THESIS

THE IMPACT OF TIME-RESTRICTED EATING ON CIRCULATING FACTORS, INSULIN SENSITIVITY AND CIRCADIAN RHYTHMS

Submitted by

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ABSTRACT

THE IMPACT OF TIME-RESTRICTED EATING ON CIRCULATING FACTORS, INSULIN SENSITIVITY AND CIRCADIAN RHYTHMS

Purpose: Obesity has been steadily increasing over several decades. In 2008, prevalence rates of obesity were reported at over 3oo million people, defined as a body mass index of >30kg/m^2. For years, scientists have tried to find "solutions" to obesity. While obesity prevention measures taken in childhood might result in decreased adulthood obesity, childhood prevention measures are not common, and obesity is often a health issue in adulthood. Negative energy balance and caloric restriction is most effective for reducing body weight, and studies have reported beneficial effects such as reduced fasting glucose and insulin, reductions in body weight [1], significantly higher insulin sensitivity, significantly lower BMI [2], reduced β-cell sensitivity [3], and reduced fasting glycemia and fasting insulinemia [4]; however, long-term adherence to caloric restriction is low.

Certain fasting practices are emerging as promising possible solutions to help combat obesity. Fasting practices have resulted in improvements in cardiometabolic health including but not limited to protection from obesity [5], improved LDL and HDL cholesterol, reduced HbA1c and c-reactive proteins, [6], cell proliferation, and body weight [7].

Intermittent fasting is one method by which an individual can reduce body weight but also improve numerous cardiometabolic factors. However, research exploring intermittent fasting (IF), specifically time-restricted eating (TRE), as a method of improving cardiometabolic health is limited. Circadian rhythms might be the reason that aligning feeding windows to earlier in the day

is showing these benefits. Currently, a gap in the knowledge exists as to whether circadian rhythms play a role in contributing to the metabolic benefits that are conferred by TRE, or if the timing of the food intake/duration is what results in the benefits. Therefore, our objective was to examine the effects of TRE on 24-hour glucose homeostasis and nighttime patterns of circulating factors (glucose, insulin, free fatty acids, triglycerides, and glycerol) as well as insulin sensitivity and the central circadian clock.

Methods and results: This study employed a consecutive design. Eight healthy adults (6F; 27±4 y; 22.6±2.1 kg/m2; mean ± SD) completed a 2-week protocol. During Week 1 participants were instructed to consume their daily calories over a 13h period (control condition). In Week 2, participants were instructed to consume their daily calories over an 8h period (TRE condition). Specified mealtimes were pre-determined based on the habitual sleep and wake time for each individual participant. At the end of each week, participants were admitted to the Sleep and Metabolism Laboratory for an overnight stay that involved hourly blood samples. Plasma samples were analyzed for glucose, insulin, free fatty acids (FFA), lactate, triglycerides, and glycerol. The plasma analyses indicated that TRE decreased glucose variability during sleep (p=0.03), reduced nighttime insulin concentrations (p=0.005), increased nighttime FFA levels (p=0.04), increased nighttime triglycerides (p=0.006) and increased nighttime glycerol (p=0.02). TRE did not impact glucose variability during wakefulness (p = 0.49), nighttime glucose (p = 0.39), insulin sensitivity (MATSUDA-ISI, p = 0.38), or central circadian rhythms.

Conclusion: The observed changes in nighttime glucose variability and insulin levels could represent mechanisms by which TRE can improve metabolic homeostasis in healthy lean individuals. Future studies are warranted to determine whether TRE can improve metabolic

homeostasis in people at risk for diabetes such as people with overweight and obesity, and impaired glucose tolerance.

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CHAPTER 1

1.1. Introduction

Obesity

Obesity has been steadily increasing over the last four decades, and over 300 million people worldwide exceed the body mass index (BMI) threshold of 30 kg/m^2 as of 2008 [8]. More than one in five adults in the United States are considered obese, and increased rates of obesity are associated with increased rates of chronic physical illnesses, mortality, and could possibly be more detrimental to health than smoking or problem drinking [9]. Obesity is induced by a chronic imbalance in energy intake and energy expenditure (with energy intake exceeding energy expenditure) and it can result in negative health outcomes including (but not limited to): cardiovascular disease, type 2 diabetes [10], depression [7], hypertension, impaired quality of life, psychosocial disturbances, limited access to quality care [8], and coronary heart disease [11].

A "solution" for obesity?

There have been numerous attempts across several decades aimed at finding the "cure" for obesity. The cause of obesity is likely multifactorial, including behavioral, structural (household), social, psychological, biological [12] and genetic [13] factors. As described above, a chronic positive energy balance plays an important role in the development of obesity. Numerous studies have been conducted on the effect of diet and/or exercise interventions on weight loss [14-19]; however, the obesity epidemic continues to grow. In order to attempt to combat the obesity epidemic, the food industry must work hand-in-hand with the government, academics, and medical communities to strengthen consumers' understanding of which nutritional strategies could assist in their weight loss; however, this, too, must be individualized as there is not one answer that would "solve" every individual's battle with obesity [20].

Negative Energy Balance and Caloric Restriction

Caloric restriction is the most effective intervention to reduce body weight and is generally defined as a 10-40% reduction in caloric intake. Caloric restriction can result in preventative effects on conditions including cancer, hypertension, diabetes, and age-related diseases in humans and non-human primates, all without making drastic changes to the nutrient content of meals [21, 22]. Caloric restriction thus induces a negative energy balance, which if continued over time will lead to a loss in body mass. Numerous clinical trials have reported the beneficial effects of caloric restriction on fasting glucose and insulin as well as reduced body weight [1-4, 23], increased insulin sensitivity [2, 3], reduced beta-cell sensitivity [3], reduced glycemia and insulinemia [4], reductions in waist circumference, visceral fat mass, systolic and diastolic blood pressure [23]. Caloric restriction has also resulted in effects on risk factors for cardiovascular disease including reductions in body weight that are associated with decreased LDL cholesterol, triglycerides, and an increase in HDL cholesterol [24]. Further, caloric restriction-induced weight loss also results in decreased inflammatory markers, increased insulin sensitivity while decreasing insulin resistance, increased serum adipokine concentrations, reduced circulating levels of resistin, leptin, serum glucose and accumulation of glycation end-products, and increased adiponectin [25]. However, long term adherence to low calorie diets is low, and often results in weight regain over time [26-28]. For many individuals, daily food intake tracking to actively reduce calories may prove unfeasible, this identification of alternative strategies is essential. Promising dietary interventions that reduce participant burden but still result in health benefits, such as different forms of fasting, are discussed below.

Fasting

Fasting can be defined as an episode of "digestive quiescence" that follows a bout of feeding [29]. Typically, fasting bouts result in *reduced* caloric intake, but not *no* caloric intake [6]. Weight loss that results from reduced caloric intake is not the only positive outcome of fasting, as reported by studies in humans that report positive health outcomes despite variable effects on weight loss [30].

Fasting has been shown to display several metabolic health improvements such as: reduced adipocyte size, cell proliferation, reduced levels of insulin-like growth factor-1, decreases in fasting insulin, improvements in inflammatory markers [including c-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), adiponectin, leptin, and brain-derived neurotrophic factor (BDNF)] [6]. Fasting has shown to result in these improvements even when caloric intake was matched across studies [6]. Furthermore, Hatori et. al. (2013) show that, in two groups of calorically matched mice, the group whose fasting window was extended was protected from obesity, hyperinsulinemia, hepatic stenosis, and inflammation [5]. Further metabolic benefits that are not as widely known include: reductions in glucose levels, improvements in LDL and HDL cholesterol levels, reduced odds of elevated HbA1c, and lower CRP concentrations in women that ate less than 30% of their daily caloric intake after 17:00 [30]. Numerous health benefits resulting from fasting practices have been observed both in mice and in humans. Mice have exhibited decreases in adipocyte size, insulin-like growth factor-1, cell proliferation, body weight, total cholesterol, improvements in insulin sensitivity, and are protected from obesity, hyperinsulinemia, hepatic stenosis, and inflammation [7].

Calorie restriction and fasting also induce autophagy, a process that occurs in all cell types that facilitates removal of clusters of proteins that are misfolded or aggregated, and allows for

recycling of damaged cells, and is also preventative of necrosis (cell death in organs) [31]. As a result, autophagy likely plays a considerable role in preventing diseases such as cardiomyopathy, diabetes, cancer, liver disease, neurodegeneration, autoimmune diseases and certain infections [31]. According to Kroemer et. al. (2010), a variety of stress stimuli can bring about autophagy including nutrient and energy stress (restriction), endoplasmic reticulum (ER) stress, danger-associated and pathogen-associated molecular patterns, redox stress, hypoxia stress, and mitochondrial damage [32]. Thus fasting that does not result in caloric restriction may achieve improved health and lifespan through increasing an individual's resistance to stress, slowing aging and increasing longevity without negative side effects from other various interventions [33-35].

Intermittent Fasting

Intermittent fasting is a broad term that involves several types of fasting interventions. These interventions can include periodic 24-hour fasts, energy restriction, and time-restricted eating [36]. Findings in rodent studies and emerging human studies show that intermittent fasting (IF) may be an effective tactic for weight reduction, a delay in aging, and optimizing health [7]. Intermittent fasting is defined as "the practice of alternating periods of eating and fasting" [37] and it is emerging as a possible therapeutic strategy for improving certain cardiometabolic outcomes in rodents [37]. Intermittent fasting studies done in humans are showing numerous benefits such as: reduced body fat, reduced body weight, reduced glucose and/or reduced insulin levels, reduced blood pressure, improvements in insulin sensitivity, improvements in lipids and reduced markers of inflammation and oxidative stress [11, 23, 37-56].

Described here are numerous methods of putting intermittent fasting into practice. Alternate day fasting (ADF) involves limiting one's caloric intake ~25% of what one would normally consume (or consuming nothing at all) on alternating days [30]. Time-restricted eating

(TRE) allows ad libitum energy consumption within certain time frames throughout the day followed by an extended fasting period [30]. Common methods of TRE include the 16/8 method (fast for 16 hours, eat for 8) and the 14/10 method (fast for 14 hours, eat for 10). Religious fasting varies per religion, but is seen in: Hinduism, Islam, Christianity, Buddhism, Judaism [7], the Church of Jesus Christ of Latter-Day Saints and some Seventh-Day Adventists [30]. Ramadan intermittent fasting (RIF) is another practice of IF in which those who practice it fast from dawn to sunset, and this time frame can range anywhere from 10-18 hours depending on the where they reside. Typically, this method is put into practice by consuming one large meal after sunset and one lighter meal before dawn [30], but some religious exceptions may be made the closer they live to the equator (where daylight hours are extended and can result in extreme fasting conditions) [7]. Other forms of IF include: the 5:2 method (involving capping one's caloric intake at 500 calories on two days a week but maintaining a healthy and normal diet on other days), the eat-stop-eat method (involving fasting for a full twenty-four hours once or twice a week), the Warrior method (fasting completely or only eating small fruits and vegetables during the day, then eating a large meal at night), and spontaneous meal skipping (fasting when convenient). For the purposes of this thesis, we will focus on the 16/8 TRE method. Though there is plenty information regarding different methods of IF to the public, there is a shortage of evidence-based information on IF that can be transferred to the general population [30].

Time-restricted eating is one form of intermittent fasting that has demonstrated health benefits that are not a result of caloric restriction. Several studies have shown that insulin levels, insulin sensitivity, and blood pressure are improved following TRE when participants ate earlier in the day (eTRE) [37, 57-62] but actually worsened these outcomes when the participants ate later

in the day [63-65] and it is possible that the circadian rhythm may be the reason that these effects seem to be dependent on the time of day.

Circadian Rhythms

Circadian rhythms are oscillations that occur across (approximately) 24 hours, and are displayed in almost all living mammals [66]. These oscillations can occur at the molecular (involving an interplay of endogenous cell autonomous circadian oscillators), physiological (daily exposure to light and darkness), and behavioral (daily patterns of feeding and fasting) levels [66] and can tune the functioning of the digestive, metabolic, immune, reproductive, endocrine, and cardiovascular systems as well as several regions of the brain [66]. The circadian system produces these rhythms through "coordinated transcriptional;-translational feedback loops involving clock genes such as BMAL1, CLOCK, PER1/2, and CRY1/2, which in turn cause oscillations in a myriad of downstream targets" [36]. Further, the daily exposure to light and darkness result in diurnal rhythms [*diurnal* meaning "awake during the day" versus mice, who are *nocturnal* (awake at night)] that can be seen in other environmental factors such as temperature and humidity [67]. According to Manoogian et. al. (2017), synchrony between these oscillations in organs and tissues is what demonstrates these rhythms, and the oscillations can "separate incompatible biochemical or physiological processes, optimize energy expenditure (as tonic production of several proteins can be costly), and synchronize function of metabolic pathways to reduce the build-up of toxic intermediates" [66]. Every cell has a clock that oscillates roughly around 24 hours and the suprachiasmatic nucleus (SCN; located in the hypothalamus of the brain) functions as the master clock that determines the rhythms of the individual oscillators in the cells [66, 68, 69]. An analysis done on circadian gene expression in neurons in the SCN, peripheral cells, and cultured fibroblasts revealed that all known clock genes have the same temporal pattern of expression even in different

cell types [70, 71], though circadian gene expression in peripheral tissues is delayed by roughly four hours [70]. While the SCN and peripheral clocks are similar, they are not identical. The SCN is the pacemaker that is able to independently generate and maintain circadian oscillations, while peripheral clocks require external signals to either maintain or synchronize their rhythms [70]. These signals may either come from the SCN (internally), or externally from behavioral patterns such as feeding times [70, 72], neural control, hormone fluctuations, physical activity, and body temperature [72].

Diurnal regulation of metabolism is important because both the circadian clock and feeding-fasting patterns help regulate anabolic and catabolic fat, glucose, cholesterol and xenobiotic metabolism [66]. When circadian rhythms are disrupted, numerous health consequences may result. These outcomes include: increased risk for cancer, cardiovascular disease, obesity, immune disorders, infertility and affective disorders [73]. Furthermore, according to Antoniadis et. al. (2000) and Craig and McDonald (2008), circadian disruption may also result in cognitive deficits such as deficits in hippocampal learning and memory, but not in fear conditioning [74, 75] and even a shortened lifespan [76].

To assess the central circadian rhythm *in vivo*, studies often assess melatonin in blood or saliva. Melatonin is one of the most powerful synchronizers of the human circadian rhythm as it plays a role in daily sleep and wake patterns in humans [77]. Melatonin is a hormone that is secreted from the pineal gland, and is specifically controlled by the suprachiasmatic nucleus (SCN) in the brain. The secretion of melatonin occurs in response to external light cues when light hits the retinal photoreceptors in the eye [77, 78]. After the retina is exposed to light, light travels across the optic nerve via terminals in the retinohypothalamic tract which then release glutamate on to the SCN [79], influencing the output of melatonin from the SCN. The secretion of melatonin is

regulated by a multisynaptic pathway that originates in the SCN and ends at the pineal gland [80-82]. Direct photic input from retinal ganglion cells (RGCs) is how the period and phase of the circadian clock is calibrated [83-86]. In other words, the exposure to external light sources causes suppression of melatonin and the absence of external light sources results in an increase in melatonin. Its secretion is related to the duration of the time spent in darkness and will adjust to the light/dark cycle that one is exposed to on a daily basis [87, 88]. Measuring melatonin can serve as a diagnostic tool for detecting circadian rhythms and sleep disorders [87] because the SCN contains cells that are "inherently rhythmic" and expresses genetic rhythms that remain close to 24 hours [77]. Light exposure during the nighttime, whether unexpected or intentional, can cause suppression of melatonin and therefore phase shifts in circadian rhythms [77].

It is important to note that any amount of light does not immediately and completely suppress melatonin. According to Laakso et. al. (1993), humans require exposure to approximately 500 lux (a measurement of illuminance) for ~1 hour before nocturnal melatonin is suppressed [89], though the actual amount of light exposure required to suppress melatonin secretion is up for debate. Previous studies have shown that ~180 lux (approximate amount of normal room light) is sufficient enough to result in half of a phase shift that is seen when an individual is exposed to ~10,000 lux [90, 91] while other previous studies conclude that a lux level of ~2500 is required to suppress melatonin concentrations [92].

