadian Rhythmicity of Subjective Hunger and Awakening Fasted Metabolic Hormones. *Front Endocrinol (Lausanne)* 9**,** 319. doi: 10.3389/fendo.2018.00319.

McHill, A.W., Melanson, E.L., Higgins, J., Connick, E., Moehlman, T.M., Stothard, E.R., et al. (2014). Impact of circadian misalignment on energy metabolism during simulated nightshift work. *Proc Natl Acad Sci U S A* 111(48)**,** 17302-17307. doi: 10.1073/pnas.1412021111.

Mead, J.R., Irvine, S.A., and Ramji, D.P. (2002). Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med (Berl)* 80(12)**,** 753-769. doi: 10.1007/s00109-002-0384-9.

Menaker, M., and Eskin, A. (1966). Entrainment of circadian rhythms by sound in Passer domesticus. *Science* 154(3756)**,** 1579-1581. doi: 10.1126/science.154.3756.1579.

Merlino, G., Fratticci, L., Valente, M., Del Giudice, A., Noacco, C., Dolso, P., et al. (2007). Association of restless legs syndrome in type 2 diabetes: a case-control study. *Sleep* 30(7)**,** 866-871. doi: 10.1093/sleep/30.7.866.

Mills, J.N., Minors, D.S., and Waterhouse, J.M. (1978). Adaptation to abrupt time shifts of the oscillator(s) controlling human circadian rhythms. *J Physiol* 285**,** 455-470. doi: 10.1113/jphysiol.1978.sp012582.

Mistlberger, R.E. (1994). Circadian food-anticipatory activity: formal models and physiological mechanisms. *Neurosci Biobehav Rev* 18(2)**,** 171-195. doi: 10.1016/0149-7634(94)90023-x.

Monk, T.H., and Buysse, D.J. (2013). Exposure to shift work as a risk factor for diabetes. *J Biol Rhythms* 28(5)**,** 356-359. doi: 10.1177/0748730413506557.

Morgenstern, M., Wang, J., Beatty, N., Batemarco, T., Sica, A.L., and Greenberg, H. (2014). Obstructive sleep apnea: an unexpected cause of insulin resistance and diabetes. *Endocrinol Metab Clin North Am* 43(1)**,** 187-204. doi: 10.1016/j.ecl.2013.09.002.

Morin, C.M., LeBlanc, M., Daley, M., Gregoire, J.P., and Merette, C. (2006). Epidemiology of insomnia: prevalence, self-help treatments, consultations, and determinants of help-seeking behaviors. *Sleep Med* 7(2)**,** 123-130. doi: 10.1016/j.sleep.2005.08.008.

Morino, K., Petersen, K.F., Dufour, S., Befroy, D., Frattini, J., Shatzkes, N., et al. (2005). Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest* 115(12)**,** 3587-3593. doi: 10.1172/JCI25151.

Mrosovsky, N. (1988). Phase response curves for social entrainment. *J Comp Physiol A* 162(1)**,** 35-46. doi: 10.1007/BF01342701.

Mrosovsky, N. (1996). Locomotor activity and non-photic influences on circadian clocks. *Biol Rev Camb Philos Soc* 71(3)**,** 343-372. doi: 10.1111/j.1469-185x.1996.tb01278.x.

Natalucci, G., Riedl, S., Gleiss, A., Zidek, T., and Frisch, H. (2005). Spontaneous 24-h ghrelin secretion pattern in fasting subjects: maintenance of a meal-related pattern. *Eur J Endocrinol* 152(6)**,** 845-850. doi: 10.1530/eje.1.01919.

Nath, R.D., Bedbrook, C.N., Abrams, M.J., Basinger, T., Bois, J.S., Prober, D.A., et al. (2017). The Jellyfish Cassiopea Exhibits a Sleep-like State. *Curr Biol* 27(19)**,** 2984-2990 e2983. doi: 10.1016/j.cub.2017.08.014.

Nedeltcheva, A.V., Kessler, L., Imperial, J., and Penev, P.D. (2009). Exposure to recurrent sleep restriction in the setting of high caloric intake and physical inactivity results in increased insulin resistance and reduced glucose tolerance. *J Clin Endocrinol Metab* 94(9)**,** 3242-3250. doi: 10.1210/jc.2009-0483.

Ness, K.M., Strayer, S.M., Nahmod, N.G., Chang, A.M., Buxton, O.M., and Shearer, G.C. (2019). Two nights of recovery sleep restores the dynamic lipemic response, but not the

reduction of insulin sensitivity, induced by five nights of sleep restriction. *Am J Physiol Regul Integr Comp Physiol* 316(6)**,** R697-R703. doi: 10.1152/ajpregu.00336.2018.

Netzer, N.C., Stoohs, R.A., Netzer, C.M., Clark, K., and Strohl, K.P. (1999). Using the Berlin Questionnaire to identify patients at risk for the sleep apnea syndrome. *Ann Intern Med* 131(7)**,** 485-491. doi: 10.7326/0003-4819-131-7-199910050-00002.

Nguyen, J., and Wright, K.P., Jr. (2010). Influence of weeks of circadian misalignment on leptin levels. *Nat Sci Sleep* 2**,** 9-18. doi: 10.2147/nss.s7624.

Niazi, R.K., Sun, J., Have, C.T., Hollensted, M., Linneberg, A., Pedersen, O., et al. (2019). Increased frequency of rare missense PPP1R3B variants among Danish patients with type 2 diabetes. *PLoS One* 14(1)**,** e0210114. doi: 10.1371/journal.pone.0210114.

NobelPrize.org "Nobel Prize Outreach AB 2022". (<https://www.nobelprize.org/prizes/medicine/2017/press-release/>).

Novials, A., Vidal, J., Franco, C., Ribera, F., Sener, A., Malaisse, W.J., et al. (1997). Mutation in the calcium-binding domain of the mitochondrial glycerophosphate dehydrogenase gene in a family of diabetic subjects. *Biochem Biophys Res Commun* 231(3)**,** 570-572. doi: 10.1006/bbrc.1997.6147.

Oberlinner, C., Ott, M.G., Nasterlack, M., Yong, M., Messerer, P., Zober, A., et al. (2009). Medical program for shift workers--impacts on chronic disease and mortality outcomes. *Scand J Work Environ Health* 35(4)**,** 309-318. doi: 10.5271/sjweh.1332.

Ohayon, M.M. (2002). Epidemiology of insomnia: what we know and what we still need to learn. *Sleep Med Rev* 6(2)**,** 97-111. doi: 10.1053/smrv.2002.0186.

Osei, K., Cottrell, D.A., Adenuwon, C.A., Ezenwaka, E.C., Akanji, A.O., and O'Dorisio, T.M. (1993). Serum insulin and glucose concentrations in people at risk for type II diabetes. A comparative study of African Americans and Nigerians. *Diabetes Care* 16(10)**,** 1367-1375. doi: 10.2337/diacare.16.10.1367.

Owens, D.S., Macdonald, I., Benton, D., Sytnik, N., Tucker, P., and Folkard, S. (1996). A preliminary investigation into individual differences in the circadian variation of meal tolerance: effects on mood and hunger. *Chronobiol Int* 13(6)**,** 435-447. doi: 10.3109/07420529609020914.

Pacini, G., and Bergman, R.N. (1986). MINMOD: a computer program to calculate insulin sensitivity and pancreatic responsivity from the frequently sampled intravenous glucose tolerance test. *Comput Methods Programs Biomed* 23(2)**,** 113-122. doi: 10.1016/0169-2607(86)90106-9.

Pamidi, S., Aronsohn, R.S., and Tasali, E. (2010). Obstructive sleep apnea: role in the risk and severity of diabetes. *Best Pract Res Clin Endocrinol Metab* 24(5)**,** 703-715. doi: 10.1016/j.beem.2010.08.009.

Pamidi, S., Wroblewski, K., Broussard, J., Day, A., Hanlon, E.C., Abraham, V., et al. (2012). Obstructive sleep apnea in young lean men: impact on insulin sensitivity and secretion. *Diabetes Care* 35(11)**,** 2384-2389. doi: 10.2337/dc12-0841.

