Meal timing alone alters lipid oxidation rate without affecting corticosterone in mice and humans

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DEDICATION

To Michelle Moon, my wonderful supportive fiancé who remains my most treasured discovery.

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Introduction

Preface

Circadian rhythms are the body's oscillations of behavioral and molecular traits, resulting from interactions between many genes, hormones, and metabolic processes that require precise regulation on a day-to-day basis. Disruption of these rhythms can have adverse consequences on subjects, ranging from behavioral defects (such as depression/seasonal affective disorder) to metabolic syndrome (including increased blood pressure/obesity). People affected by circadian disruption on a daily basis are individuals on a shift work schedule, who alter their typical activity and feeding behavior in accordance with the time schedule of their work (Scheer *et al.*, 2009). It is believed that circadian disruption is responsible for shift workers' increased risk for obesity. While at a casual glance this observation clearly implicates circadian disruption as the cause of these metabolic conditions, the individuals on shift work schedules also have altered eating preferences, amounts of calories consumed, stress, and hormone levels (Reeves *et al.*, 2004, Scheer *et al.*, 2009, Wefers *et al.*, 2019). Another important distinction that needs to be made is whether affecting food timing alone can affect obesity risk and metabolic syndrome in a similar manner to shifting activity by altering the light:dark cycle. There is a long-held belief that eating late leads to weight gain, but with little scientific basis for this belief. It has been difficult for circadian researchers to prove that a circadian disruption caused by activity or feeding misalignment alone is sufficient to cause weight gain and obesity in humans. By understanding how disruption

of rhythms can adversely affect metabolism, we may discover new approaches for providing therapy and treatment of metabolic disorders.

The effects of circadian disruption of metabolism has been investigated through strict control of both the light:dark cycle and restricting feeding to set times in an attempt to mimic shift work feeding behavior. The latest research involving timing of feeding studied nocturnal mice, rats, and even some humans (diurnal) conducting shift work. The results from the mouse work, in particular, has shown that restricting feeding to the mice's normally inactive phase causes weight gain, despite similar activity and caloric intake levels (Arble *et al.*, 2009). While the change to high fat diet (HFD) caused both groups of mice to gain weight, the mice fed HFD during the day had a significantly higher weight gain than mice fed HFD during the night. This disparity in weight gain suggests that the timing of food intake is the critical determinant of the difference in metabolism between the mice. Research from Hatori *et al.* (2012) further suggests that this is likely due to metabolic genes linked to the circadian clock, genes such as BMAl1, REVERBα, and PPARγ. Similar results found in humans, such as those reported in McHill *et al.* (2014), show that shifting a subject's light:dark cycle and meals by eight hours caused a decrease in energy expenditure. However, the heavy manipulation of feeding behavior in these studies raised some issues regarding their interpretations. In the case of the Hatori paper, the mice are exposed to a 16-hour fast every day. Fasting has long been used as a measure to test stress response in rodents and it is likely that a 12-hour or longer fast is causing a behavioral stress response in addition to the disruption of the feed-timing clock oscillator. For instance, short-term stress can affect energy homeostasis, creating an increase in weight gain in mice due to the influence of

cortisol's (the primary stress hormone's) effects on neuropeptide Y secretion and its action as an insulin antagonist (Luque *et al.*, 2007). This controlled lighting and feeding paradigms may also influence the findings in the human experiments. While the research from McHill *et al.* suggests that a disruption of the circadian rhythm through changes in the light:dark cycle (LD) are enough to alter the metabolic state in humans, it is unclear whether shifting meal times alone would induce a similar disruption in energy expenditure. Indeed, much of the current research monitoring the effect of evening snacking on energy expenditure [such as in Sato *et al.* (2011) and Hibi *et al.* (2012)] shows no significant effects. However, both studies used young adults, who may be less susceptible to altered meal timing challenges than older subjects. Furthermore, these studies also use different individuals for their different meal timing challenges, which likely adds to the variation in response and may make it difficult to detect differences in metabolism between groups. In order to address these concerns, I have developed a mouse model that shifts feeding behavior using restricted HFD access at specific times while providing regular chow *ad libitum* to eliminate the issue of a possible corticosterone-induced fasting response. In this mouse model, I show how metabolism changes in response to access to food early during the night phase versus later during the day. This is recapitulated in my human studies, where subjects (aged 50 or above) we monitored energy expenditure when given a breakfast, lunch, and dinner and when given a lunch, dinner, and a late-night snack. Using these methods, we sought to determine whether the findings from previous research are the result of the circadian disruption alone, the behavioral stress associated with food restriction, or a combination of the two effects. Answering this question is extremely important for

understanding the effects of shifting food intake timing in humans and how to modulate food intake in individuals conducting shift work as well as help the general populace understand the metabolic effects of their daily eating times. I hypothesize that shifting meal provision to an irregular time causes a short-term metabolic stress response, resulting in a temporary increase in excess caloric storage/adipocyte formation in both restricted and unrestricted feeding conditions.

Clock genes and their interactions and feedback with metabolism

Circadian biology is defined by sets of regulatory genes that control gene expression in a daily pattern. In mammals, the core clock genes are *CLOCK*, *BMAL,* *PER,* and *CRY*. *CLOCK* and *BMAL* act as positive regulators by binding to the E-box promoter regions, causing the transcription of a large number of genes (Buhr and Takahashi, 2013). *CLOCK* and *BMAL* also form a *CLOCK:BMAL* dimer that can transcribe its own inhibitors *PER* and *CRY*. *PER* and *CRY* also dimerize and after reaching a certain threshold the *PER:CRY* dimer is phosphorylated by casein kinase, moving it to the nucleus where it removes *CLOCK:BMAL* from the E-box domain, thus stopping transcription of *CLOCK* and *BMAL*. This defines what is known as the transcriptional translational feedback loop (TTFL) which is highly conserved in eukaryotes (Buhr and Takahashi, 2013). The inherent delay and the negative feedback in the TTFL allow for clock genes to be self-sustaining and allow for oscillations of gene expression even under constant conditions. Another critical aspect of the group of genes associated with the core clock complex is the ability to respond and entrain to external stimuli, primarily light:dark cycles but also temperature and feeding. In response to the stimuli,

the core clock complex maintains a steady oscillation that is temperature-independent (known as temperature compensation) (Pittendrigh, 1954).