Furthermore, it is often mistaken that exposure to dark increases melatonin concentrations, when rather, the SCN controls the circadian rhythm of melatonin and levels begin to rise approximately 2 hours before habitual bedtime [93]. If constant routines and positions are maintained, melatonin levels will continue a pattern of rising and falling without the influence of light [93]. Melatonin is easily measured in saliva, blood, and urine, therefore, saliva samples

should be collected every thirty to sixty minutes while under dim light conditions [93] so as not to suppress melatonin. Melatonin levels above 10 pg/mL is what defines the beginning of melatonin onset, therefore, lux levels below 8 lux are ideal to avoid even the most acute suppression [93].

Circadian Rhythms and Time-restricted Eating

As previously mentioned, time-restricted eating allows *ad libitum* energy consumption within certain time frames throughout the day followed by an extended fasting period [11]. The bulk of scientific evidence regarding the health benefits of fasting in general come from studying rodents, though studies focusing on human interventions in regard to types of religious intermittent fasting are increasing [30]. In regard to TRE, studies in rodents have shown that TRE can restore circadian rhythms in mice that are considered "clock mutants," (i.e. lacking a circadian rhythm) [94]. Furthermore, in a rodent study done by Sherman et. al., 2012 [95] demonstrated that mutation of the clock proteins: CLOCK, BMAL1, PER1, PER2, CRY1, and CRY2 lead to metabolic disturbances, but that TRE restored the circadian phases of some clock proteins (and phases-advanced others). When compared to the rodent group that was allowed the same amount of food intake but was not limited to the timing of the intake, the TRE group showed improved insulin sensitivity, fat oxidation, decreased body weight and fat profiles, and decreased inflammation [95]. TRE studies conducted on humans have been largely observational [30] but studies measuring metabolic parameters are slowly surfacing. Thus, the benefits of TRE may be due, at least in part, to alignment of the feeding window with appropriate diurnal metabolic patterns.

A study done by Chaix et. al. (2014) in mice revealed that mice who were exposed to diverse nutritional challenges were protected from the negative metabolic effects that result from obesogenic diets, and benefits that were seen from TRE interventions attenuated metabolic diseases [96]. The diverse nutritional challenges involved 4 conditions: 1.) longer durations of

TRE interventions with access to high-fat diets across 9 hours, 12 hours, and 15 hours, 2.) 12 weeks of alternating between TRE and ad libitum feeding (ALF) involving TRE on 5 weekdays and ALF on weekends, 3.) 13 weeks of exposure to TRE and then switched to 12 weeks of ALF, and 4.) leaving a subset of mice in each condition which were switched to TRE feeding regimens after being allowed ad libitum feeding for 26 weeks to observe the therapeutic potential of TRE in reversing body weight gain in pre-existing diet-induced obesity [96]. The results revealed that TRE protected the mice from excessive body weight gain but without affecting caloric intakes independent of feeding times, diet, or the mice's initial body weight, improved glucose tolerance, reduced insulin resistance, improved nutrient homeostasis and restored cholesterol homeostasis [96]. This study also found that the benefits of TRE were proportional to the length of the fasting window that was implemented, the progression of metabolic diseases was stabilized and reversed, and high-fat diets were mitigated by TRE [96].

Several studies implementing TRE in humans are observing changes such as increased insulin sensitivity, increased β-cell function, decreased postprandial insulin, decrease blood pressure [37, 57-63, 65], decreased oxidative stress, decreased appetite [36, 37], improved nocturnal glucose homeostasis, decreased fasting glucose and insulin, increased cortisol in the morning but decreased in the evening, increased evening BDNF levels [36], improvements in body weight [37, 57, 59-63, 65, 97], decreased 24h levels of glucose [36], and increased morning LDL and HDL concentrations [36], without affecting energy expenditure [36, 62].

Twenty-four-hour rhythms exhibited in insulin sensitivity and the thermic effect of food, as well as plasma lipids, cortisol, insulin and growth hormone all peak in the morning [98-101]. A study on the timing of TRE feeding windows was conducted by Elizabeth Sutton and colleagues in 2018. In this study, men with prediabetes were randomized into two groups: early-Time-

restricted feeding (eTRF; 6-hour feeding window involving eating dinner before 15:00) and a control condition (12-hour feeding windows) for 5 weeks each separated by a 7-week washout period. The results from this study revealed that the eTRF schedule reduced insulin levels and improved insulin sensitivity and β-cell responsiveness, decreased blood pressure, decreased oxidative stress, and decreased appetite [37]. This suggests that food intake might be most optimal in the morning in order to align with one's circadian rhythm. Though scientific evidence in humans is increasing, human studies are lacking the rigorous control that is seen in rodent studies. A review done by Patterson et. al. (2017) found only four human studies that investigated the impact of TRE and prolonged nighttime fasts. Thus, there remains a gap in the knowledge as to whether circadian rhythms play a role in the metabolic benefits conferred by TRE, or if the timing of the food intake/duration of the fast is what provides the benefits.

Data from previous studies on TRE report weight reductions, but either didn't look at (or very minimally looked at) circulating metabolic factors [30]. Further, it is unknown as to whether changing the timing of food intake impacts diurnal profiles of circulating factors (i.e. glucose, insulin, free fatty acids, triglycerides, and glycerol), and only one study previously examined the impact of TRE on central circadian rhythms. The current study was therefore conducted to test the hypothesis that time-restricted eating will improve 24-hour glucose homeostasis and alter nighttime patterns of circulating factors and insulin sensitivity in healthy individuals without impacting the central circadian clock.

CHAPTER 2

2.1. Methods

Overview

Participants underwent two weeks of outpatient monitoring and stayed overnight in the Sleep and Metabolism Laboratory (SAM Lab) on two occasions (see Figure 1 for protocol schematic). During the first week, the participants followed what resembled a normal feeding schedule but with assigned meal times based on their habitual sleep and wake times. All meal times were controlled and prescribed, and are described below in further detail in section 2.5. Participants' interstitial glucose levels were measured with a minimally-invasive Continuous Glucose Monitor (CGM). Following one week of normal feeding habits, the participants spent one night in the lab involving overnight hourly blood draws, followed by two resting metabolic rate (RMR) assessments and an oral glucose tolerance test (OGTT) the next morning. At discharge in the morning, participants were instructed to restrict their feeding window to eight hours following the HSA protocol described below. In the TRE condition, participants were asked to keep the amount and type of food as similar as possible to the control condition to avoid confounding results that might arise from differing macronutrient content and/or caloric intake. The content of the meals was monitored through the use of photos sent to the lab (described below). Following one week of time-restricted eating habits, the participants again spent a night in the lab allowing for overnight hourly blood draws as well as two RMR assessments and OGTT the next morning (following the same protocol as the first overnight stay). Participants were given detailed informed consent before beginning the study, and the study was approved by the Colorado State University Institutional Review Board. Of the sixteen who were excluded from the study, nine withdrew, and

seven failed the health screening that followed the informed consent visit. Common reasons for withdrawal included failure to comply with the study protocol, scheduling conflicts, relocating, unresponsiveness, personal health concerns, or other personal matters unrelated to the study.

2.2. Experimental Design (study protocol)

Figure 1 is a representation of the protocol based on a participant who follows a 00:00-08:00 sleeping schedule. Each participant participated in the study for a total duration of roughly 2-2.5 weeks. The first week served as the fourteen-hour control condition and the second week served as the TRE condition.

2.3. Inclusion/exclusion criteria

The main inclusion criteria included: healthy men and women between the ages of 18-65. The exclusion criteria required that the participant not be participating in any other research studies. The research team confirmed that participants were not involved in other research studies that also required blood draws before they were enrolled in the current study. The participants were asked to maintain physical activity routines during participation. Participants could not have any active illness at the time of the study. Participants could not have a clinically significant, unstable medical condition within the last year (treated or untreated), including but not limited to: endocrine (diabetes, hyper/hypothyroid, adrenal), metabolic, respiratory (excluding asthma), cardiovascular (hypertension), kidney, neurological (migraines, seizures, stroke), connective tissue/joint, musculoskeletal (carpal tunnel), immune (rheumatoid arthritis, lupus, graves), chronobiological, hematopoietic, neoplastic (tumor, cancer), infectious disease (hepatitis), or a diagnosed sleep disorder. Participants could not have any clinically significant psychiatric condition including but not limited to: depression, ADHD, schizophrenia, anxiety, or personality disorder [participants

with mood disorders were excluded]. Participants were not allowed the use of prescription medication other than oral contraceptives.

Participants could not have any diagnosed stomach or intestinal diseases including but not limited to: ulcers, acid reflux, irritable bowel syndrome (IBS), or gastrointestinal disorders. Participants could not have asthma or need an inhaler at the time of the study. Participants could not consume >400mg of caffeine per day and could not consume caffeine past five hours after waking. Participants could not consume >14 alcoholic beverages a week, or > 4 alcoholic beverages in a single day over the past month for men; >7 alcoholic beverages a week, or >3 alcoholic beverages in a single day over the past month for women. It was required that participants' habitual sleep duration be between 7-9.25 hours per night. Participants were excluded if they had a history of parasomnia as an adult (abnormal or unusual behavior of the nervous system during sleep); history of insomnia [sleep latency >30 minutes, inability to maintain sleep within >60 minutes of wakefulness], including but not limited to: sleep apnea, periodic limb movements, and narcolepsy. Participants were not allowed previous travel outside the Mountain Time zone within the three months prior to participation in the study. Participants could not have a history of shift work within six months prior to participation in the study. Participants must have lived at the altitude of Denver or higher for a minimum of three months prior to participation in the study. Participants could not have given blood within thirty days prior to participation, or given birth/currently be breastfeeding within one year prior to participation. The participants were required to be free of illicit drugs for a minimum of one month prior to participation. The participants' Beck Depression Inventory (BDI) scores could not exceed >13, Beck Anxiety Inventory (BAI) scores could not exceed >10, and Sleep Disorders Questionnaire (SDQ) sleep apnea scores could not exceed >26 in men or >19 in women.

2.4. Visits to the lab

Each participant visited the lab on four occasions: Visit 1 (informed consent meeting), Visit 2: equipment pick up and DEXA scan, Visit 3: first overnight stay, and Visit 4: second overnight stay. During Visit 1, participants were given detailed descriptions of participation expectations, given a tour of the SAM Lab, and completed several health history questionnaires including: Sleep Disorders Questionnaire (SDQ; measures sleep disturbances and sleep habits during the previous month), Functional Outcomes of Sleep Questionnaire (FOSQ; measures one's functional status that results from sleepiness), Morningness Eveningness Questionnaire (MEQ; determines whether an individual's circadian rhythm produces peak alertness in the morning or evening), Three Factor Eating Questionnaire (TFEQ; measures human eating behaviors and patterns based on cognitive restraint, disinhibition, and hunger), Beck Anxiety Inventory (BAI; measures the severity of anxiety in an individual), Beck Depression Inventory (BDI; measures characteristics and attitudes that exhibit symptoms of depression), Epworth/Excessive Sleepiness Scale (ESS; assesses daytime sleepiness), and the Berlin questionnaire (OSA; identifies middle-aged and older individuals who are at high risk for Obstructive Sleep Apnea). Visit 2 occurred a few days after Visit 1, approximately one week before the participant checked in for the first overnight stay. At Visit 2, the researchers obtained a DEXA scan of each participant, and the participant received equipment such as an ActiWatch, ActivPAL, and Continuous Glucose Monitor (CGM). Each participant was also provided with sleep and wake logs, food logs, and two Visual Analog Scale (VAS) questionnaires (Appendix G) to complete thirty minutes pre-dinner and post-dinner on the day that they checked in for their first overnight stay. Visit 3 (the first overnight stay) consisted of verifying the participant's drug-free status, equipping the participant with an IV line to be used for blood draws, obtaining the blood draws, and overnight hourly blood sampling. The next morning

consisted of saliva samples, two Resting Metabolic Rate (RMR) assessments, an Oral Glucose Tolerance Test (OGTT), and several repeated VAS and Karolinska Sleepiness Scale (KSS) questionnaires (Appendix F). Four VAS questionnaires were given before the in-lab sleep opportunity. These questionnaires took place at hours since awake: 10:30, 11:45, 13:15, and 15:30 (week 1 – control condition) and 8:30, 9:30, 11:00, and 15:30 (week 2 – TRE condition). While the participant slept, the research team downloaded the data from the participant's ActiWatch and ActivPAL and reset the equipment to continue recording for the second week of the study. The following morning, VAS questionnaires were given at minutes 10, 50, and 245 post-wake. The participants were instructed to implement the 8h eating window following discharge from the first overnight stay. One week later, the participant checked in Visit 4 (the second overnight stay), and an identical protocol was followed for Visit 4 as was followed for Visit 3.

2.5. Hours since awake (HSA) prescribed meal times for each condition

All feeding times were controlled and were relative to each participant's habitual sleep and wake times. In the control condition, meals were spread across 13 hours, and specific meal times were prescribed at +1 hour since awake (HSA; breakfast), +6 HSA (lunch), +11 HSA (dinner), and +14 HSA (snack). After the participants' first overnight stay in the lab, the participants were then instructed to limit their food intake to an 8-hour feeding window during the second week of the study, again assigned based on relative habitual sleep and wake times. . In the TRE condition, specific meal times were prescribed at +1 HSA (breakfast), +5 HSA (lunch and snack), and +9 HSA (dinner). The participants were asked to match their caloric intake in week two to week one to prevent data that might be skewed from caloric differences. The research team instructed participants to stop caffeine consumption by 5 hours post-wake to avoid negative effects on circadian rhythms that are associated with consuming caffeine too close to habitual bedtime.

2.6. Dietary records and photos sent to lab

Participants were given food logs to log each meal, the time the meal was consumed, and the contents of the meal during each day during participation in the study. These records remained with the research team. The participants were asked to send photos of their meals to the SAM Lab staff by texting the lab's Google voice account. The purpose of this detail was to allow for the participant to reference their meals at certain times on certain days and ensure that comparable meals were consumed in each condition. The research team then used these texts/photos to track the timing of the participants' meals on a cumulative data sheet containing the timing of these texts, and then compared the time the texts were sent to the participant's assigned mealtime to determine compliance. Example pictures of meals are provided in Appendices H and I.

2.7. Sleep and wake logs/texting the lab

All participants were asked to follow a strict eight-hour sleep schedule throughout participation and were only allowed 30-minute windows before and after their assigned times to wake up and go to bed. Participants were allowed to select the sleep and wake times that fit best with their current habitual schedule, and then were asked to follow that sleep schedule for the entire study. Participants were instructed to log their sleep and wake times as well as an approximated amount of time that it took for the participant to fall asleep. Participants also logged the last time of caffeine consumption for the day. Participants were asked to text the SAM Lab's Google voice account as the last thing they did before going to sleep and the first thing they did when they woke up. The timing of these texts were then compared the assigned sleep and wake times to monitor compliance. The sleep and wake log is attached as Appendix J.

2.8. Exercise requirements during participation

Participants were asked to maintain similar exercise routines that they were following before beginning the study and not to dramatically change their routines. This was meant to control for acute changes from differences in exercise types or intensities. Furthermore, participants were asked to stop all exercise 24h before checking in to the lab for the overnight stays.

2.9. ActiWatch & ActivPAL

Participants were given ActiWatches and ActivPALs to wear starting at Visit 2 (equipment pick-up visit) and throughout the remainder of their participation in the study. The ActiWatch is as an accelerometer used to monitor participants' sleep and wake. The participants were instructed to wear the ActiWatch on their non-dominant wrist and to roll up long sleeves to allow for light exposure to the ActiWatch. Participants were asked to use the button on the side of the ActiWatch to mark an event in the data collection at the same time that they texted the lab their sleep and wake times. This information was then compared to their assigned sleep and wake times to monitor compliance.

Participants wore ActivPALs on the non-dominant leg approximately a third of the way down between the hip and the knee starting the day they picked up their equipment and throughout the remainder of their participation. ActivPALs recorded participants' body positions, and were used to assess physical activity throughout the study.

2.10. Continuous Glucose Monitors (CGMs)

Participants were equipped with CGMs at Visit 2, which remained in place for the remainder of the study. These were placed just above the iliac crest on the side of their body that they slept on the least.