Pan, A., Schernhammer, E.S., Sun, Q., and Hu, F.B. (2011). Rotating night shift work and risk of type 2 diabetes: two prospective cohort studies in women. *PLoS Med* 8(12)**,** e1001141. doi: 10.1371/journal.pmed.1001141.

Panda, S., Hogenesch, J.B., and Kay, S.A. (2002). Circadian rhythms from flies to human. *Nature* 417(6886)**,** 329-335. doi: 10.1038/417329a.

Papanas, N., Steiropoulos, P., Nena, E., Tzouvelekis, A., Maltezos, E., Trakada, G., et al. (2009). HbA1c is associated with severity of obstructive sleep apnea hypopnea syndrome in nondiabetic men. *Vasc Health Risk Manag* 5**,** 751-756. doi: 10.2147/vhrm.s7057.

Patrick, G.T.W., and Gilbert, J.A. (1896). Studies from the psychological laboratory of the University of Iowa: On the effects of loss of sleep. *Psychological Review* 3(5)**,** 469-483.

Peppard, P.E., Young, T., Palta, M., Dempsey, J., and Skatrud, J. (2000). Longitudinal study of moderate weight change and sleep-disordered breathing. *JAMA* 284(23)**,** 3015-3021. doi: 10.1001/jama.284.23.3015.

Petersen, K.F., Dufour, S., Befroy, D., Garcia, R., and Shulman, G.I. (2004). Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350(7)**,** 664-671. doi: 10.1056/NEJMoa031314.

Petersen, M.C., and Shulman, G.I. (2018). Mechanisms of Insulin Action and Insulin Resistance. *Physiol Rev* 98(4)**,** 2133-2223. doi: 10.1152/physrev.00063.2017.

Pfluger, P.T., Kampe, J., Castaneda, T.R., Vahl, T., D'Alessio, D.A., Kruthaupt, T., et al. (2007). Effect of human body weight changes on circulating levels of peptide YY and peptide YY3-36. *J Clin Endocrinol Metab* 92(2)**,** 583-588. doi: 10.1210/jc.2006-1425.

Phielix, E., Schrauwen-Hinderling, V.B., Mensink, M., Lenaers, E., Meex, R., Hoeks, J., et al. (2008). Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. *Diabetes* 57(11)**,** 2943-2949. doi: 10.2337/db08-0391.

Puchalski, S.S., Green, J.N., and Rasmussen, D.D. (2003). Melatonin effects on metabolism independent of gonad function. *Endocrine* 21(2)**,** 169-173. doi: 10.1385/ENDO:21:2:169.

Qian, J., Block, G.D., Colwell, C.S., and Matveyenko, A.V. (2013). Consequences of exposure to light at night on the pancreatic islet circadian clock and function in rats. *Diabetes* 62(10)**,** 3469-3478. doi: 10.2337/db12-1543.

Qian, J., Dalla Man, C., Morris, C.J., Cobelli, C., and Scheer, F. (2018). Differential effects of the circadian system and circadian misalignment on insulin sensitivity and insulin secretion in humans. *Diabetes Obes Metab* 20(10)**,** 2481-2485. doi: 10.1111/dom.13391.

Qian, J., Morris, C.J., Caputo, R., Garaulet, M., and Scheer, F. (2019a). Ghrelin is impacted by the endogenous circadian system and by circadian misalignment in humans. *Int J Obes (Lond)* 43(8)**,** 1644-1649. doi: 10.1038/s41366-018-0208-9.

Qian, J., Morris, C.J., Caputo, R., Wang, W., Garaulet, M., and Scheer, F. (2019b). Sex differences in the circadian misalignment effects on energy regulation. *Proc Natl Acad Sci U S A* 116(47)**,** 23806-23812. doi: 10.1073/pnas.1914003116.

Rafalson, L., Donahue, R.P., Stranges, S., Lamonte, M.J., Dmochowski, J., Dorn, J., et al. (2010). Short sleep duration is associated with the development of impaired fasting glucose: the Western New York Health Study. *Ann Epidemiol* 20(12)**,** 883-889. doi: 10.1016/j.annepidem.2010.05.002.

Rajaratnam, S.M., and Redman, J.R. (1998). Entrainment of activity rhythms to temperature cycles in diurnal palm squirrels. *Physiol Behav* 63(2)**,** 271-277. doi: 10.1016/s0031-9384(97)00440-x.

Rao, M.N., Neylan, T.C., Grunfeld, C., Mulligan, K., Schambelan, M., and Schwarz, J.M. (2015). Subchronic sleep restriction causes tissue-specific insulin resistance. *J Clin Endocrinol Metab* 100(4)**,** 1664-1671. doi: 10.1210/jc.2014-3911.

Raskind, M.A., Burke, B.L., Crites, N.J., Tapp, A.M., and Rasmussen, D.D. (2007). Olanzapine-induced weight gain and increased visceral adiposity is blocked by melatonin replacement therapy in rats. *Neuropsychopharmacology* 32(2)**,** 284-288. doi: 10.1038/sj.npp.1301093.

Rasmussen, D.D., Boldt, B.M., Wilkinson, C.W., Yellon, S.M., and Matsumoto, A.M. (1999). Daily melatonin administration at middle age suppresses male rat visceral fat, plasma leptin, and plasma insulin to youthful levels. *Endocrinology* 140(2)**,** 1009-1012. doi: 10.1210/endo.140.2.6674.

Rechtschaffen, A., Kales, A. (1968). *A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects.* Washington, D.C.: United States Government Printing Office.

Reppert, S.M., and Weaver, D.R. (2001). Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* 63**,** 647-676. doi: 10.1146/annurev.physiol.63.1.647.

Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian timing in mammals. *Nature* 418(6901)**,** 935-941. doi: 10.1038/nature00965.

Reynolds, A.C., Dorrian, J., Liu, P.Y., Van Dongen, H.P., Wittert, G.A., Harmer, L.J., et al. (2012). Impact of five nights of sleep restriction on glucose metabolism, leptin and testosterone in young adult men. *PLoS One* 7(7)**,** e41218. doi: 10.1371/journal.pone.0041218.

Rizzi, M., Barrella, M., Kotzalidis, G.D., and Bevilacqua, M. (2011). Periodic Limbic Movement Disorder during Sleep as Diabetes-Related Syndrome? A Polysomnographic Study. *ISRN Endocrinol* 2011**,** 246157. doi: 10.5402/2011/246157.

Robertson, M.D., Russell-Jones, D., Umpleby, A.M., and Dijk, D.J. (2013). Effects of three weeks of mild sleep restriction implemented in the home environment on multiple metabolic and endocrine markers in healthy young men. *Metabolism* 62(2)**,** 204-211. doi: 10.1016/j.metabol.2012.07.016.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1)**,** 139-140. doi: 10.1093/bioinformatics/btp616.

Roden, M., Price, T.B., Perseghin, G., Petersen, K.F., Rothman, D.L., Cline, G.W., et al. (1996). Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 97(12)**,** 2859-2865. doi: 10.1172/JCI118742.

Rodrigues, N.R., Macedo, G.E., Martins, I.K., Gomes, K.K., de Carvalho, N.R., Posser, T., et al. (2018). Short-term sleep deprivation with exposure to nocturnal light alters mitochondrial bioenergetics in Drosophila. *Free Radic Biol Med* 120**,** 395-406. doi: 10.1016/j.freeradbiomed.2018.04.549.

Roenneberg, T., and Merrow, M. (2000). Circadian clocks: Omnes viae Romam ducunt. *Curr Biol* 10(20)**,** R742-745. doi: 10.1016/s0960-9822(00)00739-9.

Ronksley, P.E., Hemmelgarn, B.R., Heitman, S.J., Hanly, P.J., Faris, P.D., Quan, H., et al. (2009). Obstructive sleep apnoea is associated with diabetes in sleepy subjects. *Thorax* 64(10)**,** 834-839. doi: 10.1136/thx.2009.115105.

Rusak, B., and Zucker, I. (1979). Neural regulation of circadian rhythms. *Physiol Rev* 59(3)**,** 449-526. doi: 10.1152/physrev.1979.59.3.449.

Rynders, C.A., Morton, S.J., Bessesen, D.H., Wright, K.P., Jr., and Broussard, J.L. (2020). Circadian Rhythm of Substrate Oxidation and Hormonal Regulators of Energy Balance. *Obesity (Silver Spring)* 28 Suppl 1**,** S104-S113. doi: 10.1002/oby.22816.