There are many advantages to having a clock complex, but this dissertation will focus on the role of circadian genes/proteins on metabolism. Clock genes are responsible for many aspects of metabolism. Knock-out studies of clock genes have shown that processes such as glucose metabolism, lipid metabolism, and cholesterol metabolism are disrupted by the loss of function of the clock complex (Rudic *et al.*, 2004, Zhong *et al.*, 2018, Pan *et al.*, 2013). This is controlled both through tissue-specific rhythmic gene expression as well as through rhythmic expression of key metabolic hormones such as ghrelin, leptin, insulin, and corticosterone (Qian *et al.*, 2005, Scheer *et al.*, 2009).

While circadian genes regulate a large number of metabolic processes, it is important to note that metabolic genes/proteins can affect the clock's function. As stated before, meal timing itself has been found to be an external element for clock entrainment (Damiola *et al.*, 2000). Diet composition, such as HFD in mice, has been shown to cause arrhythmic behavior (Kohsaka *et al.*, 2007, Pendergast *et al.*, 2013). There is also the uncertainty surrounding the food entrainable oscillator, because some researchers report an anticipation behavior to meal timing that can occur in animals who have had their core clock removed either through knockouts of the core clock genes (such as PER1/PER2/PER3) or ablation of the suprachiasmatic nucleus (the nuclei in mammals involved in entraining the rest of the body to the light cycle) (Landry *et al.*, 2006, Pendergast *et al.*, 2012). Furthermore, changes in meal timing that conflict with the light cycle can cause tissue-specific changes to entrainment (Pendergast *et al.*, 2013). While these effects are well characterized, it is unclear what underlying mechanism controls

these effects on metabolism. What is clear from this research, however, is that while the core clock is critical to regulating metabolism, metabolic pathways and genes can feedback onto the clock changing cellular processes and organismal behavior. Currently, this is an important region of study in the circadian field, as elucidating the influence of metabolism on the clock could help us understand how metabolic signals that are in conflict with the brain clock, such as meal timing, would affect humans. This would be useful because treating metabolic targets known to influence the clock could be a viable therapy in order to prevent metabolic defects such as metabolic syndrome and obesity.

Effects of shift work on metabolism

Currently in the United States, obesity is a primary health concern. The percentage of adults that are obese in the United States has been increasing since the 1970s (Flegal *et al.*, 1998). Currently, around 35% of Americans are obese (CDC, 2018). This has major implications on health as obesity causes type II diabetes and metabolic syndrome greatly increases the risk for heart disease and stroke (Eckel *et al.*, 2005). Researchers have tried to determine and characterize risk factors associated with obesity in order to develop preventative and therapeutic measures to combat the obesity epidemic.

From this research, shift work was identified as a major risk factor associated with an increased likelihood for obesity (Pan *et al.*, 2011). Shift workers, in this context, are defined as employees in any job whose hours are outside of the traditional 9AM-5PM work schedule and include professions such as police officers, firefighters, and nurses. It has been found that many aspects of metabolism are disrupted in subjects under shift

work, including changes to appetite, food intake, and food preference (Reeves *et al.*, 2004, Crispim *et al.*, 2011). This leads many to believe that a large part of the metabolic disruption is due to the shift in meal timing and activity against the subject's entrained schedule, similar to the effects reported in jet lag models (McHill *et al.*, 2014). In simulated shift work in both rats and humans, researchers show a decrease in energy expenditure during the inactive phase when undergoing the shift (Caron and Stephenson, 2010, McHill *et al.*, 2014). In a study performed on nurses undergoing shift work, researchers found that the core body temperature rhythms of subjects were misaligned with their new work schedule, providing a possible mechanism accounting for changes in energy expenditure seen in other shift work and jet lab studies (McHill *et al.*, 2014, Roskoden *et al.*, 2017, Wefers *et al.*, 2018).

The studies on shiftwork listed above also note hormonal imbalances consistent with a circadian misaligned state, in particular cortisol and melatonin. Cortisol is a rhythmic hormone considered to be part of the stress response. Cortisol can elicit an immune response, is elevated in the morning to stimulate wakefulness, and has roles in metabolism associated with the fasting response (Debono *et al.*, 2009, Rose *et al.*, 2010). Melatonin is also a rhythmic hormone, one that is critical for sleep and sleep quality (Rajaratnam *et al.*, 2003). Unsurprisingly, both sleep duration and quality has been shown to decrease in shift work and jet lag studies (Santhi *et al.*, 2007, Wright *et al.*, 2013, Wright *et al.*, 2015, McHill *et al.*, 2019). This is also important in a metabolic context, as there have been many correlations between disrupted sleep and risk for obesity (Watanabe *et al.*, 2010, Xiao *et al.*, 2013, Grandner *et al.*, 2014). Ghrelin and leptin, rhythmic hormones involved with hunger and satiety, have also been shown to be

circadian misaligned in shift work studies, likely contributing to the changes in appetite and increase in food intake discussed above (Scheer *et al.*, 2009, McHill *et al.*, 2014). In summary, from the data collected from many shift work and jet lag studies there is a large body of evidence suggesting that circadian misalignment has a significant affect on metabolism in mammals.

Meal timing studies in mice and humans are confounded by altered fasting period

While the research discussed in the previous section shows evidence of large metabolic disruption in shift work and jet lag models, one issue of the models is the inability to distinguish which effects on metabolism are caused by changes to sleep and which are caused by changes to circadian misalignment. It is also unclear from shift work studies whether metabolic effects seen in shift work are caused by a circadian misalignment of the light:dark cycle or the circadian meal timing, both of which are disrupted in these models. Consequently, it is difficult to use shift work/ jet lag models to determine which factors play the strongest role in the risk for obesity seen in shift workers. To separate circadian effects on metabolism from effects due to sleep, circadian researchers began to test the effect of meal timing on metabolism. Key evidence related to the affect of meal timing was derived from a study by Arble *et al.* (2009) which showed that restricting HFD feeding to the inactive phase produced greater weight gain in mice than restricting feeding to the active phase, despite similar activity and caloric intake. This prompted a large amount of research in both mice and humans on how meal timing affects metabolism. From these studies it was found that by limiting meal timing to within the active phase, misalignment of the core body