2.11. Overnight blood sampling

Upon arrival at the lab, an IV was placed in the cephalic vein in the forearm. Blood draws began hourly at +12 hours since awake. Timing was set relative to habitual wake based on Actigraphy and call-ins that were determined during Week 1. Immediately prior to bed time, the IV was attached to a 10 ft extension tube, which was run through a specially-designed port in the wall to facilitate overnight blood draws that would not disturb the participant's sleep. Blood sampling continued hourly overnight with saline flushes of the IV line occurring every 30 minutes in between the blood draws. The final sample was taken immediately prior to waking the participant. Pictures of the through-the-wall blood sampling methods are provided in Appendix K.

2.12. Oral Glucose Tolerance Test (OGTT)

One hour following wake-up in the laboratory, a 3-hour OGTT was initiated. Basal blood draws were taken at T= -15 and -5. At T=0 participants ingested a solution of glucose (75g). Blood was sampled at T=30, 60, 90, 120, 150, and 180. Blood was centrifuged and plasma was separated and frozen for later analyses of glucose and insulin.

2.13. MATSUDA Insulin Sensitivity Index (MATSUDA-ISI)

The MATSUDA-ISI was established in 1999 as a whole-body method of quantifying physiological insulin sensitivity from data taken from OGTT tests [102]. It is a method of measuring insulin sensitivity in which the area under the curve (AUC) functions as a predictive measurement of individuals' insulin sensitivity. Both the MATSUDA-ISI and the homeostasis model assessment of insulin resistance (HOMA-IR) have the ability to detect individuals that lie in the lowest quartile of insulin sensitivity [103]. The glucose and insulin responses to the OGTT were observed to assess changes in insulin sensitivity in both the control condition and the TRE condition.

2.14. Saliva samples and dim-light melatonin offset (DLMOff)

Saliva was sampled immediately upon wake and every hour for 5 hours, totaling 6 samples, and saliva was assayed for melatonin. Salivary dim-light melatonin onset (DLMO) is defined as the time point when daytime levels of melatonin rise above 3pg/mL, and DLMOff is defined as the time point when levels of melatonin return to daytime levels, falling below 3 pg/mL. During the saliva sampling, participants remained in dim light (< 8 lux). By maintaining lux levels below 8, the research team assured that the decline in melatonin concentrations was a result of the participant's natural circadian rhythm independent from external light cues.

2.15. Indirect calorimetry

Resting Metabolic Rate (RMR) was assessed 30 minutes post-wake, and again during the OGTT (T=120, or 180-minutes post-wake). Each RMR assessment lasted approximately 15 minutes and participants were asked not to do anything but breathe, and try not to fall asleep during this time. RMR assessments occurred so that the research time could observe changes in fuel utilization. These data will be analyzed for future studies.

2.16. Statistical Analyses

Data are reported as means ± SEM. CGM data are further reported as coefficient of variation. The coefficient of variation was calculated using the following standard equation: $CV = (\sigma/\mu)*100$. A two-sided paired *t*-test analysis was utilized to test statistically significant differences between the control condition and TRE condition. Significance was set *a priori* at an α level of p=0.05. All analyses were conducted using SAS software version 9.4. Each segment was analyzed as area under the curve (AUC) calculated using the trapezoid rule.

The continuous glucose monitor data were divided into three glucose segments: mean daytime, nighttime, and 24h. All other outcomes were only collected during the nighttime segment.

The daytime segment was defined as the time period from when the participant woke to the beginning of the allowed sleep opportunity. The nighttime segments (excluding continuous glucose monitor data) are defined by the time just prior to and including the 8h sleep opportunity; and it was characterized in the lab from the time that the lights were turned off to when the lights were turned on (with exactly 8 hours in-between). Twenty-four-hour segments were defined by the 24 hours in between when a participant woke on one day to the time the participant woke on the following day.

CHAPTER 3

3.1. Results

Participants

In total, one-hundred and ninety-seven applicants were assessed for eligibility for this study. Thirty participants signed the consent form. Nine participants withdrew from the study and seven participants failed the health screening. At the time of the study, three participants were in progress and three were on hold. Eight participants successfully completed the study protocol. The participants identified their races as White (*n*=6, 75% of participants), American Indian/Alaskan Native (*n*=1, 12.5% of participants), and as "more than once race" (*n*=1, 12.5% of participants).

3.2. Participant Characteristics

Clinical participant characteristics are listed in Table 1. There were no changes in Body Mass Index (BMI) between the first overnight stay and the second overnight stay ($p = 0.17$). Here, the participants' age, sex, race, and BMI from the first and second overnight stay is listed. Sex and race are listed as *n* and percentages.

The participant recruitment flowchart is depicted in Figure 2. One hundred and ninety-seven participants were screened for eligibility. Eight completed the study and were included in the final analysis.

3.3. Interstitial Glucose

Figure 3 represents the average interstitial glucose across 24h during the control and TRE conditions. In the 8h TRE condition (indicated by the red line), participants had glucose concentrations that were reduced from the 13h control condition (indicated by the black line). During the TRE intervention, glucose levels began declining following the last meal of the day

(shown in Figure 3 at 17:00) and continued declining throughout the night until a fasting level of glucose was reached. Glucose levels remained at fasting level until breakfast was consumed the following morning.

3.4. Glucose Variability

Figure 4 shows the glucose variability during wakefulness and sleep as measured by the CGM. The coefficient of variance (CV%) indicated that there was not a significant difference between conditions during wakefulness (p=0.49) but there was a significant difference between conditions during sleep (p=0.03).

3.5. Summary of Interstitial Glucose

Figure 5 represents the area under the curve (AUC) values of 24h interstitial glucose (panel A), daytime interstitial glucose (panel B), and nighttime interstitial glucose (panel C). Twenty-four-hour segments were defined by the 24 hours in between when a participant woke on one day to the time the participant woke the following day. The daytime segment was defined as the time period from when the participant woke to the beginning of the sleep opportunity. The nighttime segment was defined in the lab by the time that the lights were turned off during the participants' overnight stays to when the lights were turned on (exactly 8 hours in-between). Each segment was analyzed as area under the curve (AUC) calculated using the trapezoid rule. No significant differences were found between conditions in any of the three comparisons.

3.6. Circulating Nighttime Factors

The average nighttime FFAs are shown in Figure 6. A significant difference was found between the control condition and the TRE condition (p=0.04), exhibiting that the TRE condition significantly elevated levels of nighttime FFAs compared to the control condition. This could indicate a change in fuel utilization overnight.

The average nighttime concentrations of glucose (top panel) and insulin (bottom panel) are shown in Figure 7. Average nighttime concentrations of glucose were not found to be significant (p=0.39). The average nighttime insulin measurements, however, were found to be significantly different following the TRE condition (p=0.005).

Figure 8 represents the average nighttime lactate concentrations. The findings here do not show that a significant difference was found in circulating nighttime lactate concentrations between the control condition and TRE condition (p=0.14).

The average nighttime triglyceride levels are represented in Figure 9. The findings show that the TRE condition resulted in significantly reduced nighttime triglyceride levels when compared to the control condition (p=0.006).

Average nighttime glycerol concentrations are shown in Figure 10. Our results show that a significant difference was found between the conditions with the TRE condition exhibiting significantly lower nighttime glycerol concentrations than the control condition (p=0.02).

3.7. Insulin Sensitivity

Figure 11 represents the glucose and insulin responses to the oral glucose challenge. This figure indicates that the TRE intervention did not affect glucose concentrations (top panel; p=0.34) or insulin concentrations (bottom panel; p=0.50) in response to the 180-minute oral glucose tolerance test. Following the OGTT, glucose and insulin levels increased in both conditions and then were gradually cleared at a rate that was shown to be insignificant. The MATSUDA insulin sensitivity index (MATSUDA-ISI), which is a representation of whole-body insulin sensitivity, was also not different between conditions (bottom panel; p=0.19).

3.8. Central Circadian Rhythm

Figure 12 depicts salivary melatonin concentrations starting immediately after waking the participant, and then occurring every hour for 5 hours. The blue triangles are indicative of the time points at which melatonin levs dropped and remained below 3pg/mL (DLMOff) in each condition. There was no significant difference DLMOff between conditions, indicating no change in circadian phase due to the TRE intervention.

CHAPTER 4

4.1. Discussion

The current study was conducted to examine the impact of time-restricted eating on circulating factors, insulin sensitivity, and circadian rhythms. The primary findings were that TRE significantly reduced interstitial glucose variability during the nighttime period, defined here as the habitual sleep opportunity TRE also significantly reduced average nighttime insulin, triglycerides, and glycerol concentrations, and significantly increased average nighttime free fatty acids. TRE did not impact insulin sensitivity or central circadian rhythms.

Increasing amounts of research indicate that intermittent fasting in general [104-107] as well as TRE specifically [37, 61, 65, 108] are both becoming popular topics in research and as possible tools to combat the obesity epidemic that continues to worsen. Fasting is associated with multiple cardiometabolic health benefits including reduced glucose levels, improvements in low-density lipoprotein cholesterol levels, improvements in high-density lipoprotein cholesterol levels, reductions in HbA1c levels, and lowered C-reactive proteins (CRP) concentrations [30], and these benefits are observed even when caloric intake is matched [6] and no weight loss occurs.

The average interstitial glucose, measured by CGM, showed that there was not a significant difference across 24h between the control condition and the TRE condition. It is possible that this was observed since participants were asked to match food intake between week 1 and week 2 as closely as possible. Several studies conducted on intermittent fasting have shown similar results in that glucose levels were not affected to the extent that insulin levels were affected [38, 43, 45, 46, 50, 53, 55, 56], though there are limited studies on interstitial glucose concentrations. It is possible that the individuals studied here did not show great changes in CGM glucose because a healthy population was studied.

Glucose variability was similar during scheduled wake in both the control condition and TRE condition but was significantly reduced during the sleep opportunity in the TRE condition (p=0.03). This is likely explained by the cessation of food intake earlier in the day in the TRE condition. For example, during the control condition, dinner was consumed much closer to habitual sleep onset compared to in the TRE condition. Food intake ceased much earlier in the day in the TRE condition, which would allow for glucose during sleep to be lower and less variable. High glucose variability (and lack of glucose predictability) are common problems seen in people with Type 1 and Type 2 diabetes (T2D). Since lowering blood glucose is the primary goal of treating diabetes [109, 110], it is possible that implementing a TRE lifestyle could represent a strategy to improve glucose levels—specifically at night. Since people with Type 1 diabetes are at high risk of hypoglycemia [109, 110]. Thus a TRE intervention in this population will require close monitoring, as small snacks might be required to avoid nocturnal hypoglycemia [111].

TRE in T2D is not out of reach. As reported by Sutton and colleagues, TRE was successful in improving certain aspects of cardiometabolic health in clinical populations [37]. Sutton et. al. (2018) found that TRE was efficacious in treating prediabetes in men (a condition which often leads to diabetes), as well as hypertension [37]. The results from the current study in combination with Sutton et. al. (2018)'s findings suggest TRE earlier in the day might prove to be beneficial in the treatment of T2D. However, more research is needed in the field of implementing TRE into the lives of diabetic populations.

In the current study, we found that the TRE condition significantly reduced nighttime insulin levels compared to the control condition. Knowing that calories were matched to the best of the participant's abilities, this indicates that the metabolic benefits observed are not a result of reduced caloric intake, but rather, of the implementation of the TRE protocol. Sutton et. al. (2018)

examined ten weeks of early time-restricted eating (eTRE) that required participants follow an eTRE protocol for five weeks (eating dinner before 15:00) as well as a control condition (eating all meals within twelve hours) for five weeks [37]. These conditions were separated by a washout period of seven weeks, and the researchers found that insulin levels were drastically lowered following the TRE condition, but glucose levels were not improved [37]. This is similar to what was found in the current study. Future studies might assess glucose and insulin changes while also experimenting with the quality of the food intake.

It is possible that aligning eating windows with circadian rhythms is responsible for the results that were seen here after implementing the TRE condition. Energy metabolism and fuel utilization are regulated by circadian system [98, 112]. Further, insulin sensitivity, ß-cell responsiveness and the thermic effect of food are higher in the morning [98, 112-114]. It is therefore possible that the results observed in the nighttime plasma insulin and nighttime plasma glucose levels were results of implementing the TRE condition in alignment with each participants' habitual circadian rhythms (rather than a blanket 12pm – 8pm feeding window – a common method of implementing TRE but a method which does not take lifestyle factors into consideration).

In the current study, nighttime plasma FFA concentrations were significantly elevated in the TRE condition when compared to the control condition. Previous studies in mice have found that fatty acids are reduced following TRE without reducing the caloric intake [5], though overnight concentrations of free fatty acids have not been evaluated. The elevated nighttime FFA concentrations in the TRE condition likely indicate that participants were utilizing higher levels of fat as a fuel source during the nighttime compared to the control condition. This is likely due to the fact that feeding was stopped earlier in the day, allowing for glucose and insulin levels to return

to fasting levels, and therefore resulting in increased amounts of FFAs being oxidized throughout the night. This is not a surprising find when considering that participants stopped eating 4 hours before the initial blood draw in the TRE condition, but consumed dinner only one hour prior to the initial blood draw as well as a snack 2 hours after the initial blood draw in the control condition. This may also explain the significantly lower triglyceride levels and glycerol levels in the TRE condition as it is known that circulating glycerol and triglycerides in plasma are largely driven by food intake [115].

In this context, elevated FFAs may be considered a metabolically healthy outcome. Here, we see elevated nighttime FFA in conjunction with reduced glucose variability and nighttime insulin concentrations following the TRE intervention. This suggests that lipid oxidation was increased during TRE, and nighttime fuel utilization favored higher fat oxidation during the TRE condition. Moreover, Chaix et. al. (2018) found in mice that TRE enhances lipolysis and β-oxidation, therefore resulting in a reduction in the FFA pool in the liver, and reduced inflammation in mice [116]. Hatori et. al. (2012) also found that TRE increased the expression of genes associated with lipid metabolism such as cytoplasmic carnitine acyltransferase (Crat), malic enzyme (Me1; reducing production of NADPH for fat synthesis), and monoacylglycerol O-acyltransferase (Mogat1) and also found that lipid oxidation was elevated in the nocturnal fasting period during TRE [5].

The findings in the average nighttime glucose and insulin (Figure 7) was not surprising, as previous studies have also reported that insulin is more greatly affected by intermittent fasting practices than is glucose [38, 43, 45, 46, 50, 53, 55, 56]. Further, there were no differences in concentrations of average nighttime lactate levels between conditions, which is consistent with

comparable glucose levels between conditions. The extent to which insulin was affected is significant enough that it could indicate health improvements in individuals with T2D.

Nighttime concentrations of triglycerides were similar at the time of the first blood draw; however, concentrations were found to be significantly lower in the TRE condition than in the control condition (Figure 9; p=0.006). It is possible that this was observed due to the fact that the control condition had a feeding window that ran later into the evening that did the TRE condition. Changes in triglycerides levels are influenced by meal times [115], and it is expected that the TRE condition would result in reduced triglycerides because the amount of time that passed since the last meal in this condition was much longer. It is assumed that the participants' last meals in the control condition were responsible for the elevated triglyceride levels into the evening, and would also explain the lower levels of nighttime triglycerides seen in the TRE condition. The most likely explanation for this significant finding is the fact that the TRE condition required cessation of meal intake much earlier than did the control condition.

Average nighttime glycerol concentrations were found to be statistically significantly different between the two conditions (Figure 10; p=0.02). It is likely that significantly reduced concentrations of glycerol following the TRE condition were observed in conjunction with the changes in concentrations of triglycerides as it is known that triglycerides and glycerol concentrations are influenced by meal times [117]. This would also indicate that glycerol levels are more strongly influenced by meal times and are less influenced by circadian rhythms.

The oral glucose challenge showed that the plasma glucose and insulin were not affected by TRE using this method to assess insulin sensitivity. Both glucose and insulin follow a pattern in that they increased following the ingestion of the 75g glucose drink, and then exhibited a similar

clearance rate across the subsequent 3 hours. It is likely that these results did not yield a difference following the TRE intervention due to the fact that a healthy population was studied.