Rynders, C.A., Pereira, R.I., Bergouignan, A., Kealey, E.H., and Bessesen, D.H. (2018). Associations Among Dietary Fat Oxidation Responses to Overfeeding and Weight Gain in Obesity-Prone and Resistant Adults. *Obesity (Silver Spring)* 26(11)**,** 1758-1766. doi: 10.1002/oby.22321.

Saba, G.C., Materazzi, F., and Hoet, J.J. (1963). Diurnal Rhythm of the Adrenal Cortical Activity and of the Cortisol Metabolism in Normal Subjects. *Folia Endocrinol* 16**,** 611-616.

Sachs, S., Zarini, S., Kahn, D.E., Harrison, K.A., Perreault, L., Phang, T., et al. (2019). Intermuscular adipose tissue directly modulates skeletal muscle insulin sensitivity in humans. *American Journal of Physiology-Endocrinology and Metabolism* 316(5)**,** E866-E879. doi: 10.1152/ajpendo.00243.2018.

Sala, D., Ivanova, S., Plana, N., Ribas, V., Duran, J., Bach, D., et al. (2014). Autophagy-regulating TP53INP2 mediates muscle wasting and is repressed in diabetes. *J Clin Invest* 124(5)**,** 1914-1927. doi: 10.1172/JCI72327.

Salgado-Delgado, R.C., Saderi, N., Basualdo Mdel, C., Guerrero-Vargas, N.N., Escobar, C., and Buijs, R.M. (2013). Shift work or food intake during the rest phase promotes metabolic disruption and desynchrony of liver genes in male rats. *PLoS One* 8(4)**,** e60052. doi: 10.1371/journal.pone.0060052.

Saner, N.J., Lee, M.J., Kuang, J., Pitchford, N.W., Roach, G.D., Garnham, A., et al. (2021). Exercise mitigates sleep-loss-induced changes in glucose tolerance, mitochondrial function, sarcoplasmic protein synthesis, and diurnal rhythms. *Mol Metab* 43**,** 101110. doi: 10.1016/j.molmet.2020.101110.

Sargent, C., Zhou, X., Matthews, R.W., Darwent, D., and Roach, G.D. (2016). Daily Rhythms of Hunger and Satiety in Healthy Men during One Week of Sleep Restriction and Circadian Misalignment. *Int J Environ Res Public Health* 13(2)**,** 170. doi: 10.3390/ijerph13020170.

Savvidis, C., and Koutsilieris, M. (2012). Circadian rhythm disruption in cancer biology. *Mol Med* 18**,** 1249-1260. doi: 10.2119/molmed.2012.00077.

Scheer, F.A., Chan, J.L., Fargnoli, J., Chamberland, J., Arampatzi, K., Shea, S.A., et al. (2010). Day/night variations of high-molecular-weight adiponectin and lipocalin-2 in healthy men studied under fed and fasted conditions. *Diabetologia* 53(11)**,** 2401-2405. doi: 10.1007/s00125-010-1869-7.

Scheer, F.A., Hilton, M.F., Mantzoros, C.S., and Shea, S.A. (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc Natl Acad Sci U S A* 106(11)**,** 4453-4458. doi: 10.1073/pnas.0808180106.

Scheer, F.A., Morris, C.J., and Shea, S.A. (2013). The internal circadian clock increases hunger and appetite in the evening independent of food intake and other behaviors. *Obesity (Silver Spring)* 21(3)**,** 421-423. doi: 10.1002/oby.20351.

Schernhammer, E.S., Kroenke, C.H., Laden, F., and Hankinson, S.E. (2006). Night work and risk of breast cancer. *Epidemiology* 17(1)**,** 108-111. doi: 10.1097/01.ede.0000190539.03500.c1.

Schober, A.K., Neurath, M.F., and Harsch, I.A. (2011). Prevalence of sleep apnoea in diabetic patients. *Clin Respir J* 5(3)**,** 165-172. doi: 10.1111/j.1752-699X.2010.00216.x.

Schuld, A., Hebebrand, J., Geller, F., and Pollmacher, T. (2000). Increased body-mass index in patients with narcolepsy. *Lancet* 355(9211)**,** 1274-1275. doi: 10.1016/S0140-6736(05)74704-8.

Schwartz, M.W., Woods, S.C., Seeley, R.J., Barsh, G.S., Baskin, D.G., and Leibel, R.L. (2003). Is the energy homeostasis system inherently biased toward weight gain? *Diabetes* 52(2)**,** 232-238. doi: 10.2337/diabetes.52.2.232.

Senaratna, C.V., English, D.R., Currier, D., Perret, J.L., Lowe, A., Lodge, C., et al. (2016). Sleep apnoea in Australian men: disease burden, co-morbidities, and correlates from the Australian longitudinal study on male health. *BMC Public Health* 16(Suppl 3)**,** 1029. doi: 10.1186/s12889-016-3703-8.

Senaratna, C.V., Perret, J.L., Lodge, C.J., Lowe, A.J., Campbell, B.E., Matheson, M.C., et al. (2017a). Prevalence of obstructive sleep apnea in the general population: A systematic review. *Sleep Med Rev* 34**,** 70-81. doi: 10.1016/j.smrv.2016.07.002.

Senaratna, C.V., Perret, J.L., Matheson, M.C., Lodge, C.J., Lowe, A.J., Cassim, R., et al. (2017b). Validity of the Berlin questionnaire in detecting obstructive sleep apnea: A systematic review and meta-analysis. *Sleep Med Rev* 36**,** 116-124. doi: 10.1016/j.smrv.2017.04.001.

Shakespeare, W. (1992). *Macbeth.* Ware, England: Wordsworth Editions.

Shea, S.A., Hilton, M.F., Orlova, C., Ayers, R.T., and Mantzoros, C.S. (2005). Independent circadian and sleep/wake regulation of adipokines and glucose in humans. *J Clin Endocrinol Metab* 90(5)**,** 2537-2544. doi: 10.1210/jc.2004-2232.

Shepherd, P.R., and Kahn, B.B. (1999). Glucose Transporters and Insulin Action — Implications for Insulin Resistance and Diabetes Mellitus. *New England Journal of Medicine* 341(4)**,** 248-257. doi: 10.1056/nejm199907223410406.

Shields, M. (2002). Shift work and health. *Health Rep* 13(4)**,** 11-33.

Shimmura, T., and Yoshimura, T. (2013). Circadian clock determines the timing of rooster crowing. *Curr Biol* 23(6)**,** R231-233. doi: 10.1016/j.cub.2013.02.015.

Simoneau, J.A., Colberg, S.R., Thaete, F.L., and Kelley, D.E. (1995). Skeletal muscle glycolytic and oxidative enzyme capacities are determinants of insulin sensitivity and muscle composition in obese women. *FASEB J* 9(2)**,** 273-278.

Sinha, M.K., Ohannesian, J.P., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Magosin, S., et al. (1996). Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. *J Clin Invest* 97(5)**,** 1344-1347. doi: 10.1172/JCI118551.

Sokwalla, S.M., Joshi, M.D., Amayo, E.O., Acharya, K., Mecha, J.O., and Mutai, K.K. (2017). Quality of sleep and risk for obstructive sleep apnoea in ambulant individuals with type 2 diabetes mellitus at a tertiary referral hospital in Kenya: a cross-sectional, comparative study. *BMC Endocr Disord* 17(1)**,** 7. doi: 10.1186/s12902-017-0158-6.

Spengler, C.M., Czeisler, C.A., and Shea, S.A. (2000). An endogenous circadian rhythm of respiratory control in humans. *J Physiol* 526 Pt 3**,** 683-694. doi: 10.1111/j.1469-7793.2000.00683.x.

Spiegel, K., Leproult, R., L'Hermite-Baleriaux, M., Copinschi, G., Penev, P.D., and Van Cauter, E. (2004). Leptin levels are dependent on sleep duration: relationships with sympathovagal balance, carbohydrate regulation, cortisol, and thyrotropin. *J Clin Endocrinol Metab* 89(11)**,** 5762-5771. doi: 10.1210/jc.2004-1003.