temperature rhythm (as seen in shift work models) does not occur. Changes that do occur are glucose tolerance and insulin resistance, and these are dependent on circadian meal timing (Sato *et al.*, 2011, Hibi *et al.*, 2013). There is also strong evidence that energy expenditure is elevated in mice whose feeding duration (the length of time between the first meal to the last meal in a day) is limited to the active phase compared to mice that eat *ad libitum* throughout the day (Hatori *et al.*, 2012, Chaix *et al.*, 2014). From these data, the current understanding in the field is that the duration of feeding dictates how carbohydrates and lipids are metabolized and that shorter feeding duration leads to an overall increase in energy expenditure and weight loss. This has been corroborated in human studies such as Nas *et al.* (2017), Kobayashi *et al.*, (2014), and Gill *et al.* (2015), but many other human circadian meal timing studies only report differences in the increase of energy expenditure in response to a feeding event (known as the thermic effect of food) which was dependent on the time of the feeding event. However, the changes these studies report on the thermic effect of food didn't affect the daily energy expenditure or show no change in energy expenditure at all (Sato *et al.*, 2011, Hibi *et al.*, 2013, Ravussin *et al.*, 2019). However, in both human and mouse studies, there are a wide variety of feeding paradigms being used to test circadian meal timing. Similarly, there has been evidence of the respiratory quotient (a measure of VCO_2/VO_2 used to determine if carbohydrates or lipids are being oxidized) being shifted in circadian meal timing studies in both mice and humans but with varying degrees of significance making interpretation difficult (Sato *et al.*, 2011, Hatori *et al.*, 2012, Hibi *et al.*, 2013). However, it is important to note that these paradigms affect one or more aspects of circadian meal timing: the onset of meal times (first meal presented), the

offset of meal times (the last meal presented), the frequency of meals, the feeding duration (and consequently the length of fasting), and the proportion of calories in each meal (Sato *et al.*, 2011, Hibi *et al.*, 2013, Gill and Panda, 2015, Nas *et al.*, 2017, Ravussin *et al.*, 2019). With this in mind, it seems likely that factors such as feeding duration may have a very different effect on metabolism than shifting the onset and offset of feeding. Research investigating the nuances between the many factors associated with circadian meal timing will be crucial to our understanding and interpretation of how circadian meal timing affects metabolism.

Corticosterone fasting response can affect weight gain similar to circadian meal timing

In the previous section, we discussed how the various feeding paradigms used for circadian meal timing may cause the variation in responses seen in human circadian meal timing studies. One potential mechanism where this could be possible is through the corticosterone/cortisol response. Corticosterone in mice and cortisol in humans is involved in immune and fasting responses (Dinkel *et al.*, 2002, Rose *et al.*, 2010). Cortisol has been found to have an endogenous rhythm showing that circadian rhythmicity affects cortisol expression. Corticosterone and cortisol are known as "stress" hormones and are elevated in response to both behavioral and physical stressors causing changes in immune function (Dinkel *et al.*, 2002, Bowers *et al.*, 2008). However, there is a large literature on how corticosterone is elevated in fasting and controls metabolic functions such as stimulating glucose release and inhibiting lipid oxidation under fasting conditions (Challet *et al.*, 1995, Makimura *et al.*, 2003, Luque *et*

al., 2007, Rose *et al.*, 2010). As mentioned in the previous section on shift work, in many of the human studies cortisol was found to be mistimed (Scheer *et al.*, 2009, Wright *et al.*, 2015, Resuehr *et al.*, 2019). These metabolic effects are very similar to the effects seen in time restricted feeding studies. Interestingly, mice and rats under fasting response also show changes in energy expenditure thought to be due to the increase in corticosterone (Poggiolli *et al.*, 2013, Namvar *et al.*, 2016). These findings make it likely that corticosterone has an effect on metabolism not due to "stress" but rather due to the changes in the length of fasting seen in mice under many circadian meal timing regimes. While corticosterone levels have not been analyzed in most circadian meal timing studies, it is tempting to think that the discrepancies in energy expenditure found in human research were due to changes in corticosterone response brought on by the differences in the length of fasting tied to the protocols. Clearly, the fact that corticosterone has been linked with altered metabolism during fasting means that corticosterone response should be assessed as part of a circadian meal timing protocol.

Summary

In summary, metabolism and circadian rhythms are intricately linked. While the mechanism is currently not well understood, there is clear evidence the circadian timing of meals influences changes in circadian rhythms and expression, at an organismal and cell levels. The effect of changing meal timing alone without affecting activity levels, food intake levels, or entrainment to the light:dark cycle is enough to alter metabolism through changes in hormone levels, carbohydrate and lipid oxidation, and energy

expenditure. While there has been much progress in characterizing the effects of circadian meal timing on metabolism, there is still the question of what key components of the meal timing feeding paradigms are responsible for the effects seen in mouse and human studies. Furthermore, it also is unclear from current feeding paradigm studies what role corticosterone has in circadian meal timing, compared to the effects it is known to have in fasting and shift work/jet lag models. By answering these questions, the circadian and metabolism fields will have better insight on the mechanisms behind circadian meal timing. This work will also further our ability to provide valuable information on meal timing and eating habits related to the prevention of obesity.

Chapter previews

In this dissertation, I studied the effects of both timing of feeding and time restricted feeding on mouse and human metabolism.

Chapter II will cover my mouse metabolic research. The goal of this project was to isolate weight gain effects due to as fast versus effects caused by feeding at a nonoptimal circadian time. This chapter will go over the methods, results and conclusions of my long-term weight gain projects, feeding/activity data, and metabolic rate analysis through the use of the Vanderbilt metabolic Phenotyping core. In brief, I will discuss how weight gain, feeding, and activity are altered depending on whether mice are allowed regular chow *ad libitum* or not in conjunction with their restricted HFD feeding. I will also show that shifting HFD access influences weight gain as seen in other research, with mice given HFD near the end of the active phase showing increased weight gain compared to mice given HFD during the beginning of the active

phase. I will also describe how mice given regular chow *ad libitum* display altered weight gain when shifting the time of HFD feeding. The findings from this project are that the fasting duration plays an important role in the weight gain effects seen in mice. Specifically, differential weight gain based on time of feeding and shifts in the majority of calories via HFD restriction is observed, even with minimal fasting.