Salivary melatonin concentrations were not different between conditions. Melatonin levels increase approximately two hours before habitual bedtime, peak in the nighttime, and decrease in the morning [93]. It is not surprising that in both conditions melatonin exhibited elevated concentrations upon waking. It is to be expected, as melatonin levels are controlled by the suprachiasmatic nucleus in response to light exposure. However, the study protocol required that the lux levels in the room remain below 8 lux, therefore, it is known that there were not influences on melatonin levels from external light cues and the declines in melatonin seen in the morning were a result of the participants' natural circadian rhythms. Some studies have found that lux levels below 30 are sufficient enough to suppress melatonin synthesis [118]; however, the lower the lux levels, the less it is likely that even acute suppression will occur. Therefore, light exposure was not the driving influence on melatonin suppression and an accurate measurement of participants' circadian rhythms was observed. Again, this indicates that the TRE condition did not influence the central circadian rhythm, and this could be explained by the fact that healthy participants were studied, and also by the fact that the study protocol required that participants follow a rigorous sleep schedule and sampling was relative to habitual sleep and wake.

CHAPTER 5

5.1. Strengths and Limitations

There were several strengths to the current study. One strength includes the tightly controlled and tightly followed protocol as was laid out by the researchers, and is represented on the flowsheets (Appendices A and B). This protocol was strictly followed with each participant with each overnight stay.

Another strength is that the timing of each participant's feeding window was aligned to their habitual behavior. Aligning each participant's feeding window with their habitual wake and sleep times avoided confounding effects from having to alter personal schedules to adjust to generalized feeding times (e.g. a blanket 12-8pm feeding window). Doing this also avoided dramatic changes in lifestyle habits, such as sleep times and wake times, which might've also altered participants' circadian rhythms.

Participants were instructed to mark an "event" on their ActiWatches at the same time that they texted the lab when they were going to sleep and waking up. The timing of the "events" that appeared on the ActiWatch data was compared to the timing that the texts were sent, which were then compared to the assigned sleep schedules to assure that participants were being compliant with the study protocol. Additionally, the Continuous Glucose Monitors allowed the research team to observe the time that the last meal of the day was consumed as a way to further examine compliance.

The inclusion and exclusion criteria were incredibly specific and detailed. This allowed for the research team to see changes that were solely due to the TRE feeding condition rather than confounding effects from possible pre-existing medical conditions. The results were found in a

healthy population, thus future research is needed to determine whether unhealthy populations might see similar or even greater health improvements.

The participants were instructed to send texts to the research team through the SAM lab's Google text account. The timing of the texts were compared to their assigned sleep and wake times, their assigned meal times, and the "events" marked by the ActiWatches. These text messages were also forwarded to the lab manager's personal cell phone in case of emergencies outside of times that we were available in the lab.

The flowsheets (Appendix A and B below) as well as sample logs for each sampled variable (Appendices C-E) instructed the research team as to exactly what time each sample should be taken, and the lab personnel responsible for the sample initialed for the sample so that every sample was accounted for. Adverse events were also recorded on a separate event log (Appendix L).

The lux levels in the room were maintained below 8 lux for the duration of the participants' stay in the lab. This allowed for the research team to see the natural decline in morning melatonin (DLMOff) without the influence of external light cues therefore ensuring that the participants' circadian rhythms were not influenced by light and the results seen in the current study were true findings from the TRE implementation.

Lastly, an important difference between this study and previous TRE studies worth mentioning is that the feeding window implemented in this study was 8 hours, while in other studies, smaller feeding windows of 6 [37] and 4 [119] hours have been tested. Thus, the observations of health improvements using a longer feeding window make translation into everyday life of the general population even more feasible.

A number of limitations were also present in this study, including the small number of participants that successfully completed the study (n=8). More participants would likely result in

smaller margins of error in the plasma concentrations of each variable, as well as a higher statistical power to detect significant differences between conditions. Future studies should include a larger number of participants.

Another limitation was the duration of the study. The study consisted of one week of control feeding conditions and one week of TRE feeding conditions. Some of the differences seen (or not seen) might be a result of the short duration of this study. Future studies should consider implementing the same protocol for a longer duration of time.

5.2. Future Directions

Now that we know that TRE can greatly alter certain factors in healthy individuals, future studies are needed in clinical populations, as it is possible that they will see similar or even greater health improvements than was seen here in a healthy population. Because Type I and Type II diabetics have problems regulating glucose overnight, and the glucose variability data suggest that we can improve nighttime glucose homeostasis, future studies should attempt to implement TRE into these populations.

Subjective hunger and appetite were recorded using the VAS questionnaires (Appendix G) but were not analyzed for this study. These questionnaires will be analyzed as it is important to understand the feasibility of implementing TRE feeding schedules. If hunger is unbearable, the lifestyle cannot be maintained.

If a TRE intervention is not feasible in some populations, future studies could also assess the impact of restricted the feeding window of specific macronutrients to determine whether TRE of only specific nutrients could confer metabolic benefits.

Finally, future studies might consider using controlled timing of food intake when participants are circadian misaligned. This would involve keeping the food intake to the biological

day in populations such as shift workers, those with altered sleep schedules and those who travel often.

CHAPTER 6

6.1. Conclusion

Overall, we found significant differences in a variety of circulating nighttime factors (interstitial glucose variability during sleep, nighttime plasma insulin, nighttime FFA, nighttime triglycerides, and nighttime glycerol), but saw no effect on nighttime glucose, insulin sensitivity or central circadian rhythms. Since caloric intake was matched between conditions, we attribute these findings to a shorter eating window, suggesting that TRE may be a potential method to improve nighttime glucose variability and circulating nighttime insulin levels in clinical populations such as people with diabetics. Further, improved stability of glucose during the night could also represent a mechanism by which TRE improves glucose homeostasis in people at risk for diabetes.

Table 1. Clinical Participant Characteristics.

Variable		Mean ± SD
Age, y		27 ± 3.8
Sex		
	Female, n (%)	6 (75)
	Male, n (%)	2 (25)
Race		
	African American, n (%)	0 (0)
	Asian, n (%)	0 (0)
	White, n (%)	6 (75)
	Hispanic, n (%)	0 (0)
	American Indian/Alaskan Native, n (%)	1 (12.5)
	More than one race, n (%)	1 (12.5)
BMI, kg/m^2		
	1^{st} OVN Stay (control)	22.6 ± 2.1
	2^{nd} OVN Stay (TRE)	22.4 ± 2.1

Table 2. Fasting Values in the Control Condition and TRE Condition.

Variable	1st OVN Stay (control)	2nd OVN Stay (TRE)
	Mean ± SEM	Mean ± SEM
Glucose, mg/dL	79.7 ± 3.55	78.6 ± 2.72
Insulin, mU/L	2.24 ± 0.54	1.86 ± 0.44
Free fatty acids, mmol	0.37 ± 0.06	0.36 ± 0.05
Lactate, mg/dL	8.4 ± 0.81	7.8 ± 0.55
Triglycerides, mmol	0.42 ± 0.04	0.44 ± 0.05
Glycerol, mmol	0.07 ± 0.01	0.06 ± 0.01

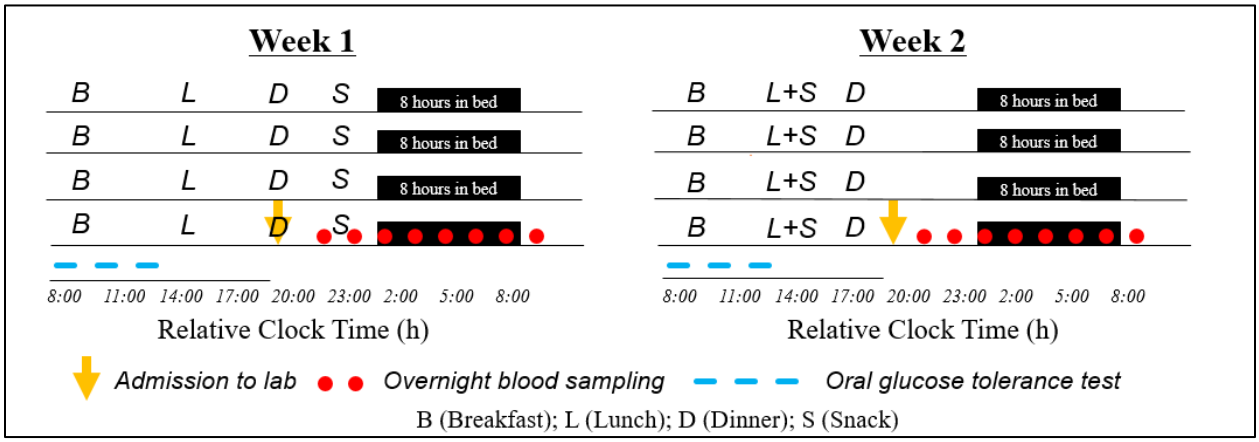


Figure 1. Study Protocol. Each participant served as their own control as they underwent a two-week crossover time-restricted eating (TRE) intervention. Illustrated above is an example of a participant that follows a 00:00-8:00 sleep schedule and the corresponding scheduled meal times associated with an 8am wake time. During the first week of the study, participants followed a 13-hour control condition in which meals were consumed at the following hours post-wake: breakfast +1 HSA, lunch +6 HSA, dinner +11 HSA, and snack +14 HSA. During the second week of the study, participants implemented the 8-hour TRE condition, eating at the following times post-wake: breakfast +1 HSA, lunch & snack +5 HSA, and dinner +9 HSA. Upon admission into the lab, all participants participated in overnight blood draws (indicated by the red dots) and an oral glucose tolerance test the following morning (indicated by the blue dashed lines). The yellow arrows indicate the time that the participant arrived at the lab.

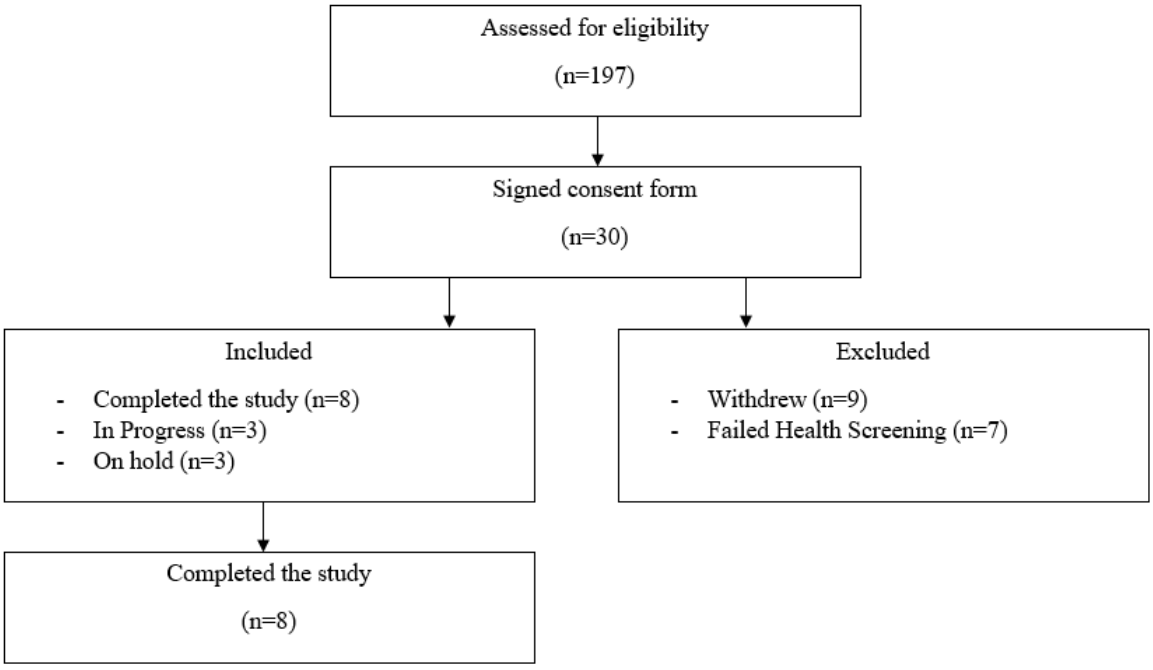


Figure 2. Participant Recruitment Flowchart. One-hundred and ninety-seven potential participants applied and were assessed for eligibility for participation in the study. Of that one-hundred and ninety-seven, thirty applicants signed the consent form and then completed health history questionnaires (based on a range of scores) which resulted in the exclusion of sixteen participants. The questionnaires utilized in the health screening are listed and explained above in the methods: SDQ, FOSQ, MEQ, TFEQ, BAI, BDI, ESS and the Berlin questionnaire. Following the health screening, eight participants qualified, successfully completed the study, and were compensated for their participation.

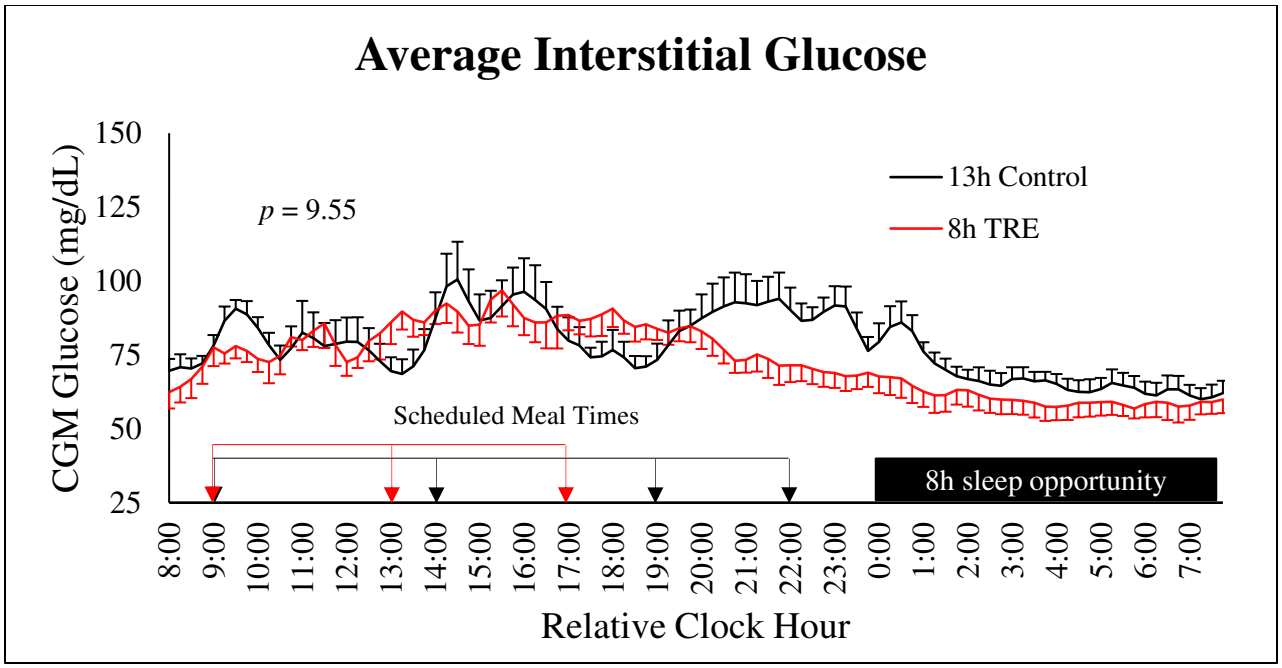


Figure 3. Average Interstitial Glucose During TRE and Control Conditions Across 24 hours. The black line indicates interstitial glucose across 24 hours during the control condition while the red line indicates interstitial glucose across 24 hours during the TRE condition. Significant differences were not found between the two conditions across 24 hours (p = 9.55). The red line representing the TRE condition suggests that interstitial glucose was steadier in this condition. This is complementary of the glucose variability results (Figure 4) in that interstitial glucose was steadier throughout the sleep opportunity. This is not surprising as the TRE condition required that participants stop meal consumption much earlier allowing for glucose concentrations to steady.