Spiegel, K., Leproult, R., and Van Cauter, E. (1999). Impact of sleep debt on metabolic and endocrine function. *Lancet* 354(9188)**,** 1435-1439. doi: 10.1016/S0140-6736(99)01376-8.

Spiegel, K., Tasali, E., Leproult, R., and Van Cauter, E. (2009). Effects of poor and short sleep on glucose metabolism and obesity risk. *Nat Rev Endocrinol* 5(5)**,** 253-261. doi: 10.1038/nrendo.2009.23.

St-Pierre, J., Vohl, M.C., Brisson, D., Perron, P., Despres, J.P., Hudson, T.J., et al. (2001). A sequence variation in the mitochondrial glycerol-3-phosphate dehydrogenase gene is associated with increased plasma glycerol and free fatty acid concentrations among French Canadians. *Mol Genet Metab* 72(3)**,** 209-217. doi: 10.1006/mgme.2000.3144.

Stamatakis, K.A., and Punjabi, N.M. (2010). Effects of sleep fragmentation on glucose metabolism in normal subjects. *Chest* 137(1)**,** 95-101. doi: 10.1378/chest.09-0791.

Steiropoulos, P., Papanas, N., Nena, E., Tsara, V., Fitili, C., Tzouvelekis, A., et al. (2009). Markers of glycemic control and insulin resistance in non-diabetic patients with Obstructive Sleep Apnea Hypopnea Syndrome: does adherence to CPAP treatment improve glycemic control? *Sleep Med* 10(8)**,** 887-891. doi: 10.1016/j.sleep.2008.10.004.

Stevens, R.G., Blask, D.E., Brainard, G.C., Hansen, J., Lockley, S.W., Provencio, I., et al. (2007). Meeting report: the role of environmental lighting and circadian disruption in cancer and other diseases. *Environ Health Perspect* 115(9)**,** 1357-1362. doi: 10.1289/ehp.10200.

Stevens, R.G., and Rea, M.S. (2001). Light in the built environment: potential role of circadian disruption in endocrine disruption and breast cancer. *Cancer Causes Control* 12(3)**,** 279-287. doi: 10.1023/a:1011237000609.

Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y., and Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science* 291(5503)**,** 490-493. doi: 10.1126/science.291.5503.490.

Stothard, E.R., McHill, A.W., Depner, C.M., Birks, B.R., Moehlman, T.M., Ritchie, H.K., et al. (2017). Circadian Entrainment to the Natural Light-Dark Cycle across Seasons and the Weekend. *Curr Biol* 27(4)**,** 508-513. doi: 10.1016/j.cub.2016.12.041.

Straif, K., Baan, R., Grosse, Y., Secretan, B., El Ghissassi, F., Bouvard, V., et al. (2007). Carcinogenicity of shift-work, painting, and fire-fighting. *Lancet Oncol* 8(12)**,** 1065-1066. doi: 10.1016/S1470-2045(07)70373-X.

Suwazono, Y., Sakata, K., Okubo, Y., Harada, H., Oishi, M., Kobayashi, E., et al. (2006). Long-term longitudinal study on the relationship between alternating shift work and the onset of diabetes mellitus in male Japanese workers. *J Occup Environ Med* 48(5)**,** 455-461. doi: 10.1097/01.jom.0000214355.69182.fa.

Sweeney, E.L., Peart, D.J., Kyza, I., Harkes, T., Ellis, J.G., and Walshe, I.H. (2020). Impaired Insulin Profiles Following a Single Night of Sleep Restriction: The Impact of Acute Sprint Interval Exercise. *International Journal of Sport Nutrition and Exercise Metabolism* 30(2)**,** 139-144. doi: 10.1123/ijsnem.2019-0235.

Szymanski, J. (1918). Einige Bemerkungen über die biologische Bedeutung akustischer Reize. *Pflügers Arch* 171**,** 363-373. doi: https://doi.org/10.1007/BF01722099.

Takahashi, J.S. (2017). Transcriptional architecture of the mammalian circadian clock. *Nat Rev Genet* 18(3)**,** 164-179. doi: 10.1038/nrg.2016.150.

Tasali, E., Leproult, R., Ehrmann, D.A., and Van Cauter, E. (2008a). Slow-wave sleep and the risk of type 2 diabetes in humans. *Proc Natl Acad Sci U S A* 105(3)**,** 1044-1049. doi: 10.1073/pnas.0706446105.

Tasali, E., Van Cauter, E., Hoffman, L., and Ehrmann, D.A. (2008b). Impact of obstructive sleep apnea on insulin resistance and glucose tolerance in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 93(10)**,** 3878-3884. doi: 10.1210/jc.2008-0925.

Terman, A. (1913a). The sleep of school children, its distribution according to age, and its relation to physical and mental efficiency: Part I: The distribution of sleep according to age. *J.Educ.Psychol* 4**,** 138-147.

Terman, A. (1913b). The sleep of school children, its distribution according to age, and its relation to physical and mental efficiency: Part III: The conditions of children's sleep. *J.Educ.Psychol* 4**,** 269-282.

Torsvall, L., Akerstedt, T., Gillander, K., and Knutsson, A. (1989). Sleep on the night shift: 24-hour EEG monitoring of spontaneous sleep/wake behavior. *Psychophysiology* 26(3)**,** 352-358. doi: 10.1111/j.1469-8986.1989.tb01934.x.

Tuchsen, F., Hannerz, H., and Burr, H. (2006). A 12 year prospective study of circulatory disease among Danish shift workers. *Occup Environ Med* 63(7)**,** 451-455. doi: 10.1136/oem.2006.026716.

Tune, G.S. (1968). Sleep and wakefulness in normal human adults. *Br Med J* 2(5600)**,** 269-271.

Tune, G.S. (1969). The influence of age and temperament on the adult human sleep-wakefulness pattern. *Br J Psychol* 60(4)**,** 431-441.

van Amelsvoort, L.G., Schouten, E.G., and Kok, F.J. (1999). Duration of shiftwork related to body mass index and waist to hip ratio. *Int J Obes Relat Metab Disord* 23(9)**,** 973-978. doi: 10.1038/sj.ijo.0801028.

Van Cauter, E., Blackman, J.D., Roland, D., Spire, J.P., Refetoff, S., and Polonsky, K.S. (1991). Modulation of glucose regulation and insulin secretion by circadian rhythmicity and sleep. *J Clin Invest* 88(3)**,** 934-942. doi: 10.1172/JCI115396.

van den Berg, R., Mook-Kanamori, D.O., Donga, E., van Dijk, M., van Dijk, J.G., Lammers, G.J., et al. (2016). A single night of sleep curtailment increases plasma acylcarnitines: Novel insights in the relationship between sleep and insulin resistance. *Arch Biochem Biophys* 589**,** 145-151. doi: 10.1016/j.abb.2015.09.017.

Vetter, C., Dashti, H.S., Lane, J.M., Anderson, S.G., Schernhammer, E.S., Rutter, M.K., et al. (2018). Night Shift Work, Genetic Risk, and Type 2 Diabetes in the UK Biobank. *Diabetes Care* 41(4)**,** 762-769. doi: 10.2337/dc17-1933.

Vgontzas, A.N., Fernandez-Mendoza, J., Liao, D., and Bixler, E.O. (2013). Insomnia with objective short sleep duration: the most biologically severe phenotype of the disorder. *Sleep Med Rev* 17(4)**,** 241-254. doi: 10.1016/j.smrv.2012.09.005.

Vgontzas, A.N., Liao, D., Pejovic, S., Calhoun, S., Karataraki, M., and Bixler, E.O. (2009). Insomnia with objective short sleep duration is associated with type 2 diabetes: A population-based study. *Diabetes Care* 32(11)**,** 1980-1985. doi: 10.2337/dc09-0284.

von Maltzahn, J., Bentzinger, C.F., and Rudnicki, M.A. (2011). Wnt7a-Fzd7 signalling directly activates the Akt/mTOR anabolic growth pathway in skeletal muscle. *Nat Cell Biol* 14(2)**,** 186-191. doi: 10.1038/ncb2404.

Vosshall, L.B., Price, J.L., Sehgal, A., Saez, L., and Young, M.W. (1994). Block in nuclear localization of period protein by a second clock mutation, timeless. *Science* 263(5153)**,** 1606-1609. doi: 10.1126/science.8128247.