Chapter III describes corticosterone experiments I performed on mice under the conditions detailed in chapter II. The goal of this project was to assess whether the HFD restrictions imposed on the mice are enough to induce a stress response compared to the unfasted mice. Since fasting and the corticosterone response are intimately linked and corticosterone is a steroid hormone that directly influences glucose metabolism/storage, it was reasonable to believe that shifting the timing of the fasting period would alter corticosterone levels in mice. I will detail the experiments in assessing corticosterone through ELISA assays from collected trunk blood from mice under early or late night HFD feeding, for mice that experienced either an 18-hour fast or were given regular chow *ad libitum*. This work demonstrated that differences in fasting and shifted feeding conditions had very little effect on the corticosterone daily rhythm. Over the course of the experiment corticosterone levels were not significantly altered between treatments although there is a trend toward the mice under fasting conditions having an elevated corticosterone level as compared to their regular chow *ad libitum* counterparts. Ultimately, we cannot conclude from these data that corticosterone is a driving force behind the weight gain effects seen in time restricted fed mice.

Chapter IV will cover my human work investigating the effects of meal timing on human metabolism, which was accomplished through the use of the Vanderbilt metabolic chamber. This chapter will detail the recruitment of subjects into the study and how the metabolic chamber was used to compare a breakfast versus a snack regimen on respiratory quotient and lipid/carbohydrate oxidation. The goal of this work was to show that when individuals consume a meal in temporal proximity to their sleep episode, the abundance of available carbohydrates causes a significant decrease in lipid oxidation compared to a similar isocaloric scenario in which the meal is shifted to the early morning.

Chapter V will describe the temperature compensation properties of Rat1 and U2OS cell lines in response to various treatments influencing metabolism. This work involved the use of a Lumicycle and showed that pharmacological inhibitors/activators of the AMPK pathway (drugs include AICAR, Compound C, and Rapamycin) can influence the cell's ability to temperature compensate. I observed this deficit in temperature compensation by monitoring changes in the periodicity of cells at various temperatures. The findings from this chapter are that influencing the metabolic pathway can alter the temperature compensated period effects of both Rat1 and U2OS cell lines. This finding is exciting because it shows that metabolic pathways can feedback onto the clock and alter temperature compensation.

In Chapter VI I will detail the overall conclusions derived from my dissertation research. In brief, the circadian clock is predominantly regulated and entrained by the light dark cycle and uninfluenced by feeding time. However, this causes issues when feeding time is altered as there are optimal and nonoptimal times to metabolize

carbohydrates and lipids over the course of the day. In mice, both the time of feeding and fasting duration can affect weight gain and metabolism depending on their time of day placement. In humans, feeding in temporal proximity of the inactive phase leads to a delay in lipid oxidation in favor of metabolism of the carbohydrates available from the meal. This could potentially lead to an increase in fat accumulation without altering calories.

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CHAPTER II

Analysis of timing of feeding effects on mice with or without a fast on metabolic response.

Abstract

Interpretation of previous research on meal timing and weight gain is confounded in study designs that require an imposed fasting period. Because fasting itself has an effect on metabolism, it is difficult to conclude that the effects described in these papers are solely the result of a dyssynchronous feeding time. I have found that mice given restricted HFD with regular chow *ad libitum* will feed mostly when HFD is available, but will have snacks of regular chow during their active period when HFD is not available, accounting for 20-35% of their daily caloric intake. This provides a method to shift feeding behavior via shifting the HFD feeding restriction window without putting the mouse under a fasting protocol. I have compared the metabolic differences between mice that have HFD food restrictions without any regular chow *ad libitum* (*ad libitum*) and mice with regular chow *ad libitum* and found that the timing of the HFD presentation leads to differences in weight gain, with HFD presented at the onset of activity causing less weight gain than if presented later. Surprisingly, I also found that mice given both *ad libitum* regular chow supplement and restricted HFD show similar weight gain effects as their counterparts receiving no-ad-libitum chow. Mice with restricted HFD access and regular chow *ad libitum* do not consume more calories than their no-ad-libitum chow counterparts despite having the ability to feed throughout day. Nevertheless, the

vast majority of calories come from the HFD restriction window. This indicates that the weight gain seen in previous studies is due to disruptions of the circadian metabolic response and not due to a fasting response. For assessment of the metabolic state under different restricted feeding regimes, I performed a short-term metabolic analysis of mice on shifted restricted/unrestricted feeding protocols using the SABLE system to monitor energy expenditure (3 week study) through indirect calorimetry. We measured energy expenditure, respiratory exchange ratio (RER), and oxidation of carbohydrates/lipids based on VO_2 and VCO_2 values to evaluate the metabolic changes occurring between the breakfast and snack sessions. I found that all mice show no change in energy expenditure, replicates previous studies, and daily caloric intake was similar amongst all groups. However, I also observed a short-term disruption in RER caused by a shift in timing of carbohydrate and lipid oxidation. This is temporary and, after 5 days under the HFD restricted protocol, mice adjust their metabolic schedule accordingly.

Introduction

A growing body of research suggests that the circadian meal timing can effect metabolism. This work is critical based on the finding that subjects on shift work are much more likely to develop obesity and metabolic syndrome related disorders (Pan *et al.*, 2011). While the obesity issue is a multifactorial one, there is evidence that circadian meal timing alone can influence metabolic rate.

One of the first studies to show this effect was by Arble *et al.* (2009). They restricted HFD feeding to a 12-hour window and compared mice given a HFD restricted to their active phase or their inactive phase, finding that mice restricted to feeding in the inactive phase gained more weight than mice given food in the active phase, despite similar caloric intake and activity. Work from Hatori *et al.* (2012) found the primary feeding-associated mechanism explaining weight gain was feeding duration and mice under a larger food restriction show elevated energy expenditure as compared to their *ad libitum* counterparts. Other mouse studies on circadian meal timing have shown that the effects seen in terms of mouse weight gain are largely due to changes to insulin sensitivity, glucose tolerance, and changes in energy expenditure (Hatori *et al.*, 2012, Chaix *et al.*, 2014, Adamovich *et al.*, 2014).