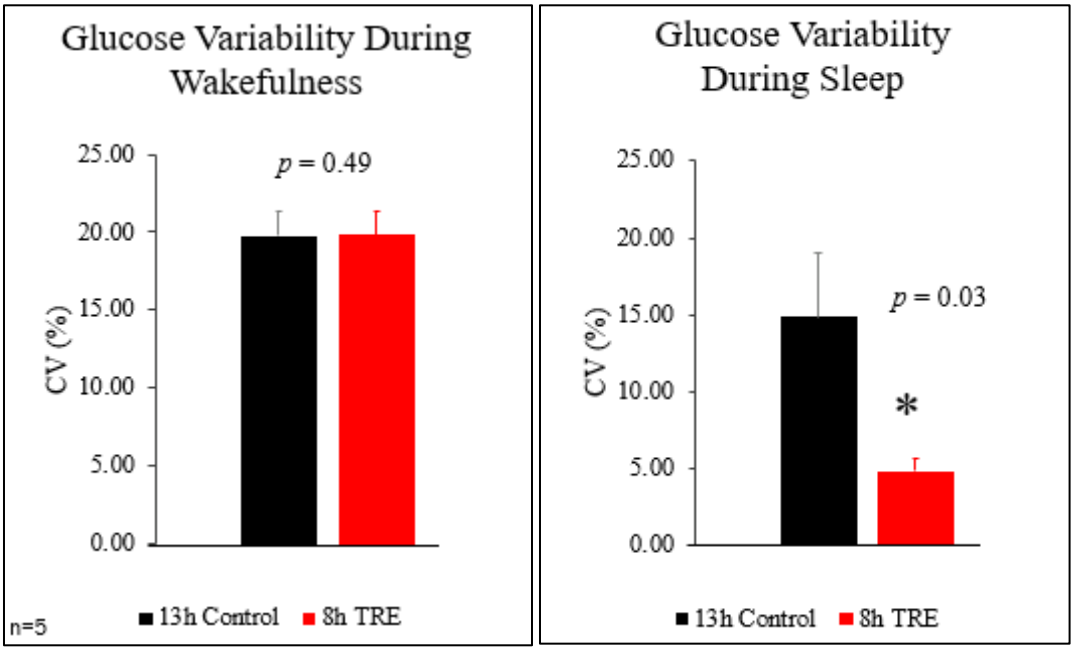


Figure 4. Glucose Variability. Shown above is glucose variability as recorded from the Continuous Glucose Monitors. The left panel indicates glucose variability during scheduled wake and the right panel indicates glucose variability during the sleep opportunity with the black bars representing the 13h control condition and the red bars representing the 8h TRE condition. The coefficient of variance (CV%) was not significant during wakefulness (p=0.49) but was found to be significantly different during sleep (p=0.03). Glucose variability is a common problem in diabetic populations. Since lowering glucose variability is a focus of treating Type II diabetes, implementing a TRE lifestyle might be a step towards treating the condition.

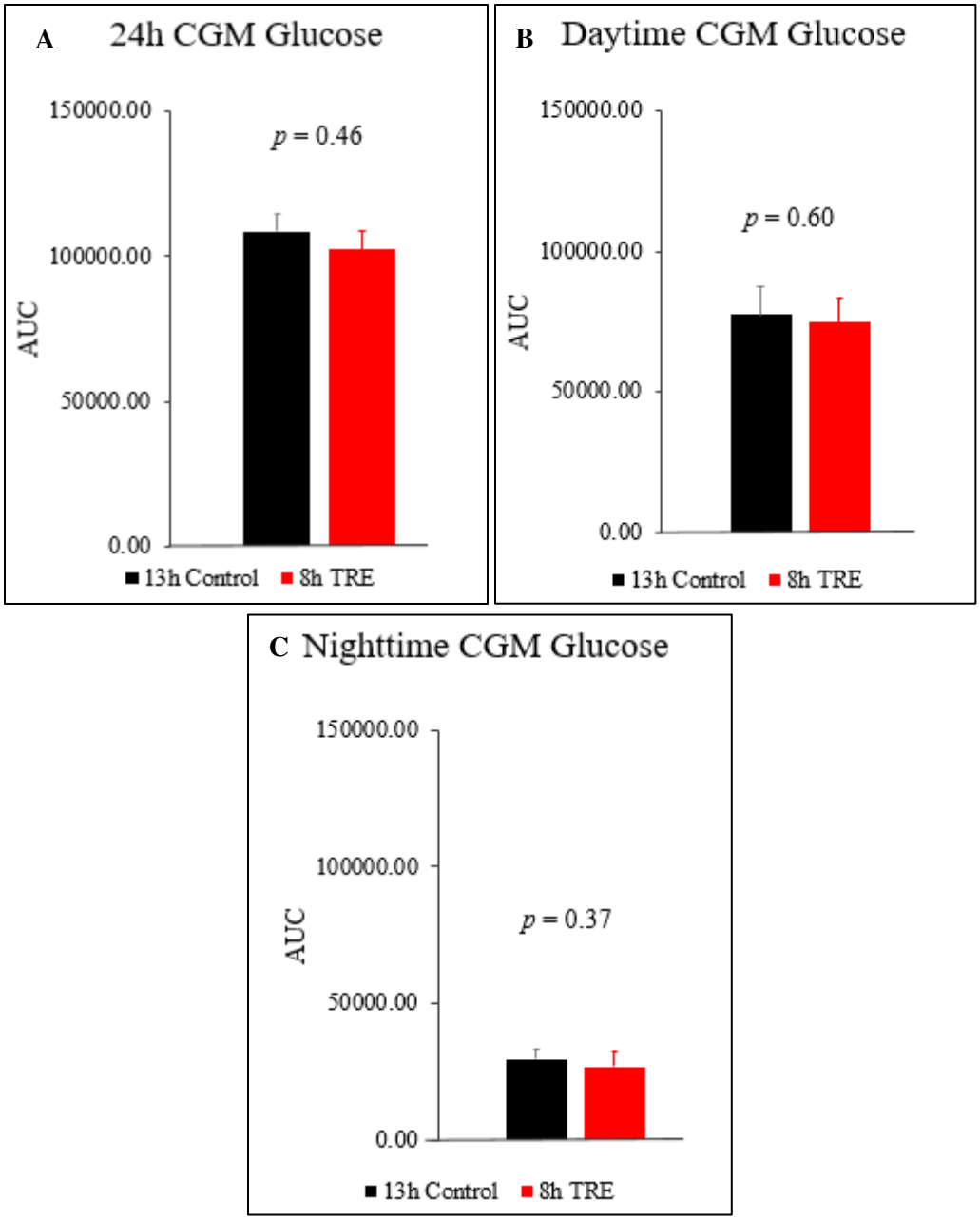


Figure 5. Summary of Interstitial Glucose. Shown here is the interstitial glucose across twenty-four-hours (A), during the daytime (B), and during the nighttime (C) as represented by total area under the curve (AUC). Twenty-four-hour segments were defined by the 24 hours in between when a participant woke on one day to the time the participant woke the following day. Each segment was analyzed as area under the curve (AUC) calculated using the trapezoid rule. The daytime segment was defined as the time period from when the participant woke to the beginning of the sleep opportunity. "Nighttime" was defined in the lab by the time that the lights were turned off during the overnight stays to when the lights were turned on (8 hours). No significant differences were found between conditions in any of the three comparisons (p=0.46, p=0.60, and p=0.37 respectively).

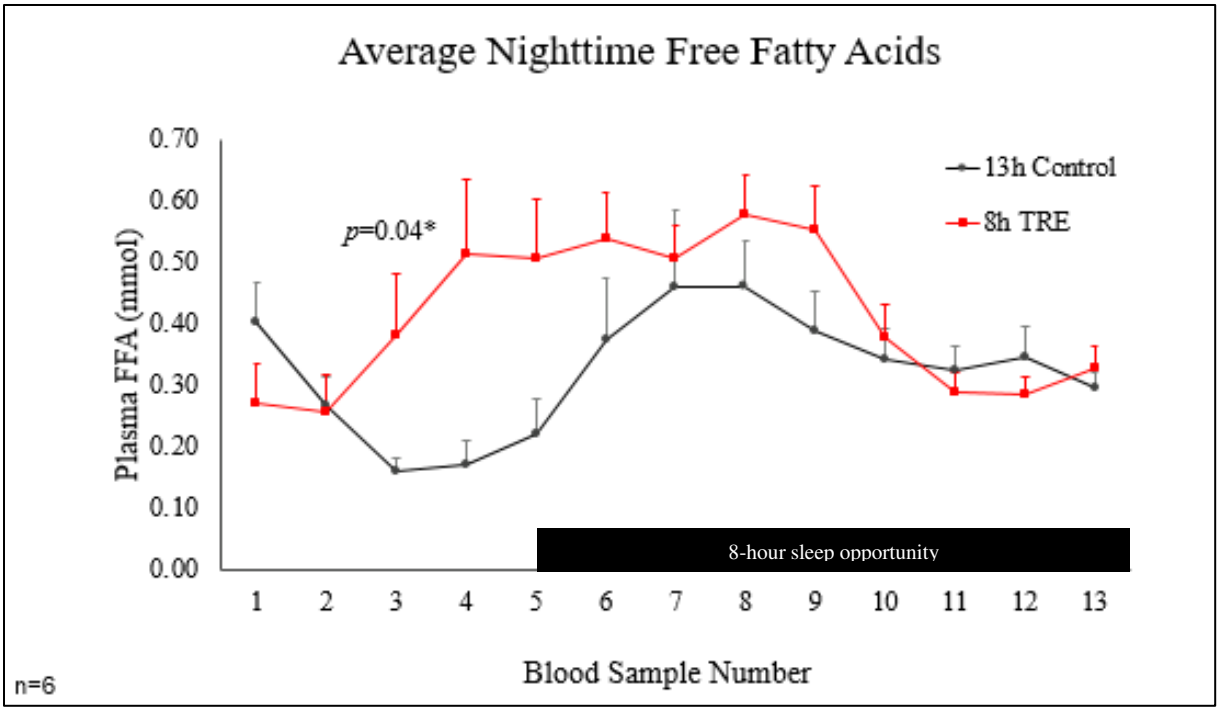
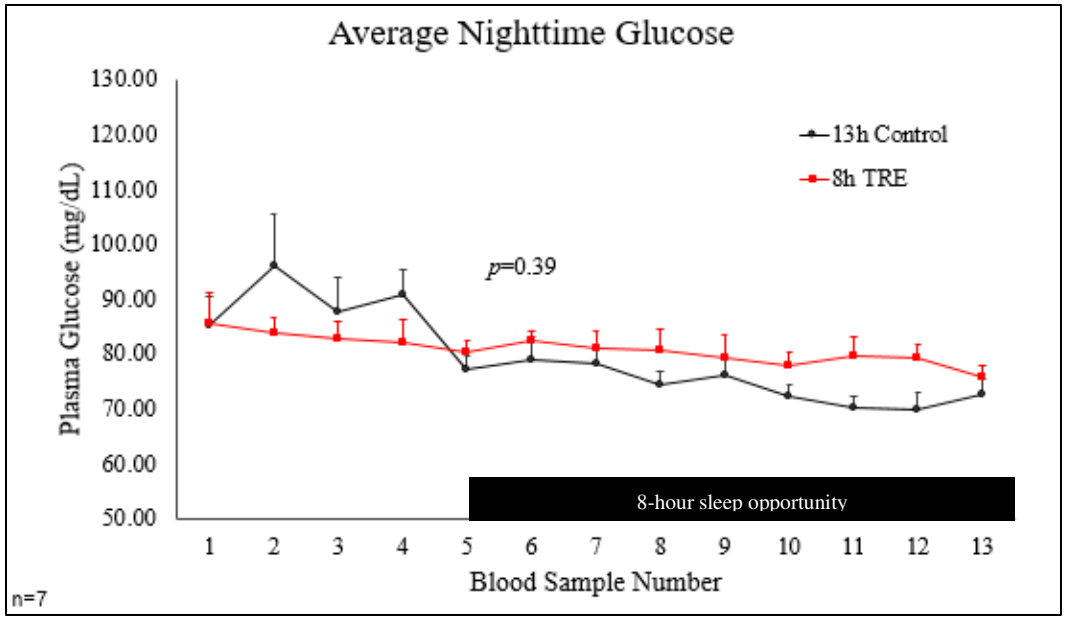


Figure 6. Average Nighttime Free Fatty Acids. Above is the average nighttime free fatty acid concentrations. The black line indicates the 13h control condition and the red line indicates the 8h TRE condition The black box indicates the sleep opportunity. "Nighttime" is defined as the average values from all blood draws (samples 1-13). The sleep opportunity began immediately following sample #5 and concluded immediately after sample #13. Nighttime FFA levels were significantly elevated in the 8h TRE condition when compared to the 13h control condition (p = 0.04). Increased nighttime FFAs are indicative of a switch in fuel utilization and nighttime lipid oxidation. Elevated FFAs are a positive outcome in this context as increased lipid oxidation reduces liver inflammation [5], and could also produce weight loss if TRE habits are continued, though the primary outcome of this study was not the use of TRE as a weight loss method.



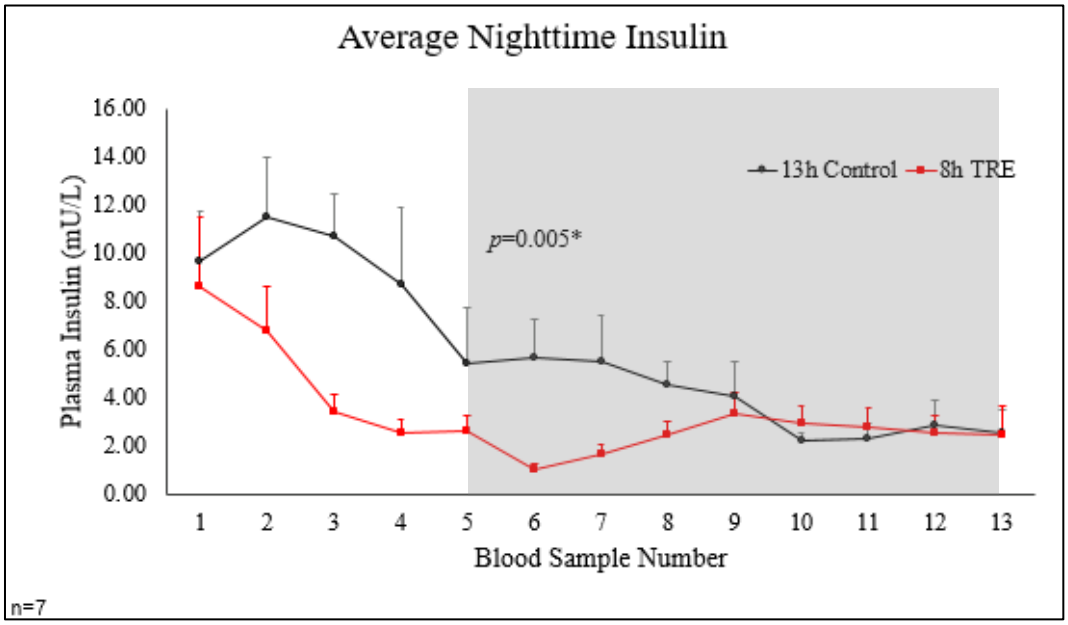


Figure 7. Average Nighttime Glucose (A) and Insulin (B). Nighttime glucose and insulin concentrations were assayed from the overnight blood draws. The black line indicates the control condition and the red line indicates the 8h TRE condition. The black box (top panel) and gray box (bottom panel) indicate the sleep opportunity. "Nighttime" is defined as the average values from all blood draws (samples 1-13). The sleep opportunity began immediately following sample #5 and concluded immediately after sample #13. There was no significant difference between the control and TRE conditions (p=0.39) in the nighttime glucose concentrations but there was a significant difference (p=0.005) in nighttime insulin concentrations. Previous studies on TRE have also found that glucose levels are not as greatly affected as insulin levels are. The decreased nighttime insulin is a promising find for clinical populations such as Type II diabetics.