Vyas, M.V., Garg, A.X., Iansavichus, A.V., Costella, J., Donner, A., Laugsand, L.E., et al. (2012). Shift work and vascular events: systematic review and meta-analysis. *BMJ* 345**,** e4800. doi: 10.1136/bmj.e4800.

Wang, X., Greer, J., Porter, R.R., Kaur, K., and Youngstedt, S.D. (2016). Short-Term Moderate Sleep Restriction Decreases Insulin Sensitivity in Young Healthy Adults. *Sleep Health* 2(1)**,** 63-68. doi: 10.1016/j.sleh.2015.11.004.

Waterhouse, J., Jones, K., Edwards, B., Harrison, Y., Nevill, A., and Reilly, T. (2004). Lack of evidence for a marked endogenous component determining food intake in humans during forced desynchrony. *Chronobiol Int* 21(3)**,** 445-468. doi: 10.1081/cbi-120038628.

Weaver, T.E., Laizner, A.M., Evans, L.K., Maislin, G., Chugh, D.K., Lyon, K., et al. (1997). An instrument to measure functional status outcomes for disorders of excessive sleepiness. *Sleep* 20(10)**,** 835-843.

Wefers, J., van Moorsel, D., Hansen, J., Connell, N.J., Havekes, B., Hoeks, J., et al. (2018). Circadian misalignment induces fatty acid metabolism gene profiles and compromises insulin sensitivity in human skeletal muscle. *Proc Natl Acad Sci U S A* 115(30)**,** 7789-7794. doi: 10.1073/pnas.1722295115.

Weir, G.C., and Bonner-Weir, S. (2004). Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes* 53 Suppl 3**,** S16-21. doi: 10.2337/diabetes.53.suppl_3.s16.

Weir, J.B. (1949). New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol* 109(1-2)**,** 1-9. doi: 10.1113/jphysiol.1949.sp004363.

Wolden-Hanson, T., Mitton, D.R., McCants, R.L., Yellon, S.M., Wilkinson, C.W., Matsumoto, A.M., et al. (2000). Daily melatonin administration to middle-aged male rats suppresses body weight, intraabdominal adiposity, and plasma leptin and insulin independent of food intake and total body fat. *Endocrinology* 141(2)**,** 487-497. doi: 10.1210/endo.141.2.7311.

Wong, P.M., Manuck, S.B., DiNardo, M.M., Korytkowski, M., and Muldoon, M.F. (2015). Shorter sleep duration is associated with decreased insulin sensitivity in healthy white men. *Sleep* 38(2)**,** 223-231. doi: 10.5665/sleep.4402.

Wright, K.J., Drake, CL., Lockley, SW (2008). "Diagnostic Tools for Circadian Rhythm Sleep Disorders," in *Handbook of Sleep Disorders (2nd ed.)*. CRC Press), 28.

Wright, K.P., Jr., Bogan, R.K., and Wyatt, J.K. (2013a). Shift work and the assessment and management of shift work disorder (SWD). *Sleep Med Rev* 17(1)**,** 41-54. doi: 10.1016/j.smrv.2012.02.002.

Wright, K.P., Jr., Gronfier, C., Duffy, J.F., and Czeisler, C.A. (2005). Intrinsic period and light intensity determine the phase relationship between melatonin and sleep in humans. *J Biol Rhythms* 20(2)**,** 168-177. doi: 10.1177/0748730404274265.

Wright, K.P., Jr., Hughes, R.J., Kronauer, R.E., Dijk, D.J., and Czeisler, C.A. (2001). Intrinsic near-24-h pacemaker period determines limits of circadian entrainment to a weak synchronizer in humans. *Proc Natl Acad Sci U S A* 98(24)**,** 14027-14032. doi: 10.1073/pnas.201530198.

Wright, K.P., Jr., Hull, J.T., Hughes, R.J., Ronda, J.M., and Czeisler, C.A. (2006). Sleep and wakefulness out of phase with internal biological time impairs learning in humans. *J Cogn Neurosci* 18(4)**,** 508-521. doi: 10.1162/jocn.2006.18.4.508.

Wright, K.P., Jr., McHill, A.W., Birks, B.R., Griffin, B.R., Rusterholz, T., and Chinoy, E.D. (2013b). Entrainment of the human circadian clock to the natural light-dark cycle. *Curr Biol* 23(16)**,** 1554-1558. doi: 10.1016/j.cub.2013.06.039.

Wurtman, R.J., Axelrod, J., and Phillips, L.S. (1963). Melatonin Synthesis in the Pineal Gland: Control by Light. *Science* 142(3595)**,** 1071-1073. doi: 10.1126/science.142.3595.1071.

Wyatt, J.K., Ritz-De Cecco, A., Czeisler, C.A., and Dijk, D.J. (1999). Circadian temperature and melatonin rhythms, sleep, and neurobehavioral function in humans living on a 20-h day. *Am J Physiol* 277(4 Pt 2)**,** R1152-1163. doi: 10.1152/ajpregu.1999.277.4.r1152.

Yi, J.S., Diaz, N.M., D'Souza, S., and Buhr, E.D. (2021). The molecular clockwork of mammalian cells. *Semin Cell Dev Biol*. doi: 10.1016/j.semcdb.2021.03.012.

Young, M.W., and Kay, S.A. (2001). Time zones: a comparative genetics of circadian clocks. *Nat Rev Genet* 2(9)**,** 702-715. doi: 10.1038/35088576.

Yu, G., Wang, L.G., Han, Y., and He, Q.Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 16(5)**,** 284-287. doi: 10.1089/omi.2011.0118.

Zehring, W.A., Wheeler, D.A., Reddy, P., Konopka, R.J., Kyriacou, C.P., Rosbash, M., et al. (1984). P-element transformation with period locus DNA restores rhythmicity to mutant, arrhythmic Drosophila melanogaster. *Cell* 39(2 Pt 1)**,** 369-376. doi: 10.1016/0092-8674(84)90015-1.

Zhang, B., Wu, J., Cai, Y., Luo, M., Wang, B., and Gu, Y. (2018). AAED1 modulates proliferation and glycolysis in gastric cancer. *Oncol Rep* 40(2)**,** 1156-1164. doi: 10.3892/or.2018.6478.

Zhang, R., Lahens, N.F., Ballance, H.I., Hughes, M.E., and Hogenesch, J.B. (2014). A circadian gene expression atlas in mammals: implications for biology and medicine. *Proc Natl Acad Sci U S A* 111(45)**,** 16219-16224. doi: 10.1073/pnas.1408886111.

Zhang, Y.X., Liu, Y., Dong, J., Wang, Y.X., Wang, J., Zhuang, G.Q., et al. (2013). An exploratory study of the association between KCNB1 rs1051295 and type 2 diabetes and its related traits in Chinese Han population. *PLoS One* 8(2)**,** e56365. doi: 10.1371/journal.pone.0056365.

Zhao, H., Wu, H., He, J., Zhuang, J., Liu, Z., Yang, Y., et al. (2016). Frontal cortical mitochondrial dysfunction and mitochondria-related beta-amyloid accumulation by chronic sleep restriction in mice. *Neuroreport* 27(12)**,** 916-922. doi: 10.1097/WNR.0000000000000631.

Zhu, J.L., Hjollund, N.H., Andersen, A.M., and Olsen, J. (2004). Shift work, job stress, and late fetal loss: The National Birth Cohort in Denmark. *J Occup Environ Med* 46(11)**,** 1144-1149. doi: 10.1097/01.jom.0000145168.21614.21.

Zielinski, M.R., Kline, C.E., Kripke, D.F., Bogan, R.K., and Youngstedt, S.D. (2008). No effect of 8-week time in bed restriction on glucose tolerance in older long sleepers. *J Sleep Res* 17(4)**,** 412-419. doi: 10.1111/j.1365-2869.2008.00673.x.

Zitting, K.M., Vujovic, N., Yuan, R.K., Isherwood, C.M., Medina, J.E., Wang, W., et al. (2018). Human Resting Energy Expenditure Varies with Circadian Phase. *Curr Biol* 28(22)**,** 3685-3690 e3683. doi: 10.1016/j.cub.2018.10.005.