While the effect on circadian meal timing seems clear in mice, when studies are translated into human research there are conflicting reports. A key discrepancy is that some studies observe changes in energy expenditure based on their circadian meal timing regime (Kobayashi *et al.*, 2014, Nas *et al.*, 2017), while others see no effect on energy expenditure (Sato *et al.*, 2011, Hibi *et al.*, 2013, Ravussin *et al.*, 2019). Depending on the study, researchers report changes in glucose tolerance and insulin resistance in humans (Hibi *et al.*, 2013, Kobayashi *et al.*, 2014, Nas *et al.*, 2017) and mouse models (Chaix *et al.*, 2014) while other circadian meal timing studies do not (Sato *et al.*, 2011, Hatori *et al.*, 2012). This apparent discrepancy between results of mice and humans has made determining the effects of circadian meal timing difficult.

However, there are clear differences in feeding paradigms used in humans versus mice that we believe may shed light on the difference in circadian meal timing

effects between the two species. Many of the mouse protocols use a much harsher feeding regime than is possible in humans, but also affect circadian meal timing via changing the onset/offset of feeding, the feeding duration, and the distribution of calories (Arble *et al.*, 2009, Hatori *et al.*, 2012, Kuroda *et al.*, 2012, Chaix *et al.*, 2014). Human studies also use a variety of protocols, although less drastic than in mice, but generally restrict feeding changes to the active period (Sato *et al.*, 2011, Hibi *et al.*, 2013, Kobayashi *et al.*, 2014, Nas *et al.*, 2017, Ravussin *et al.*, 2019). Studies like Nas *et al.* (2017) and Kobayashi *et al.* (2014), which do see an effect of energy expenditure on their feeding regime, are shifting the meal onset/offset as well as increasing the fasting duration. Similarly, studies that do not change their fasting duration do not see changes in energy expenditure (Hibi *et al.*, 2013, Sato *et al.*, 2011). Many mouse studies have explored feeding restrictions, whereas few have used protocols that change the onset/offset of feeding time. Currently, onset/offset of timing of feeding and food restriction are thought to regulate metabolism in the same way. However, the conflicting evidence on energy expenditure in human studies appears to be highly dependent on the feeding paradigm, suggesting that the field's current view may be an overgeneralization of the circadian meal timing mechanism.

In this study, we use mice to compare the metabolic effects of different onset/offset of feeding, with or without a fast. The purpose of this study was to determine if mice with altered onset/offset of feeding show a differential effect on weight gain and carbohydrate/lipid oxidation compared to mice on a fasting versus no-fasting protocol. To do this, we use a novel feeding paradigm that uses restricted HFD feeding during a six-hour feeding window either at the beginning or the end of the early night

phase. Mice on this regime, which involves an 18-hour fast were compared with mice under the same HFD feeding restriction window that were allowed to eat regular chow (RC) *ad libitum* (at all hours), eliminating the fasting but maintaining differences in the onset/offset for the six-hour HFD access, which constituted the majority of calories. We find that weight gain resistance occurs in mice under the 18-hour fast and in unfasted mice where only the onset/offset of feeding is altered. Our data suggests that onset/offset of meal timing primarily alters weight gain through differential carbohydrate/lipid oxidation while feeding duration affects metabolism through changes in energy expenditure. This study highlights how onset/offset of timing of feeding can influence weight gain in a different manner than through a feeding/fasting restriction paradigm.

Methods

Treatment groups

Eleven-week-old male C57/BL mice were housed for 1 week prior to the experiment under a cycle of 12 hours of light followed by 12 hours of dark (referred to as 12:12 LD). Mice were placed in one of the listed treatment groups: Regular Chow *ad libitum*, HFD *ad libitum*, Early Night HFD, Late Night HFD, Early Night HFD with Regular Chow *ad libitum*, and Late Night HFD with Regular Chow *ad libitum*. Early Night groups were given HFD during the first six hours of the dark phase and Late Night groups were given HFD during the last six hours of the dark phase. Mice with regular chow *ad libitum* were provided with unlimited access to regular chow in a separate feeding chamber. For Indirect calorimetry methods, mice were housed in cages from Promethion cages from SABLE Systems International that contained two food hoppers

that could be autonomously opened and closed based on their feeding regime. For both indirect calorimetry and long-term weight gain experiments, mice were monitored daily at times when HFD food access was allowed/cut off to ensure that no HFD chow was left in the cage or hoarded. HFD was purchased from Research Diets (D12492) and contains 60% calories from fat.

Measurements of indirect calorimetry

Experiments in the respiratory chamber used 12-week-old C57/BL mice (n=4 for each group). Prior to experimentation, mice were weighed, divided into groups, and allowed to entrain to a 12:12 LD cycle for one week. After entrainment, mice were individually housed in a chamber provided by Vanderbilt's Mouse Metabolic Phenotyping Center (MMPC) to monitor the rates of VCO_2 and VO_2. Respiratory exchange ratio and energy expenditure were extrapolated from these data using equations shown in Frayn and Hall *et al.* (Frayn *et al.*, 1983, Hall *et al.*, 2016). Mice were weighed weekly. Chow was placed in automated feeders with a scale incorporated so quantity and time of chow consumption could be calculated. Mouse activity was monitored via beam breaks using infrared detectors.

Long-term weight gain experiments

Experiment used 12-week-old C57/BL mice (n=7-10 for each feeding group). Mice were housed in a light controlled box to maintain light cycles. Prior to experiments, mice were weighed, divided into groups, and allowed to entrain to a 12:12 LD cycle for one week. Mice were singly housed and placed into treatment groups

(above) at 12 weeks of age. For seven weeks, at the end of each week mice were weighed during the time restricted feeding regimen. For restricted HFD groups, chow was removed and added manually and cages were checked for hoarded pellets which were then removed. Chow was weighed at the beginning of the light cycle each day.