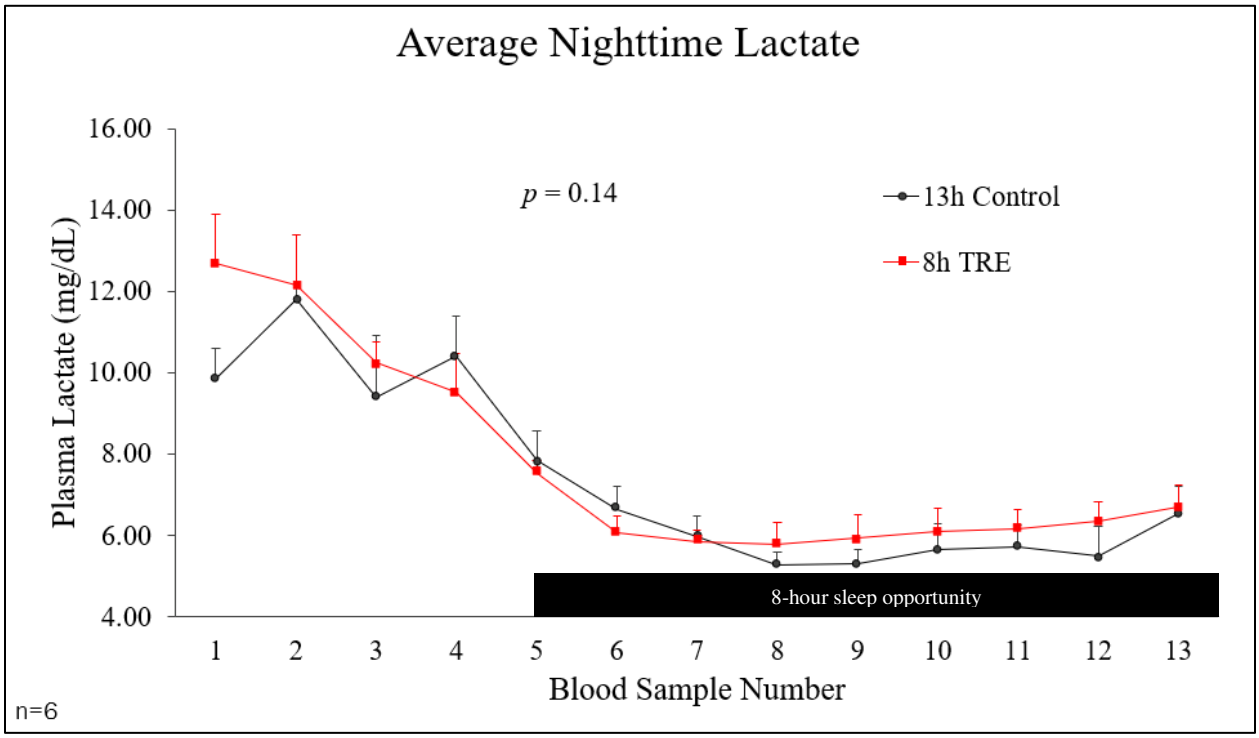


Figure 8. Average Nighttime Lactate. Above is the average nighttime lactate concentrations. The black line indicates the control condition and the red line indicates the TRE condition. The black box indicates the sleep opportunity. "Nighttime" is defined as the average values from all blood draws (samples 1-13). The sleep opportunity began immediately following sample #5 and concluded immediately after sample #13. There was not a significant difference found between the control and TRE conditions in circulating nighttime lactate concentrations (p=0.14).

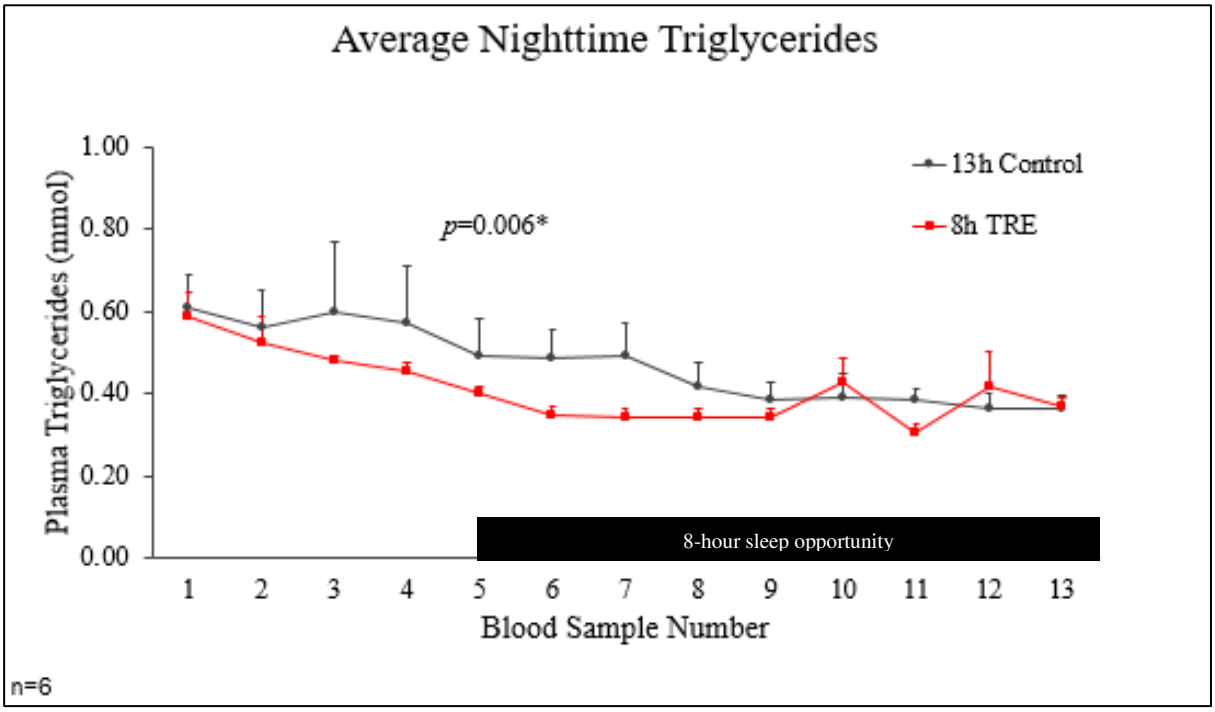


Figure 9. Average Nighttime Triglycerides. Above is the average nighttime triglyceride concentrations in which the black line indicates the control condition and the red line indicates the TRE condition. The black box indicates the sleep opportunity. "Nighttime" is defined as the average values from all blood draws (samples 1-13). The sleep opportunity began immediately following sample #5 and concluded immediately after sample #13. The TRE condition exhibited significantly lower levels of triglycerides than did the fourteen-hour control condition (p=0.006). With triglyceride levels being influenced by feeding habits, it is not surprising to find lower triglyceride levels in the TRE condition as the TRE condition required that participants stop eating much earlier in the day. These findings are to be expected.

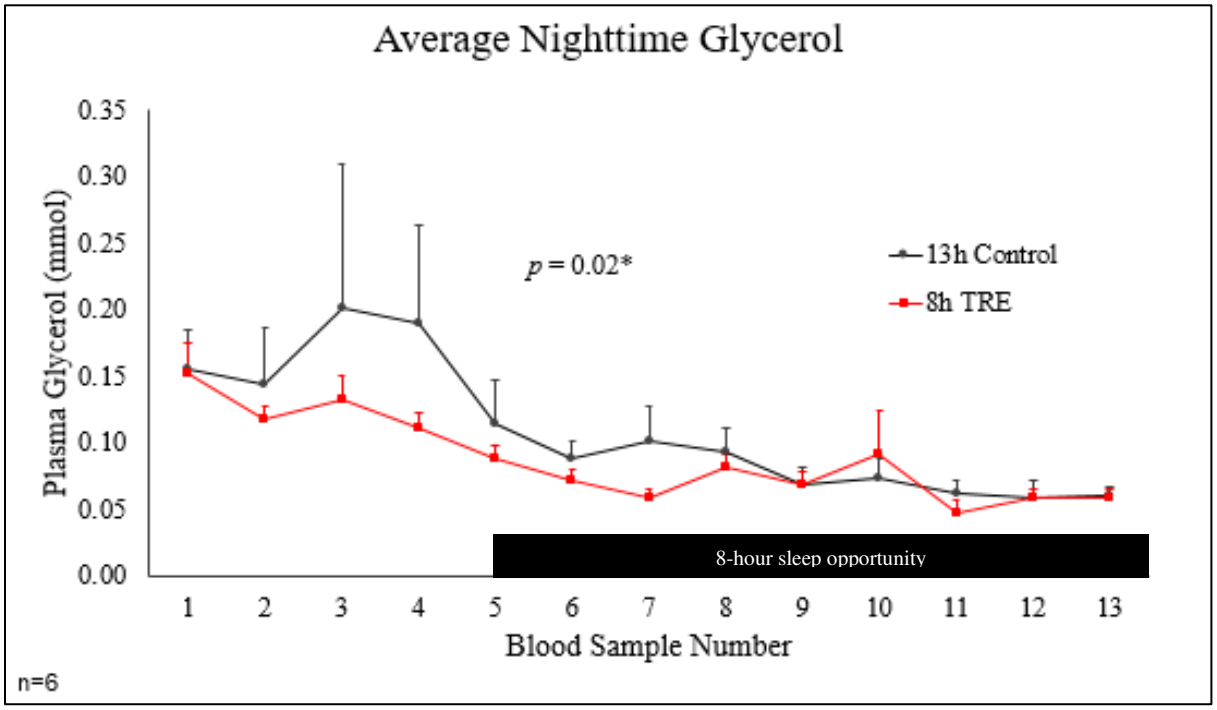


Figure 10. Average Nighttime Glycerol. Above is the average nighttime glycerol concentrations in which the black line indicates the 13h control condition and the red line indicates the 8h TRE condition. The black box indicates the sleep opportunity. "Nighttime" is defined as the average values from all blood draws (samples 1-13). The sleep opportunity began immediately following sample #5 and concluded immediately after sample #13. A significant difference was found between the two conditions in which the TRE condition exhibited significantly lower nighttime glycerol than did the control condition (p=0.02). Glycerol levels are influenced by feeding times and glycerol levels were significantly lower in the TRE condition than the control condition. The TRE condition required that participants stop meal consumption much earlier, therefore, these findings are to be expected.

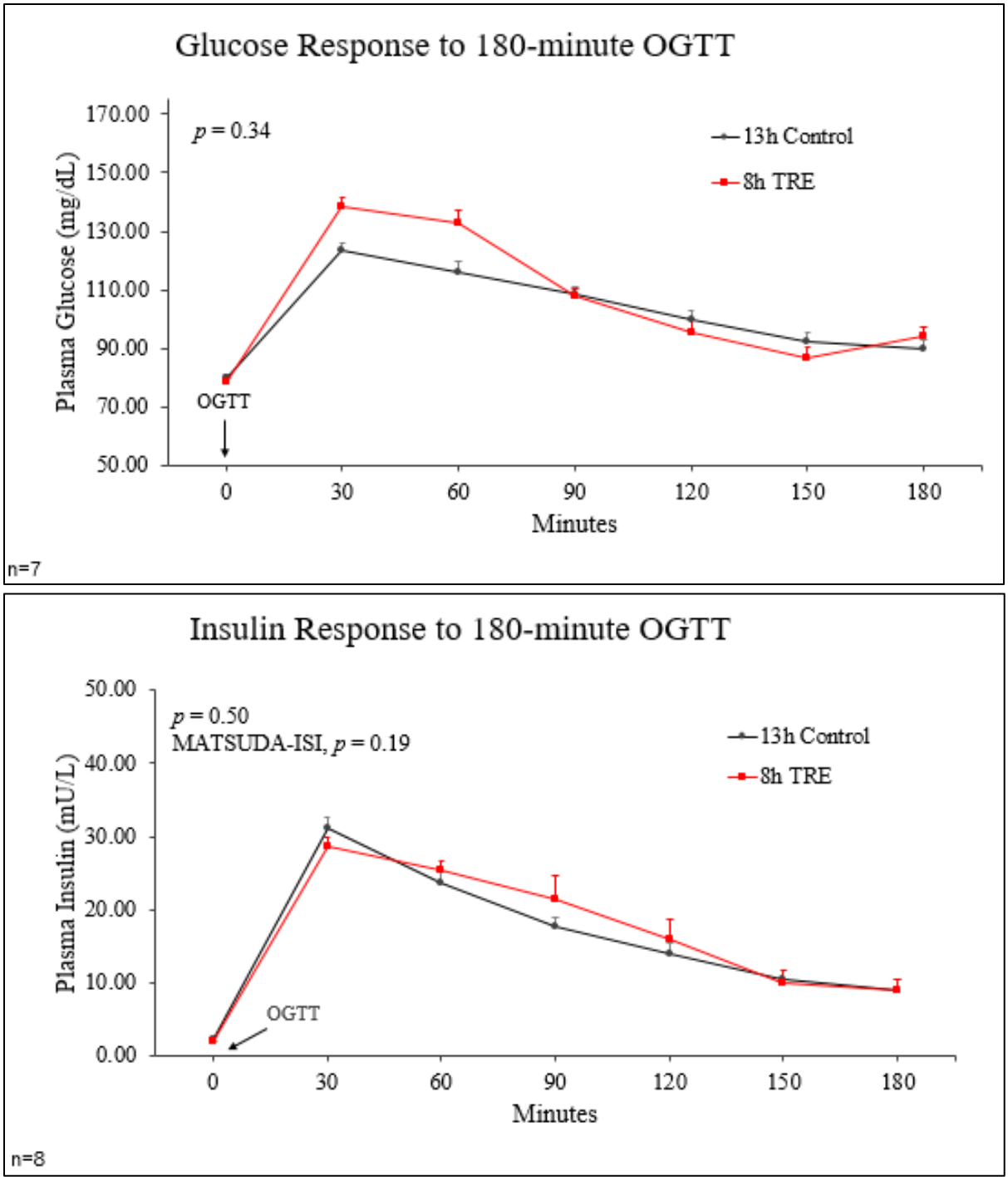


Figure 11. Glucose and Insulin Responses to 180-minute OGTT. The TRE condition did not impact the glucose (A) response or insulin (B) response during a 180-minute OGTT the morning after each overnight stay in the lab. The OGTT began sixty minutes post-wake and both conditions exhibited similar trends in glucose and insulin responses.

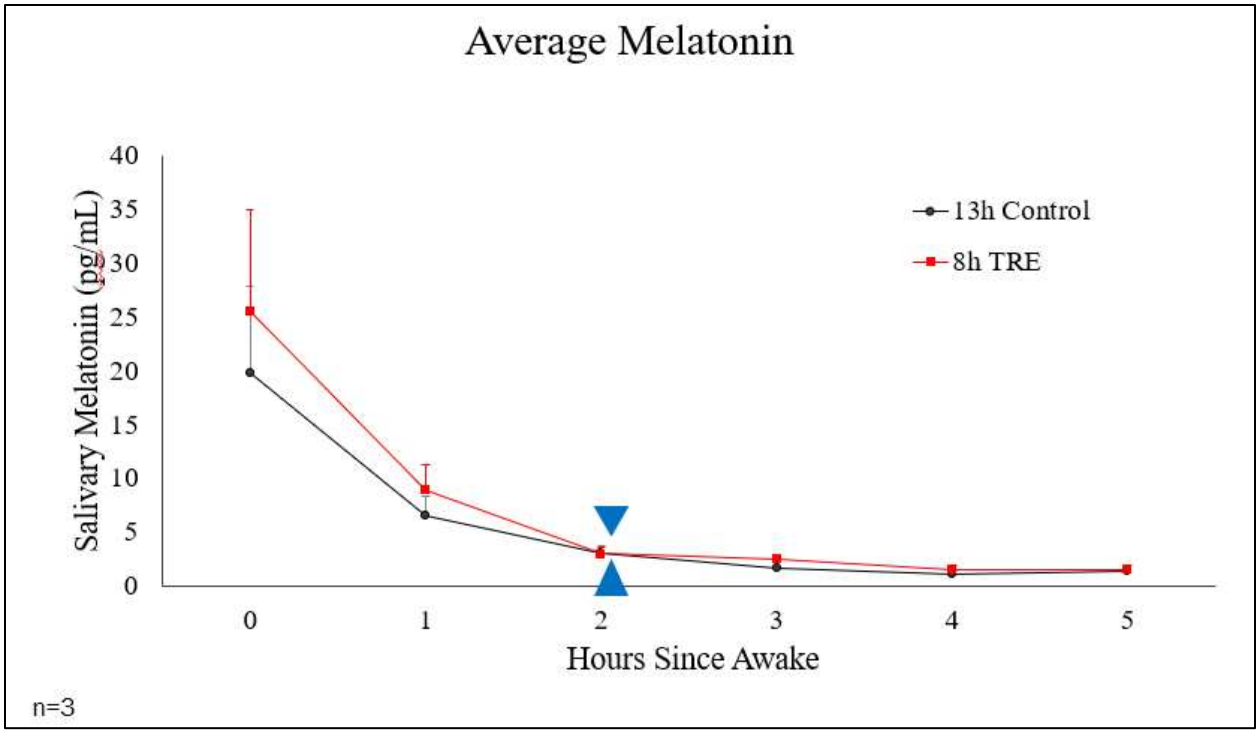


Figure 12. Average Melatonin Concentrations. Saliva collection for melatonin assessments occurred in the morning following each overnight stays. Salivary melatonin was measured in the saliva starting immediately upon waking the participant with samples occurring every hour immediately upon waking the participant and every hour until five hours were reached. The blue triangles are indicative of the Dim Light Melatonin Offset (DLMOff) at which salivary melatonin levels dropped and remained below 3pg/mL. There were no differences found between conditions in the time point at which salivary melatonin concentrations dropped. If TRE did affect circadian rhythms, we'd expect to see the red line shifted to the left to align with the earlier feeding schedule. These findings indicate that the results seen in the circulating factors were not a result of circadian misalignment, and were true results of the TRE intervention.