Zulley, J., Wever, R., and Aschoff, J. (1981). The dependence of onset and duration of sleep on th circadian rhythm of rectal temperature. *Pflugers Arch* 391(4)**,** 314-318. doi: 10.1007/BF00581514.

APPENDIX

<u>Supplemental materials</u>

<u>Participants</u>

Prior to study enrollment, participants completed a screening consisting of

a medical history evaluation, physical examination, and clinical labs (complete blood count,

comprehensive metabolic panel, and thyroid stimulating hormone). Inclusion criteria were: 18-40

years old; BMI 18.5-30 kg/m^2; low to moderate caffeine (<500 mg/day) and alcohol use

(average <2 standard drinks/day/week and ≤5 drinks in any day); free of drugs and nonsmokers;

no current or previous diagnosis of a sleep disorder. Exclusion criteria were: current or

chronic medical/psychiatric conditions; pregnancy; shift work in the previous year; dwelling

below Denver altitude (1600 m) the year prior to study; travel across more than one time zone

three weeks prior to the in-laboratory Clinical Translational Research Center (CTRC) study at

the University of Colorado Hospital; a self-reported lifetime BMI>30 kg/m^2; >5% weight change

in the past 6 months; eating disorder. Participants self-reported being medication free and a

urine toxicology test verified drug free status upon CTRC admission.

<u>Indirect calorimetry</u>

Because urine nitrogen excretion was not collected, the RQ values assume some

contribution of protein oxidation, approximately equivalent to 15% of TDEE (the protein content

of the diet). The first five minutes of each recording were excluded from analyses. Recordings

were shortened (if necessary) to minimize the chance of the participant falling asleep during the

recording (mean number of minutes used in the analyses=8.2±2.5 min; minimum=4 min, n=1

time point; maximum=14 min). Only continuous data with an associated fraction of exhaled

carbon dioxide (FECO2) between 0.9%–1.2% were included in the analyses (actual FECO2

across all valid tests was 1.0±0.08%, mean±SD; CV%=4.5±1.8%).

Analysis of circadian rhythmicity

CircWave is an extension of traditional cosinor analysis but fits one or more sinusoidal curves (harmonics) to individual data and compares this with a horizontal line through the mean of the data. CircWave analyses are included for ease of comparison between our findings and previously published reports that have used cosinor or related methods. CircWave provides the following outputs: number of sine waves contributing to the fitted curve; phase of the fitted curve; ANOVA F-statistic, ANOVA P-value (tests the significance of the number of sine waves selected to fit the data compared to a horizontal line); and the CircWave P-value and R^2 (tests the goodness of fit). In almost all cases a single harmonic fit the data (or failed to fit the data). The only exceptions were subjective hunger, "desire to eat", "pre-occupation with thoughts of food", and "desire for sweet foods" which were best fit by either one or two additional harmonics. Estimates of peak-to-nadir amplitude were also calculated for each fitted curve.

Table S2.1. Hormone Assays

Assay	Manufacturer	Method	Within-day Precision	Between-day Precision	Sensitivity
Leptin	Millipore	RIA	5.9%	5.8%	0.5 ng/mL
Total Ghrelin	Millipore	RIA	4.5%	13.5%	93 pg/mL
Peptide-YY	Millipore	RIA	5.3%	8.9%	10 pg/mL
Estradiol	Beckman Coulter	Chemiluminescent Immunoassay	4.3%	8.2%	10 pg/mL
Progesterone	Beckman Coulter	Chemiluminescent Immunoassay	4.4%	7.9%	0.1 ng/mL

Table S2.2. CircWave Fit Statistics for Each Participant, R^2 (P-Value)

Variable (% of mean)	PPT1	PPT2	PPT3	PPT4	PPT5	PPT6	PPT7
Sex (M/F)	M	F	M	F	M	M	F
EE	0.19 (0.52)	0.57 (0.07)	0.04 (0.88)	0.11 (0.80)	0.33 (0.31)	0.18 (0.68)	0.73 (0.02)
RQ	0.98 (<0.001)	0.25 (0.42)	0.92 (<0.001)	0.41 (0.35)	0.78 (0.01)	0.53 (0.22)	0.28 (0.38)
Fat Ox	0.82 (0.006)	0.23 (0.46)	0.72 (0.02)	0.55 (0.21)	0.72 (0.02)	0.17 (0.69)	0.57 (0.08)
CHO Ox	0.96 (0.004)	0.26 (0.41)	0.93 (<0.001)	0.38 (0.38)	0.76 (0.01)	0.48 (0.27)	0.31 (0.33)
% Fat Ox	0.96 (0.005)	0.25 (0.42)	0.92 (<0.001)	0.41 (0.35)	0.78 (0.01)	0.53 (0.22)	0.28 (0.38)
% CHO Ox	0.17 (0.005)	0.25 (0.42)	0.92 (<0.001)	0.41 (0.35)	0.78 (0.01)	0.53 (0.22)	0.28 (0.38)
Hunger	No data	0.74 (<0.001)	0.64 (<0.001)	0.41 (0.03)	0.24 (0.21)	0.11 (0.64)	0.57 (0.002)
Fullness	No data	0.04 (0.61)	0.07 (0.45)	0.10 (0.30)	0.23 (0.06)	0.18 (0.10)	0.10 (0.33)
Ghrelin	0.29 (0.02)	0.21 (0.07)	0.08 (0.38)	0.48 (<0.001)	0.56 (<0.001)	0.10 (0.30)	0.13 (0.21)
Leptin	0.33 (0.01)	0.05 (0.67)	0.27 (0.03)	0.05 (0.55)	0.02 (0.81)	0.08 (0.40)	0.21 (0.04)
PYY	0.35 (0.008)	0.38 (0.005)	0.27 (0.04)	0.31 (0.02)	0.04 (0.63)	0.54 (0.003)	0.16 (0.15)

Shading indicates a significant P-value <0.05. EE=Energy expenditure; RQ=Respiratory quotient; Fat Ox=Fat oxidation; CHO=Carbohydrate oxidation.

Table S2.3. Circadian Rhythmicity of Additional Dimensions of Hunger Evaluated by CircWave.

Variable	Data Mean (SEM)	LMM (P-Value)	Peak Phase Degrees (Rel. Clock Hour)	Peak to Nadir Amplitude	ANOVA: F-statistic (P-value)	CircWave: R² (P-value)
Desire to eat*	32.8 (1.5)	0.02	270° (~1400h)	37.7%	1.84 (0.02)	0.21 (<0.001)
How much could you eat right now?	40.0 (1.6)	0.33	322° (~1726h)	9.6%	1.17 (0.28)	0.02 (0.32)
Preoccupation with thoughts of food*	20.5 (1.6)	0.61	271° (~1400h)	26.4%	0.93 (0.56)	0.11 (0.006)
Desire for meat	31.9 (2.0)	<0.001	334° (~1815h)	44.0%	2.58 (<0.001)	0.27 (<0.001)
Desire for fruit	30.9 (2.4)	0.03	345° (~1900h)	35.7%	1.86 (0.02)	0.16 (<0.001)
Desire for dairy	26.9 (2.1)	0.003	353° (~1930h)	43.3%	2.33 (0.002)	0.30 (<0.001)
Desire for vegetables	27.3 (1.8)	<0.001	357° (~1945h)	68.0%	3.82 (<0.001)	0.32 (<0.001)
Desire for salty foods	29.5 (2.3)	<0.001	348° (~1910h)	53.6%	3.06 (<0.001)	0.29 (<0.001)
Desire for sweet foods*	16.7 (0.8)	0.06	49° (~2315h)	44.9%	1.64 (0.05)	0.15 (<0.001)