Statistical analyses

For the long-term weight gain experiments, a two-way repeated measures ANOVA for interactions of feeding groups and time was performed using R and Sigmaplot. For data obtained by indirect calorimetry (including kcals consumed, kcals burned, RER, carbohydrate oxidation, and lipid oxidation), a two-way repeated measures ANOVA was first performed on data from the six days mice were on regular chow, as an approach to show there were no differences in mouse treatment groups at the start of the study. After determining mice were not different at the start of the study, another two-way repeated measures ANOVA was performed on the data for the full ten day time course corresponding to the different treatments, using the average daily values for each day for each mouse. The Holms-Sidak post hoc test was used to correct for multiple comparisons. Full analyses from all two-way repeated measures ANOVAs can be found in the Supplement.

Results

In order to isolate weight effects caused by timing of feeding versus a fast, we designed a novel feeding paradigm (Figure 2.1.). In this paradigm, mice are given a six-hour window of HFD availability. This period of availability occurs at two different times;

for early night mice, it occurs at the beginning of their active phase and for the late night mice, it occurs near the end. I then compared mice on this feeding regime with mice that were on the same HFD restricted feeding, but given access to regular chow *ad libitum* feeding throughout the day. The comparison of these two protocols distinguishes effects due to an 18-hour fast versus effects due to shifting the timing of consumption of the majority of calories (i.e., between early night and late night).

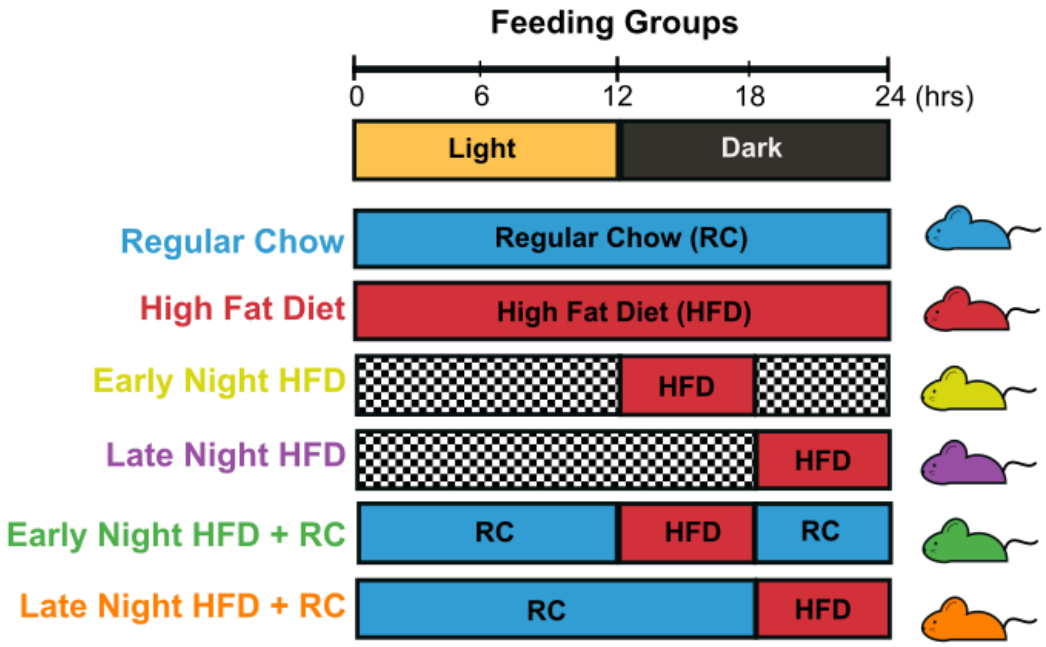

Figure 2.1. Experimental Feeding Design
The above schematic describes the feeding paradigms used during the study. Mice were subjected to 12 hours of light and 12 hours of dark (12:12 LD). Mice were given regular chow *ad libitum* (blue) or HFD *ad libitum* (red) as controls under a no feeding restriction protocol. To observe metabolic effects due to timing of feeding and fasting, mice were given HFD either the first 6 hours of the dark cycle (zeitgeber time (ZT)12-18, yellow) or the last 6 hours of the dark cycle (ZT18-24, purple). To measure effects caused solely by feeding time, mice were given early or late night HFD access but were given *ad libitum* access to regular chow (green and orange respectively).

We then compared weight gain effects with the HFD restricted fed mice with or without regular chow supplemented. Figure 2.2.A shows weight gain over time for mice

under regular chow *ad libitum*, HFD *ad libitum*, Early HFD without a RC supplement, and Late HFD without a RC supplement. Mice on the HFD *ad libitum* have the largest increase in weight gain and mice on RC maintain body weight similar to the start of the experiment as expected from previous literature (Hatori et al., 2012, Chaix et al., 2014). Mice on the restricted feeding of HFD, either early or late, show an overall lower weight gain than mice on HFD *ad libitum*. When analyzed using a two-way repeated measures ANOVA, I determined that feeding group and weeks on feeding regime had a significant interaction (P-value<0.001) on body weight while there was no significant difference in the amount of chow the mice were eating (Table S2.1.). Further analysis using a Holms-Sidak post hoc test showed that over time, the HFD *ad libitum* group gained significantly more weight than RC *ad libitum*, Early HFD, and Late HFD. However, weights of mice on the Early and Late HFD were not significantly different over the course of the study, suggesting both feeding regimes have a very similar effect on weight gain.