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Appendix A: Flowsheet – First Overnight Stay; Control Condition

Time (h.s.a.)	Clock Time	Actual Time		Initials
9:00	14:25		Complete in charge checklist	
9:00	14:25		Verify subject's consent is signed and the consent form's approval date is still valid (<1 year old).	
9:00	14:25		Turn on Axis Companion for video observation- Make sure Infrared lights work	
9:00	14:25		Call CSU Police Non-emergency line to inform them that a sleep research study is occurring in the HPCRL Building, Room B150, until [end date]. 970-491-6425	
9:00	14:25		PRA time-syncs timer and computers to time.gov	
9:00	14:25		Check room temperature using Parvo tool. Verify between 72-74 F (22-23 C). Record temp: _____	
9:00	14:25		Document lux levels in the following places: In study room with lights & TV on: Bed:_____; Antechamber: _____; Bathroom: _____	
9:00	14:25		Prepare drug/pregnancy test	
10:30	15:55		Text subject to remind them to complete VAS #1 questionnaire & bring dinner & snack	
10:45	16:10		Prep Ice Bucket for samples	
11:00	16:25		Subject Arrives, Obtain weight _____ kg	
11:00	16:25		Text Dr. Fahrner and Josie that subject has arrived	
11:00	16:25		Confirm subject brought their dinner&snack; place in fridge if needed & check if any prep is needed (microwave, etc.)	
11:00	16:25		Get urine sample from subject to do drug/preg test	
11:00	16:25		Collect completed food/sleep log, return sleep log to subject for week 2	

11:00	16:25		Collect/Download ActiWatch/ActivPAL, Download CGM; Re-initialize for Week 2 and return to subject	
11:05	16:30		PRA collects admission vitals: BP: _____ /_____; HR: _____; Temp: _____.	
11:05	16:30		Subject eats dinner	
11:15	16:40		Orient subject to sleep lab (their room, TV volume limit, bathroom protocol)	

11:15	16:40		ASAP after dinner, Start IV for Overnight Sampling (begin at distal forearm and move proximally if needed)	
11:45	17:10		Have Subject complete VAS questionnaire #2	
12:00	17:25		Blood Draw #ON 1	
12:30	17:55		Flush line	
12:30	17:55		Go over next morning procedures	
13:00	18:25		Blood Draw #ON 2	
13:15	18:40		Have Subject complete VAS questionnaire #3 (2 hrs post dinner)	
13:30	18:55		Flush line	
13:30	18:55		Explain through the wall sampling to subject; discuss how they would use the restroom at night	
14:00	19:25		Blood Draw #ON 3	
14:00	19:25		Have subject eat snack they brought from home now	
14:30	19:55		Flush line	
14:30	19:55		Check the phone/computer/tablet lux for the devices the subject wants to use in the morning	
15:00	20:25		Blood Draw #ON 4	

15:15	20:40		Prep IV through the wall, will connect before bedtime	
15:30	20:55		Flush line	
15:30	20:55		Have Subject complete VAS questionnaire #4	
15:35	21:00		Ask subject if they need to use the restroom before they go to bed/IV is connected through wall. Subject can brush their teeth.	
15:40	21:05		Connect IV through the wall; discuss with subject how to try to keep arm unbent, but shouldn't be uncomfortable.	
15:50	21:15		Lower head of bed; make sure subject's phone is off and out of room.	
15:50	21:15		Remove TABLE LAMP from room	
15:52	21:17		Turn on Sound Machine	
15:55	21:20		Blood Draw #ON 5	
16:00	21:25		LIGHTS OUT	
16:05	21:30		Text Dr. Fahrner and Josie that subject has gone to bed	
16:30	21:55		Flush line	
17:00	22:25		Blood Draw #ON 6	
17:30	22:55		Flush line	
	DATE			
Time (h.s.a.)	Clock Time	Actual Time		Initials

18:00	23:25		Blood Draw #ON 7	
18:30	23:55		Flush line	
19:00	0:25		Blood Draw #ON 8	
19:30	0:55		Flush line	
20:00	1:25		Blood Draw #ON 9	
20:30	1:55		Flush line	

21:00	2:25		Blood Draw #ON 10	
21:30	2:55		Flush line	
22:00	3:25		Blood Draw #ON 11	
22:30	3:55		Flush line	
23:00	4:25		Blood Draw #ON 12	
23:30	4:55		Flush line	
23:30	4:55		Turn on Parvo	
23:55	5:20		Blood Draw #ON 13	
0:00	5:25		WAKE UP-MUST WEAR GOGGLES BEFORE TURNING LIGHT ON	
0:00	5:25		Open door and verbally address subject to awaken subject. Turn on under the bed light on bed remote, do not allow subject to sit up or get out of bed.	
0:01	5:26		Immediately get Saliva T=0: Subject must stay laying down during sampling but can roll on side to spit. Place saliva tube on ice.	
0:01	5:26		Disconnect IV from through-the-wall (this can be done any time before first OGTT draw)	
0:05	5:30		KSS +5 _____	
0:05	5:30		PRA collects vitals: BP: _____ /_____; HR: _____; Temp: _____.	
0:05	5:30		Subject can quickly use the bathroom. Subject cannot brush teeth with toothpaste due to OGTT (toothpaste contains sugar/artificial sweeteners)	
0:05	5:30		Raise bed to approximately 30 degrees (slightly below 45 is OK since it's hard to set it exactly)	
0:05	5:30		Calibrate Flowmeter on Parvo (good for 2 hrs)	
0:10	5:35		Calibrate Gas on Parvo (good for 30 min)	

0:10	5:35		Have Subject complete VAS questionnaire #5	
0:15	5:40		KSS +15 _____	
0:15	5:40		Flush line	
0:15	5:40		Check that IV still works and start new IV if necessary	
0:30	5:55		KSS +30 _____	
0:30	5:55		Start 15 min RMR #1	
0:40	6:05		Subject needs to be in constant position for 15 min before next saliva	

0:45	6:10		Blood Draw T=-15 (can be done during RMR)	
0:50	6:15		Get glucose drink from fridge in purple room	
0:50	6:15		Have Subject complete VAS questionnaire #6	
0:55	6:20		KSS +55 _____	
0:55	6:20		Start collection Saliva T=1	
0:55	6:20		Blood Draw T=-5	
1:00	6:25		Subject drinks glucose drink, should try to drink all of it within 2-3 min (T=0). MAKE NOTE AND ADJUST TIMES ON OGTT SHEET IF DELAYED	
1:05	6:30		Text Dr. Fahrner and Josie that OGTT is underway	
1:25	6:50		PRA has subject rinse mouth for saliva sample, no eating or drinking until completion of OGTT (small sips of water OK)	
1:30	6:55		KSS +90 _____	
1:30	6:55		Blood Draw T=30	
1:45	7:10		Remind subject to stay in constant position until next saliva	
1:55	7:20		Give subject PSQ to complete	
2:00	7:25		KSS +120 _____	

2:00	7:25		Start collection Saliva T=2	
2:00	7:25		Blood Draw T=60	
2:25	7:50		Make sure Parvo is turned on	
2:30	7:55		Blood Draw T=90	
2:40	8:05		Calibrate Flowmeter & Gas on Parvo	
2:45	8:10		Remind subject to stay in constant position until next saliva	
3:00	8:25		KSS +3 hr _____	
3:00	8:25		Start collection Saliva T=3	
3:00	8:25		Blood Draw T=120	
3:05	8:30		Perform 15 min RMR #2	
3:30	8:55		Blood Draw T=150 (can be done during RMR)	
3:45	9:10		Remind subject to stay in constant position until next saliva	
3:50	9:15		UGRA prep snack for subject (microwave)	
4:00	9:25		KSS +4 hr _____	
4:00	9:25		Start collection Saliva T=4	
4:00	9:25		Blood Draw T=180	
4:01	9:26		Remove IV from subject's arm.	
4:05	9:30		Have Subject complete VAS questionnaire #7	
4:05	9:30		Provide snack to subject (eat within 20 min)	
4:25	9:50		PRA has subject rinse mouth for saliva sample, instruct subject not to eat or drink anything until finished with saliva sampling.	

4:30	9:55		Give subject timing instruction sheet for Week 2, Make sure they have all equipment & logs for week 2, create text reminders on Gmail	
4:30	9:55		Have subject complete payment forms	

4:45	10:10		Subject must remain in constant posture until next saliva sample	
5:00	10:25		KSS +5 hr _____	
5:00	10:25		Start collection Saliva T=5	
5:10	10:35		Subject may eat lunch if they would like, otherwise can send subject home	
5:10	10:35		Text Dr. Fahrner and Josie that subject has left lab	
			Follow Discharge SOP	

Appendix B: Flowsheet – Second Overnight Stay; TRE Condition

Time (h.s.a.)	Clock Time	Actual Time		Initials
8:30	13:45		Remind subject to complete VAS questionnaire #1	
9:30	14:45		Remind subject to complete VAS questionnaire #2	
9:00	14:15		Complete in charge checklist	
9:00	14:15		Verify subject's consent is signed and the consent form's approval date is still valid (<1 year old).	
9:00	14:15		Turn on Axis Companion for video observation-Make sure Infrared lights work	
9:00	14:15		Call CSU Police Non-emergency line to inform them that a sleep research study is occurring in the HPCRL Building, Room B150, until [end date]. 970-491-6425	
9:00	14:15		PRA time-syncs timer and computers to time.gov	
9:00	14:15		Check room temperature using Parvo tool. Verify between 72-74 F (22-23 C). Record temp: _____	
9:00	14:15		Document lux levels in the following places: In study room with lights & TV on: Bed:_____; Antechamber: _____; Bathroom: _____	
9:00	14:15		Prepare drug/pregnancy test	
10:45	16:00		Prep Ice Bucket for samples	
11:00	16:15		Subject Arrives, Obtain weight _____ kg	
11:00	16:15		Text Dr. Fahrner and Josie that subject has arrived	
11:00	16:15		Get urine sample from subject to do drug/preg test	
11:00	16:15		Have subject complete VAS questionnaire #3	
11:00	16:15		Collect completed food/sleep log	
11:00	16:15		Collect/Download ActiWatch/ActivPAL, Download CGM; Re-initialize for the night and return to subject	

11:05	16:20		PRA collects admission vitals: BP: _____ /_____; HR: _____; Temp: _____.	
11:15	16:30		Orient subject to sleep lab (their room, TV volume limit, bathroom protocol)	
11:15	16:30		Start IV for Overnight Sampling (begin at distal forearm and move proximally if needed)	
12:00	17:15		Blood Draw #ON 1	
12:30	17:45		Flush line	
12:30	17:45		Go over next morning procedures	
13:00	18:15		Blood Draw #ON 2	
13:30	18:45		Flush line	
13:30	18:45		Explain through the wall sampling to subject; discuss how they would use the restroom at night	

14:00	19:15		Blood Draw #ON 3	
14:30	19:45		Flush line	
14:30	19:45		Check the phone/computer/tablet lux for the devices the subject wants to use in the morning	
15:00	20:15		Blood Draw #ON 4	
15:15	20:30		Prep IV through the wall, will connect before bedtime	
15:30	20:45		Flush line	
15:30	20:45		Have Subject complete VAS questionnaire #4	
15:35	20:50		Ask subject if they need to use the restroom before they go to bed/IV is connected through wall. Subject can brush their teeth.	
15:40	20:55		Connect IV through the wall; discuss with subject how to try to keep arm unbent, but shouldn't be uncomfortable.	
15:50	21:05		Lower head of bed; make sure subject's phone is off and out of room.	
15:50	21:05		Remove TABLE LAMP from room	

15:52	21:07		Turn on Sound Machine	
15:55	21:10		Blood Draw #ON 5	
16:00	21:15		LIGHTS OUT	
16:05	21:20		Text Dr. Fahrner and Josie that subject has gone to bed	
16:30	21:45		Flush line	
17:00	22:15		Blood Draw #ON 6	
17:30	22:45		Flush line	
	DATE			
Time (h.s.a.)	Clock Time	Actual Time		Initials
18:00	23:15		Blood Draw #ON 7	
18:30	23:45		Flush line	
19:00	0:15		Blood Draw #ON 8	
19:30	0:45		Flush line	
20:00	1:15		Blood Draw #ON 9	
20:30	1:45		Flush line	
21:00	2:15		Blood Draw #ON 10	
21:30	2:45		Flush line	
22:00	3:15		Blood Draw #ON 11	
22:30	3:45		Flush line	
23:00	4:15		Blood Draw #ON 12	
23:30	4:45		Flush line	
23:30	4:45		Turn on Parvo	
23:55	5:10		Blood Draw #ON 13	
0:00	5:15		WAKE UP-MUST WEAR GOGGLES BEFORE TURNING LIGHT ON	

0:00	5:15		Open door and verbally address subject to awaken subject. Turn on under the bed light on bed remote, do not allow subject to sit up or get out of bed.	
0:01	5:16		Immediately get Saliva T=0: Subject must stay laying down during sampling but can roll on side to spit. Place saliva tube on ice.	
0:01	5:16		Disconnect IV from through-the-wall (this can be done any time before first OGTT draw)	
0:05	5:20		KSS +5 _____	
0:05	5:20		PRA collects vitals: BP: _____ /_____; HR: _____; Temp: _____.	
0:05	5:20		Subject can quickly use the bathroom. Subject cannot brush teeth with toothpaste due to OGTT (toothpaste contains sugar/artificial sweeteners)	
0:05	5:20		Raise bed to approximately 30 degrees (slightly below 45 is OK since it's hard to set it exactly)	
0:05	5:20		Calibrate Flowmeter on Parvo (good for 2 hrs)	
0:10	5:25		Calibrate Gas on Parvo (good for 30 min)	
0:10	5:25		Have Subject complete VAS questionnaire #5	
0:15	5:30		KSS +15 _____	
0:15	5:30		Flush line	
0:15	5:30		Check that IV still works and start new IV if necessary	
0:30	5:45		KSS +30 _____	
0:30	5:45		Start 15 min RMR #1	
0:40	5:55		Subject needs to be in constant position for 15 min before next saliva	
0:45	6:00		Blood Draw T=-15 (can be done during RMR)	
0:50	6:05		Get glucose drink from fridge in purple room	
0:50	6:05		Have Subject complete VAS questionnaire #6	
0:55	6:10		KSS +55 _____	

0:55	6:10		Start collection Saliva T=1	
0:55	6:10		Blood Draw T=-5	
1:00	6:15		Subject drinks glucose drink, should try to drink all of it within 2-3 min (T=0). MAKE NOTE AND ADJUST TIMES ON OGTT SHEET IF DELAYED	
1:05	6:20		Text Dr. Fahrner and Josie that OGTT is underway	
1:25	6:40		PRA has subject rinse mouth for saliva sample, no eating or drinking until completion of OGTT (small sips of water OK)	
1:30	6:45		KSS +90 _____	
1:30	6:45		Blood Draw T=30	
1:45	7:00		Remind subject to stay in constant position until next saliva	
1:55	7:10		Give subject PSQ to complete	