*Linear harmonic regression performed by CircWave determined that the profile for "Desire to eat" was best fit by 3 sinusoidal waves, whereas "Preoccupation with thoughts of food" and "Desire for sweet foods" were best fit by 2 sine waves. All other variables were described by a single sine wave. LMM= Linear mixed model effect of time; Rel. clock= Relative clock time.
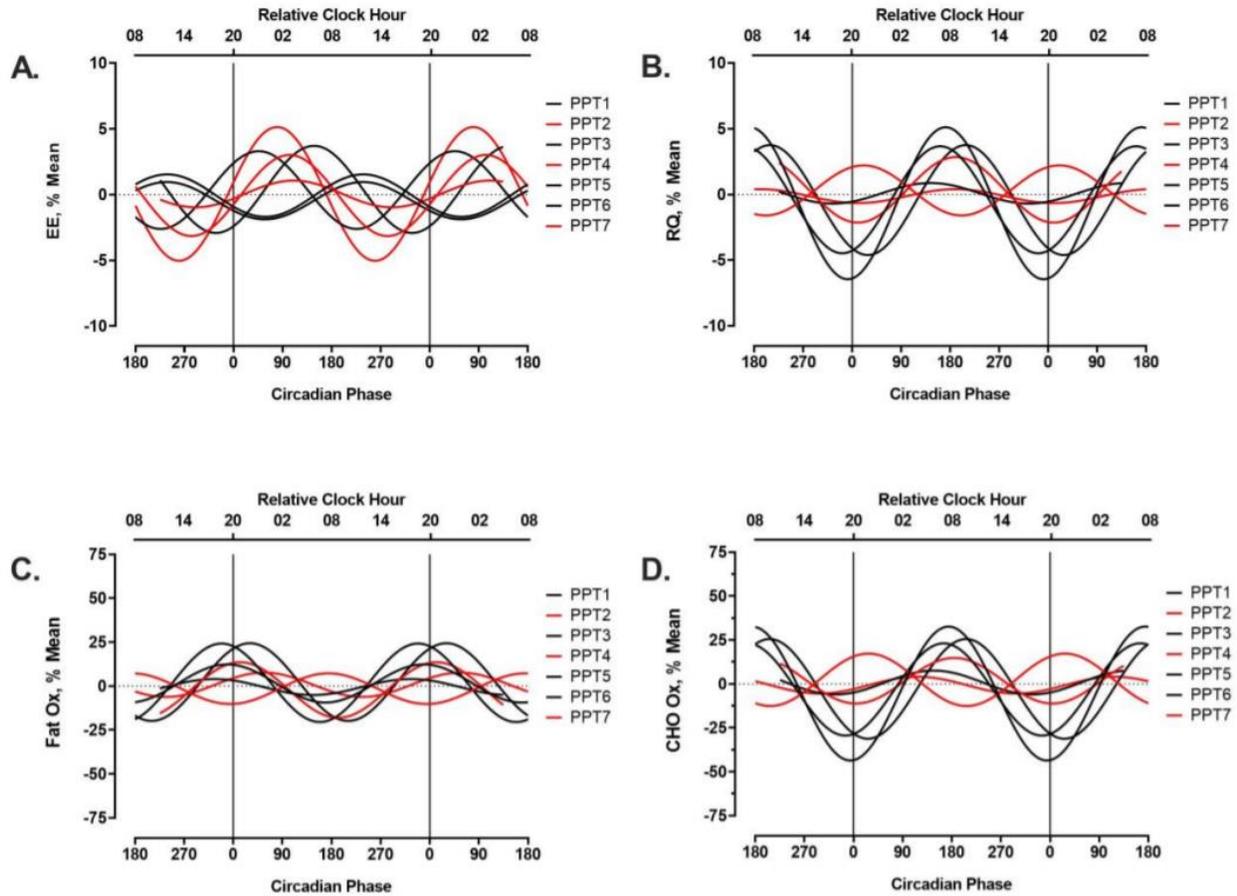
Figure S2.1. Individual Circadian Variation of Energy Expenditure, Respiratory Quotient, and Fuel Oxidation During Constant Routine Conditions. EE= Energy expenditure; RQ= Respiratory quotient (ratio of carbon dioxide production to oxygen consumption); Data are double plotted for visualization of circadian rhythms; Circadian phase, 0°= dim light melatonin onset; Female participants noted by red curves.
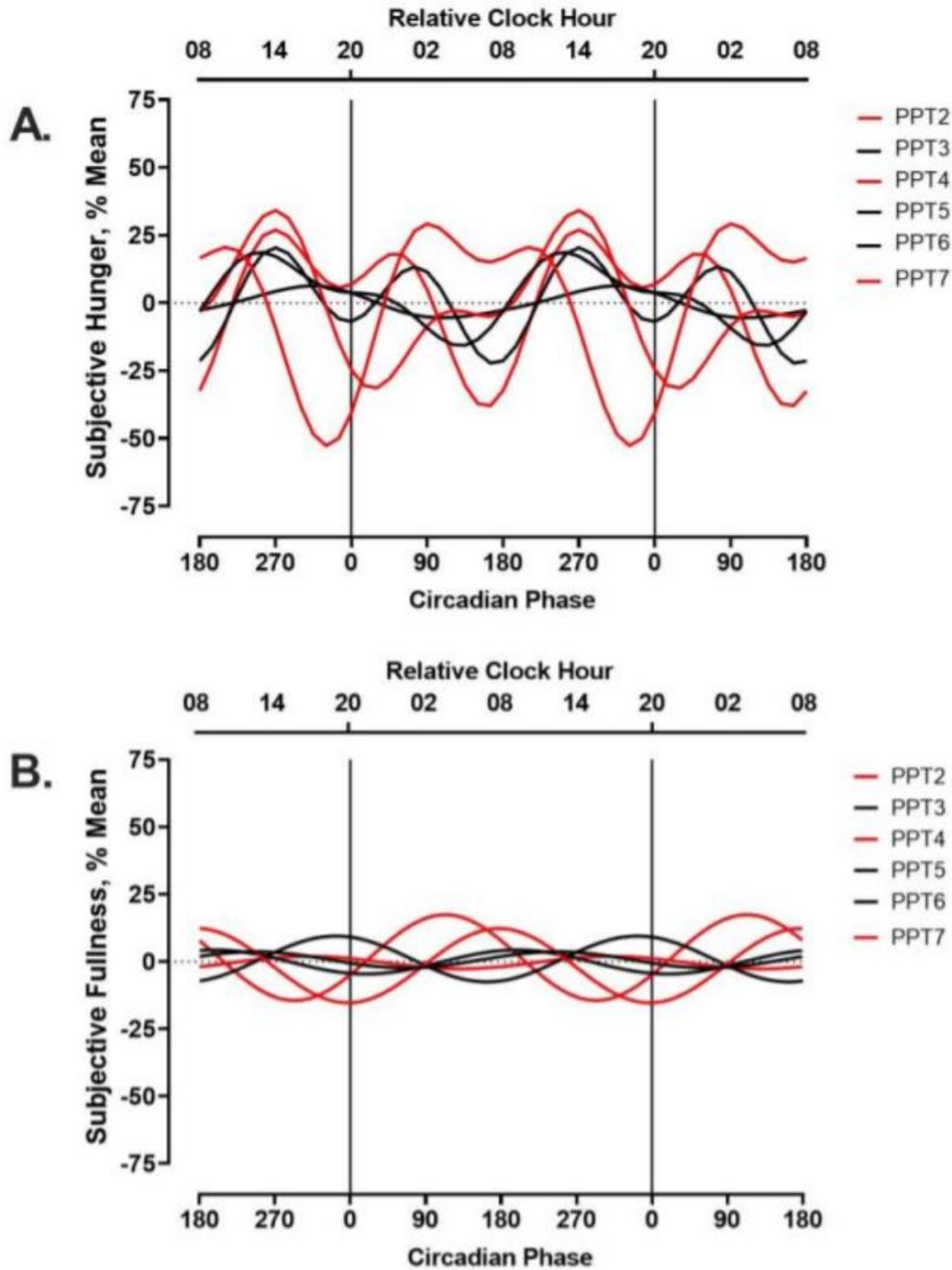