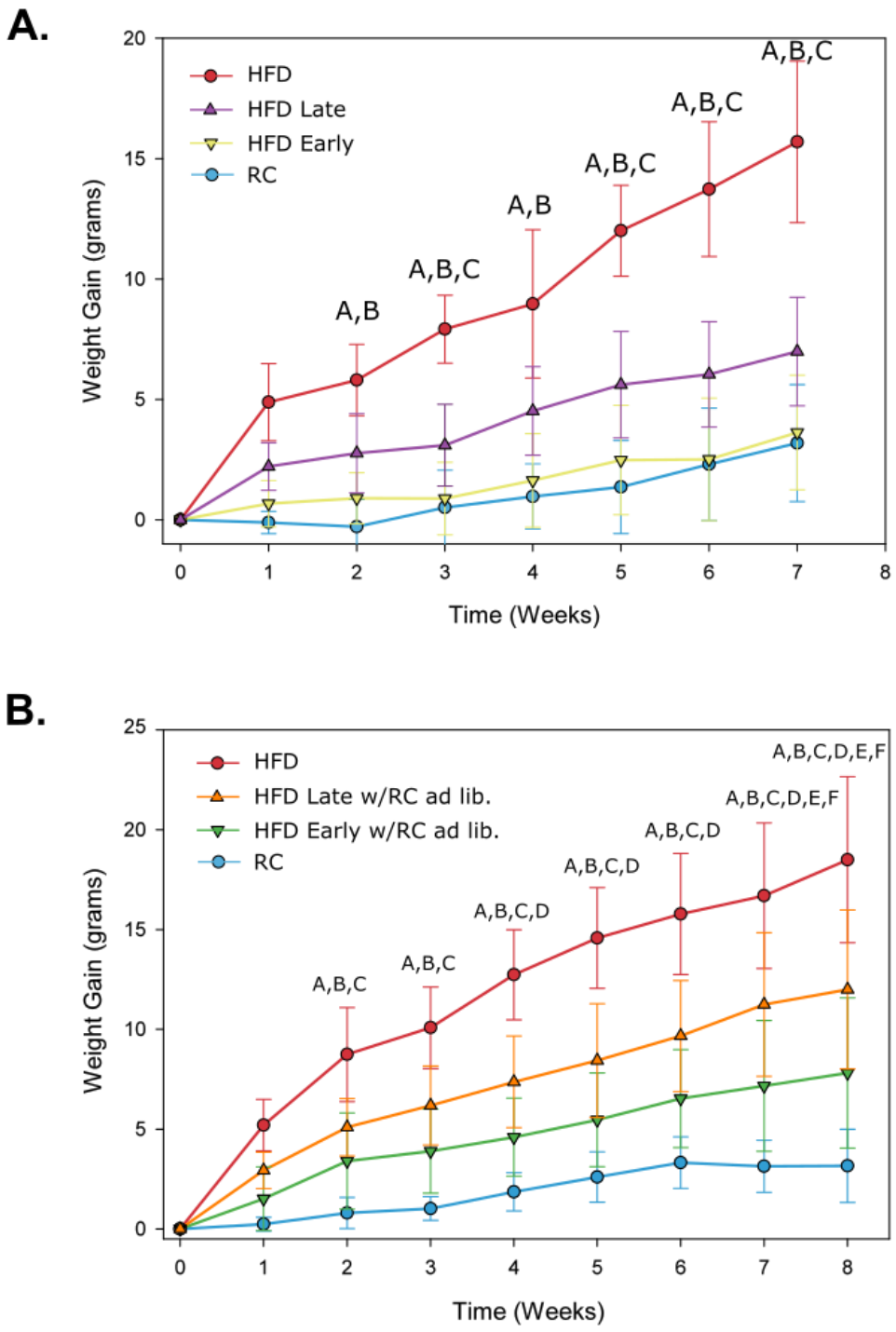

Figure 2.2. Weight gain resistance in mice is affected by allowing *ad libitum* access to regular chow.
A) Weight gain of mice given RC (blue), HFD (red), Early HFD (yellow), or Late HFD (purple) over seven weeks (n=7-10). Error bars indicates standard deviation. Letters indicate a significant effect after two-way repeated measures ANOVA analysis as

follows: A= Reg. v. HFD, B= HFD v. Early, C= HFD v. Late (see supplemental table S2.1 for detailed analysis).
B) Weight gain of mice given RC (blue), HFD (red), Early HFD with RC *ad libitum* (green), or Late HFD (orange) over eight weeks (n=7-10). Error bars indicates standard deviation. Letters indicate a significant effect after two-way repeated measures ANOVA analysis as follows: A= Reg. v. HFD, B= Reg. v. Late + Reg., C=HFD v. Early + Reg., D= HFD v. Late + Reg., E=Reg. V. Early + Reg., F= Late + Reg. v. Early + Reg. (see supplemental table S2.2 for detailed analysis)

To determine if the 18-hour fast was the primary cause of the weight gain effects, we performed another long-term weight gain experiment using RC *ad libitum* and HFD *ad libitum* compared to mice given restricted HFD access during the Early or Late night time periods. However, in this experiment, mice on the Early or Late HFD fed groups were also allowed to feed on RC *ad libitum*, thus removing any potential fasting the mice would have. Once again, there was no major difference in overall daily food intake (Table S2.2) but there was a dramatic change in the weight gain effects (Figure 2.2B). In this experiment, we once again observed HFD *ad libitum* mice gained the most weight, RC *ad libitum* gained the least weight, and both Early HFD with RC *ad libitum* and Late HFD with RC *ad libitum* show a resistance to the weight gain phenotype of HFD *ad libitum* mouse group. An analysis by two-way ANOVA also showed that there was a significant interaction between weeks on the feeding regime and feeding groups (P-value<0.001, Table S2.2). According to the ANOVA corrected by the Holms-Sidak post hoc test, by the end of the study there are significant differences between all groups with regular chow showing little to no change in weight gain compared to starting weight, and the HFD *ad libitum* group showing the largest increase in weight gain. Both the Early HFD and Late HFD restricted with regular chow *ad libitum* show a resistance to the weight gain phenotype observed in the HFD *ad libitum* group. Interestingly, the

Early HFD and Late HFD with regular chow groups are also significantly different from each other by the end of week 8 of the study, with the early night HFD fed group showing less weight gain than their late-night counterparts. From this analysis of long-term weight gain, therefore, we find that time of feeding does affect the weight gain when fasting duration is reduced.

To further determine the metabolic processes at play in mice fed the Early and Late HFD with RC *ad libitum*, we individually housed mice in specialized metabolic cages provided by the Vanderbilt Mouse Metabolic Phenotype Center (MMPC). These metabolic cages monitor locomotor activity, food/water intake, and respiration. All mice were first on RC *ad libitum* for six days in the metabolic cages, then put on their respective feeding regimes for ten days. During the first six days of the experiment, when mice were all under RC *ad libitum*, there was no difference among the groups in kcals consumed or kcals burned (Table S2.3A and 4A). Daily caloric intake remained unaltered between mouse feeding groups throughout the ten days on the experimental feeding protocol (Fig 2.3A.). This is consistent with previous reports in the literature, indicating mice will limit HFD intake due to the high caloric content but will maintain calories consumed (Hatori *et al.*, 2012). This was also true for our mice that were on HFD restricted feeding either in the early or late night where no significant difference was found between all groups (Fig 2.3A.). Kcals burned also remained at similar levels for all feeding groups although we did find a weak interaction between days on feeding regime vs. feeding group (Fig S2.4B). However, in a post hoc analysis no particular day or feeding group could be singled out as the cause to this interaction, probably because the interaction effect was so small. Taken together, it is unlikely that

either changes in kcals consumed or burned caused the weight gain differences seen in Figure 2.2.