2:00	7:15		KSS +120 _____	
2:00	7:15		Start collection Saliva T=2	
2:00	7:15		Blood Draw T=60	
2:25	7:40		Make sure Parvo is turned on	
2:30	7:45		Blood Draw T=90	
2:40	7:55		Calibrate Flowmeter & Gas on Parvo	
2:45	8:00		Remind subject to stay in constant position until next saliva	
3:00	8:15		KSS +3 hr _____	
3:00	8:15		Start collection Saliva T=3	
3:00	8:15		Blood Draw T=120	
3:05	8:20		Perform 15 min RMR #2	
3:30	8:45		Blood Draw T=150 (can be done during RMR)	
3:45	9:00		Remind subject to stay in constant position until next saliva	

3:50	9:05		UGRA prep snack for subject (microwave)	
4:00	9:15		KSS +4 hr _____	
4:00	9:15		Start collection Saliva T=4	
4:00	9:15		Blood Draw T=180	
4:01	9:16		Remove IV from subject's arm.	
4:05	9:20		Have Subject complete VAS questionnaire #7	
4:05	9:20		Provide snack to subject (eat within 20 min)	
4:25	9:40		PRA has subject rinse mouth for saliva sample, instruct subject not to eat or drink anything until finished with saliva sampling.	
4:30	9:45		Have subject complete payment forms	
4:45	10:00		Subject must remain in constant posture until next saliva sample	
5:00	10:15		KSS +5 hr _____	
5:00	10:15		Start collection Saliva T=5	
5:10	10:25		Subject may eat lunch if they would like, otherwise can send subject home	
5:10	10:25		Text Dr. Fahrner and Josie that subject has left lab	
			Follow Discharge SOP	

Appendix C: Overnight Blood Sample Log

collect 5mL **Blood Sample Log** *Change reference to B6/B3 for week 2

Sample	Date*	HAS	Scheduled Time*	Actual Time	Notes	Initials
OvN 1		12:00	17:15			
OvN 2		13:00	18:15			
OvN 3		14:00	19:15			
OvN 4		15:00	20:15			
OvN 5		15:55	21:10			
OvN 6		17:00	22:15			
OvN 7		18:00	23:15			
OvN 8		19:00	0:15			
OvN 9		20:00	1:15			
OvN 10		21:00	2:15			
OvN 11		22:00	3:15			
OvN 12		23:00	4:15			
OvN 13		23:55:00	5:10			

Appendix D: Oral Glucose Administration:
Blood Sample Log

Collect 5 mL Blood Sample Log *Change reference to B6/B3 for week 2

Sample		Date*	HAS	Scheduled Time*	Actual Time	Notes	Initials
OGTT -15			0:45	6:00			
OGTT -5			0:55	6:10			
DRINK GLUCOLA			1:00	6:15		Make note of exact glucola time and insert adjusted draw times below	
OGTT 30			1:30				
OGTT 60			2:00				
OGTT 90			2:30				
OGTT 120			3:00				
OGTT 150			3:30				
OGTT 180			4:00				

Appendix E: Saliva Sample Log

Saliva Sample Log *Change reference to B6/B3 for week 2

Sample	Date*	HAS	Scheduled Time*	Actual Time	Notes	Initials
T=0		0:01	5:16			
T=1		0:55	6:10		Do just before glucola	
T=2		2:00	7:15			
T=3		3:00	8:15			
T=4		4:00	9:15			
T=5		5:00	10:15			

NOTE: Subjects must remain in the same posture starting 15 minutes prior to sampling; mouth rinsing only needed if ate/drank something.

Appendix F: Karolinska Sleepiness Scale (KSS)

KSS Reporting

Date:

KSS q1h	Awake	Actual Time	reported #	Initials
	5 min			
	15 min			
	30 min			
	55 min			
	90 min			
	2 hour			
	3 hours			
	4 hours			
	5 hours			

Appendix G: Visual Analog Scale (VAS)

In this questionnaire, you are asked to rate the degrees or intensity of a set of symptoms, feelings, or behaviors.

At the left and right ends of each scale are words referring to your feelings. Using a pen, place a single vertical line on any position of the scale which indicates the intensity of that particular symptom as you are experiencing it NOW.

You can begin now.

1. Please rate the way you feel in terms of the dimensions given.
2. Regard the line as representing the full range of each dimension.
3. Rate your feeling as they are at this moment.

Headache

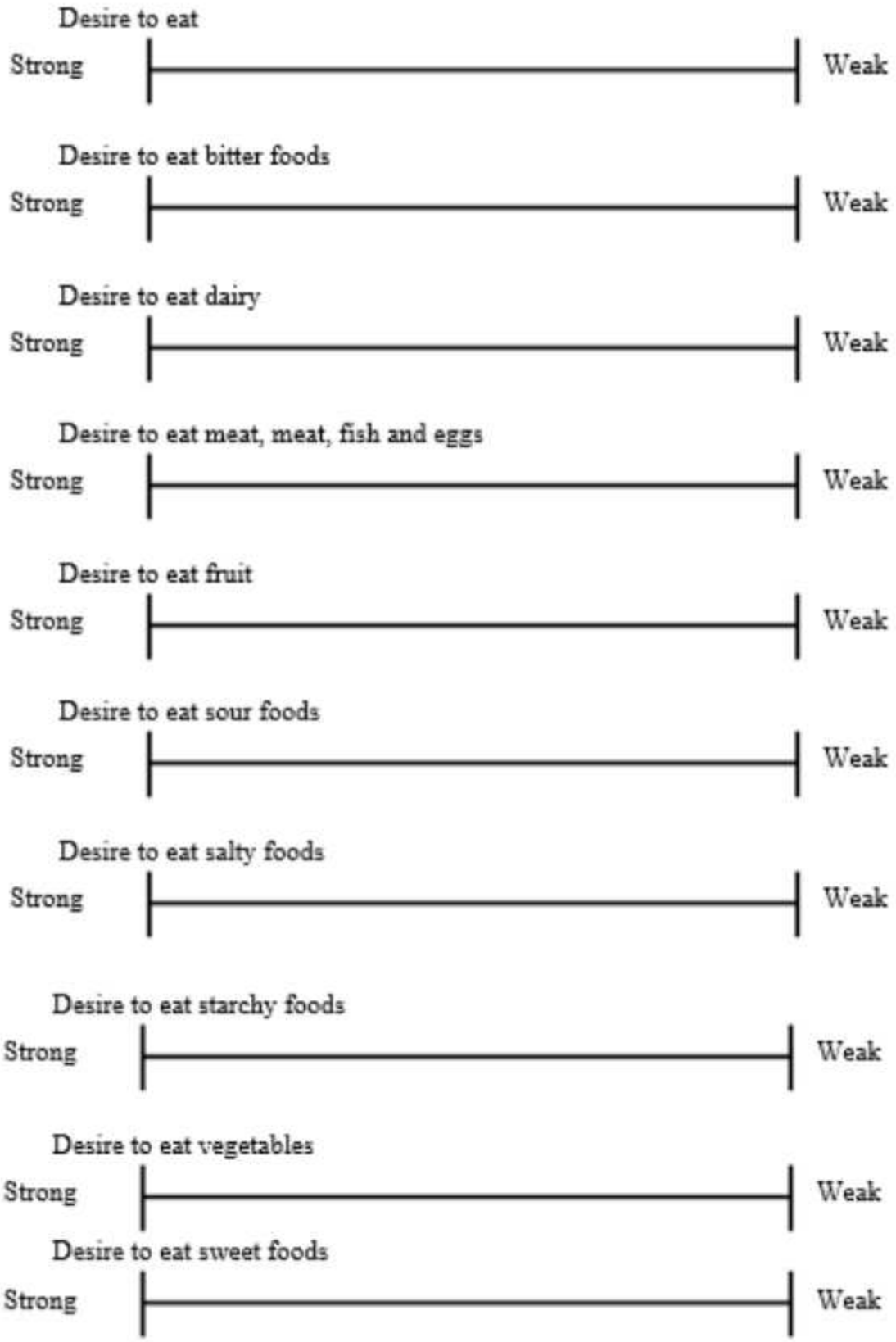
Neck pain

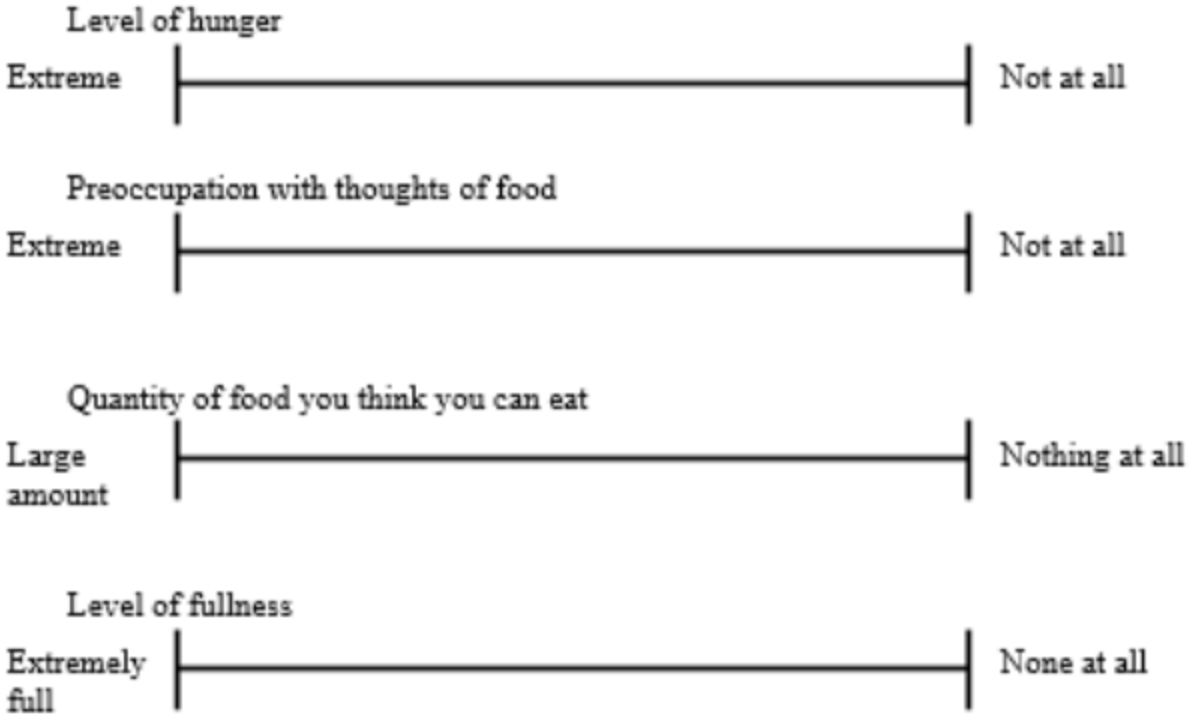
Back pain

Leg pain

Whole body pain

Stomach discomfort


Desire to eat
Strong
Weak
Desire to eat bitter foods
Strong
Weak
Desire to eat dairy
Strong
Weak
Desire to eat meat, meat, fish and eggs
Strong
Weak
Desire to eat fruit
Strong
Weak
Desire to eat sour foods
Strong
Weak
Desire to eat salty foods
Strong
Weak
Desire to eat starchy foods
Strong
Weak
Desire to eat vegetables
Strong
Weak
Desire to eat sweet foods
Strong
Weak


Level of hunger
Extreme
Not at all
Preoccupation with thoughts of food
Extreme
Not at all
Quantity of food you think you can eat
Large amount
Nothing at all
Level of fullness
Extremely full
None at all

Appendix H: Sample Photos

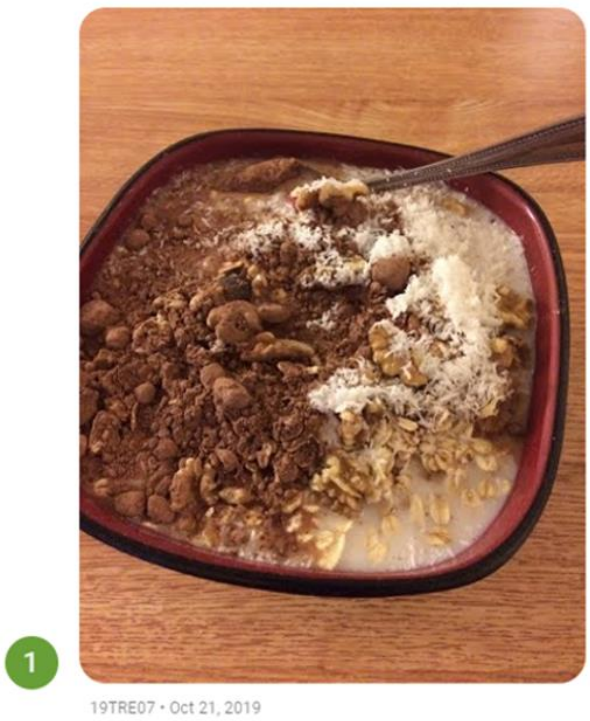
20TRE11 • Feb 14, 7:38 AM

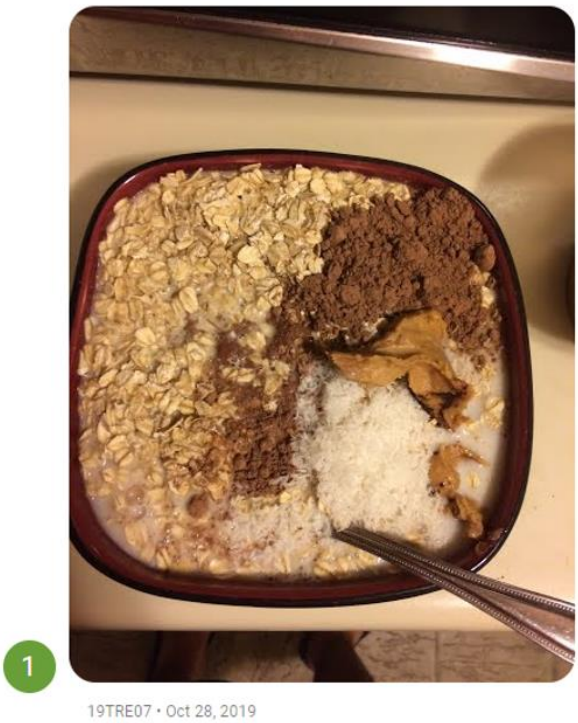
20TRE11 • Feb 14, 12:40 PM

20TRE11 • Feb 14, 5:11 PM

The above photographs are from a participant with an assigned wake time of 6:30 am, therefore, with reference to the HSA protocol, meal times were: breakfast at 7:30, lunch at 12:30, and dinner at 17:30 (with 30-minute windows before and after to consume meals).

Appendix I: Sample Photos





Sample photos are from the same participant. Pictured to the left is breakfast during the control condition and the picture to the right is breakfast during the TRE condition one week later (see date and time below photographs).

Appendix J: Sleep and Wake Log

Principal Investigator: Josiane Broussard, Ph.D. Date: 8/2/18

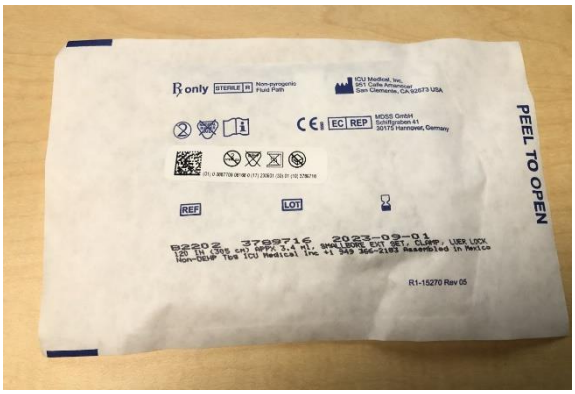
SLEEP-WAKE LOG

Subject Code:_____ Phone:_____ Date:_____

INSTRUCTIONS: Please make your form entry every night before going to bed and every morning upon awakening in addition to texting **970-775-8457.**
If you run out of forms or have any questions, please call the number above.

Date at bedtime	Day of week	Time into bed	Est. time to fall asleep	Date at wake time	Day of wake time	Wake time	Time out of bed	Caffeine

Appendix K: Through-the-wall blood sampling methods



Appendix L: Adverse Event Log

Adverse Event Reporting

Subject ID	Date	Time	Event Description	Initials