Figure S2.2. Individual Circadian Variation of Subjective Hunger and Fullness Ratings During Constant Routine Conditions. Data are double plotted for visualization of circadian rhythms; Circadian phase, 0°= dim light melatonin onset; Female participants noted by red curves.
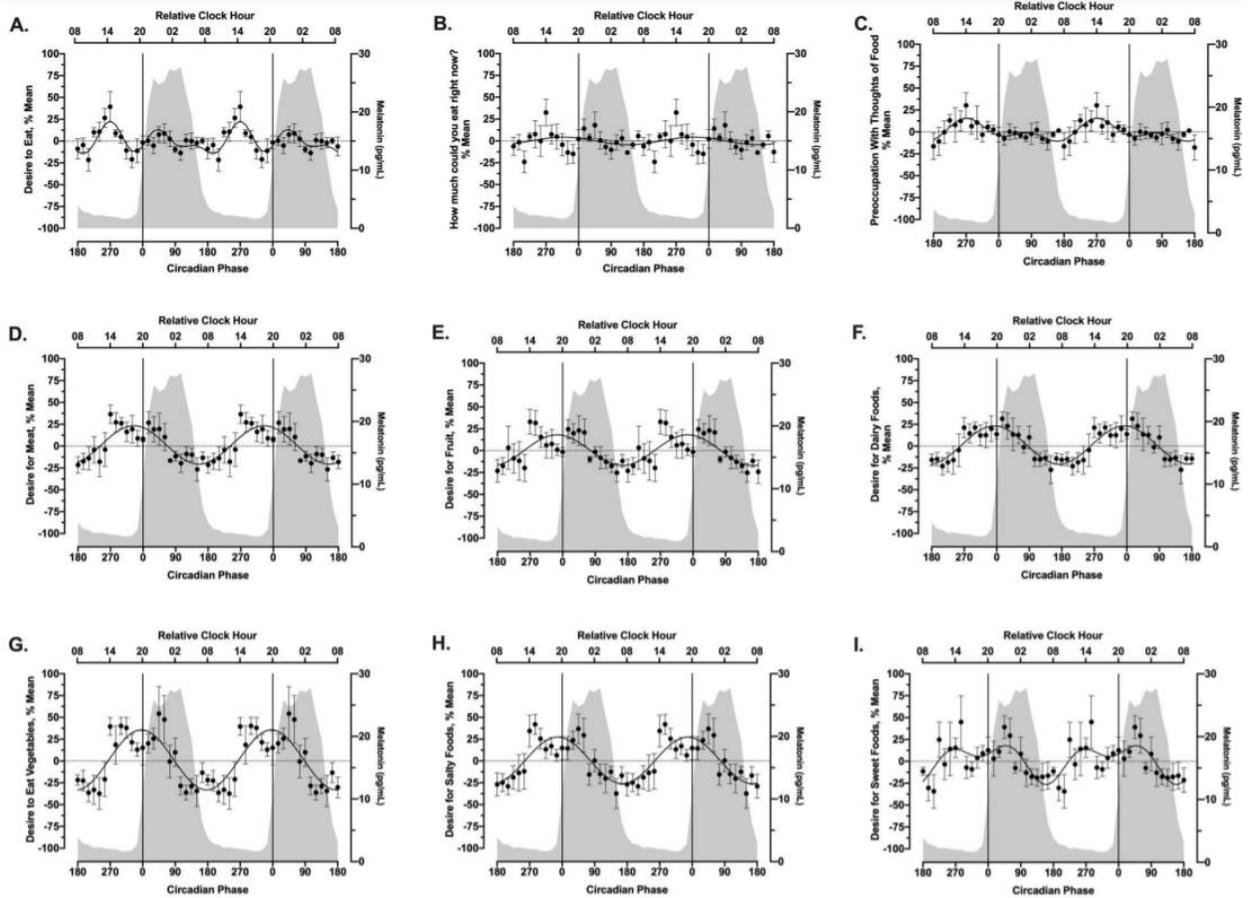
Figure S2.3. Circadian Variation in Additional Ratings of Subjective Hunger and Desire for Certain Food Groups During Constant Routine Conditions. Data are double plotted for visualization of circadian rhythms; Circadian phase, 0°= dim light melatonin onset. Shaded area indicates average salivary melatonin curve.
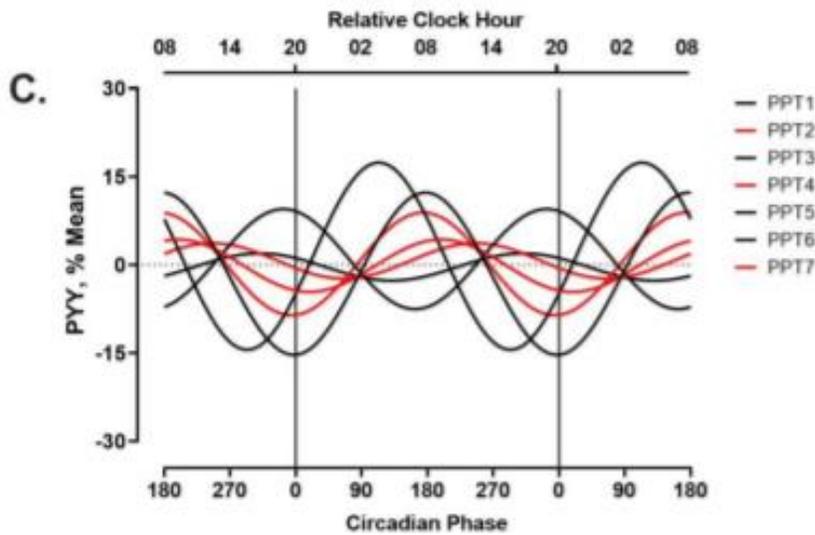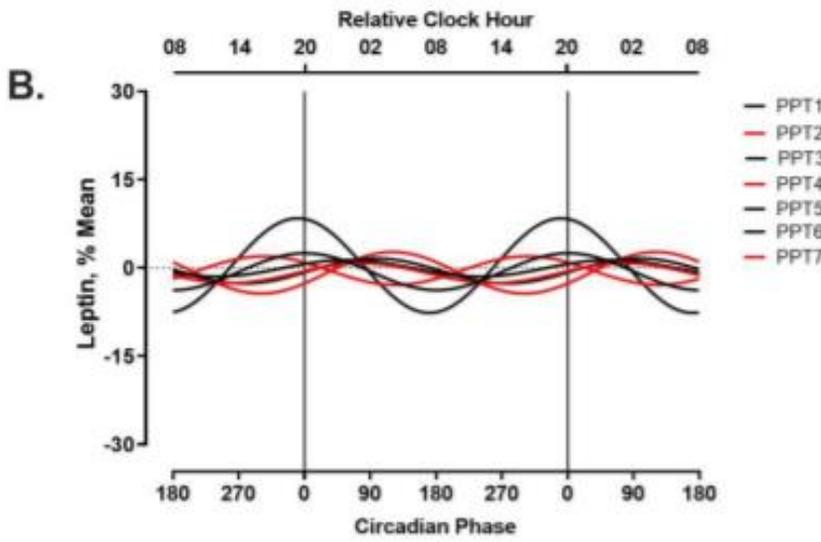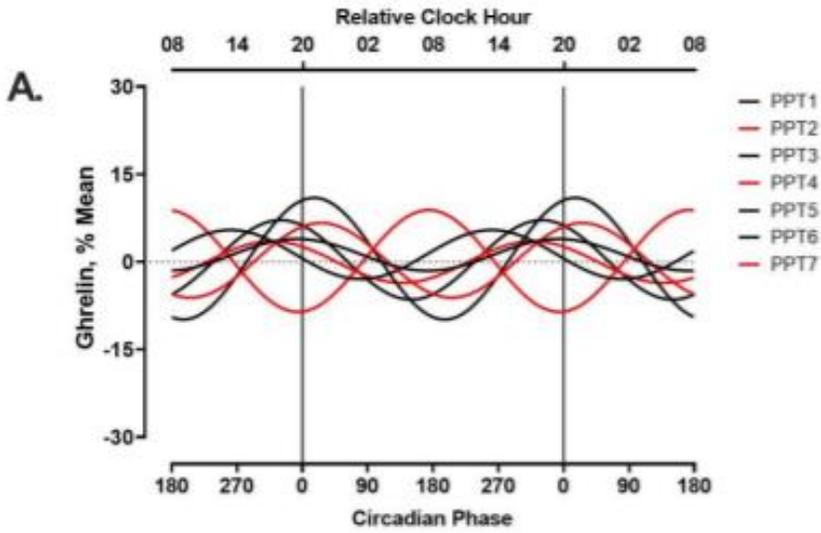
Figure S2.4. Individual Circadian Variation of Appetite-Related Hormones During Constant Routine Conditions. PYY= Peptide tyrosine (Y) tyrosine (Y); Data are double plotted for visualization of circadian rhythms; Circadian phase, 0°= dim light melatonin onset; Female participants noted by red curves.