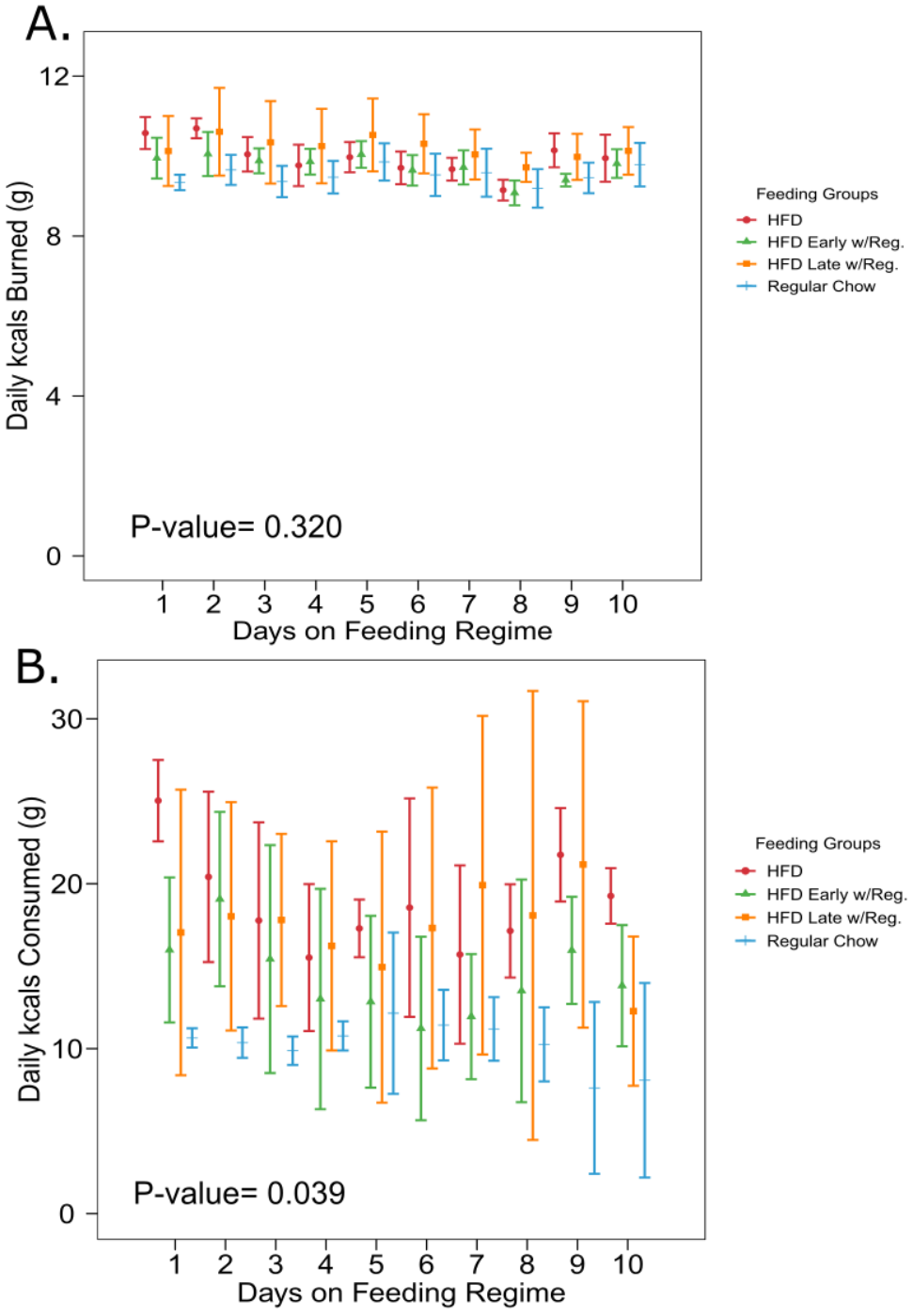

Figure 2.3. Kcals burned and consumed remain unchanged during feeding regimes.

Average daily kcals consumed (A) or burned (B) by mice on RC (blue), HFD (red), HFD Early with RC *ad libitum* (green), or HFD Late with RC *ad libitum* (orange). Bars indicated the daily average for each mouse group and error bars indicate the standard deviation within groups. P-values indicate interaction of feeding groups and days on feeding regime in a two-way repeated measures ANOVA. A weak interaction was found between days and feeding groups. However, after post hoc analysis using the Holms-Sidak test, no specific day or group could be determined as the cause of the interaction (see supplemental table S2.3 and 4 for detailed analysis).

To confirm that mice on the non-fasting HFD restricted feeding were not undergoing a self-imposed fast, I monitored timing and rate of food uptake throughout the study. Figure 2.4 shows the average hourly food uptake over a 24-hour period for each of the four feeding groups used in the MMPC. While RC *ad libitum* and HFD *ad libitum* groups were allowed access all day, they primarily fed throughout their active period during the 12 hours of darkness. We do see elevated levels of intake in the HFD *ad libitum* during the light portion as compared with the RC *ad libitum* consistent with previous literature showing that mice develop arrhythmic feeding when given *ad libitum* HFD (Koshaka *et al.*, 2007, Pendergast *et al.*, 2013). For the mice given either Early or Late night access to HFD, the shaded region indicates when mice were allowed to eat HFD (Figure 2.4C and D). We find that in both cases, mice eat throughout the 12-hour dark period in a manner similar to the RC *ad libitum* group, supplementing their diet with regular chow when HFD access was not available. We find that while the vast majority of their calories came from the HFD and that RC was mainly not eaten when HFD was available, both Early and Late night mice received between 25-35% of the kcals in their diet from RC.

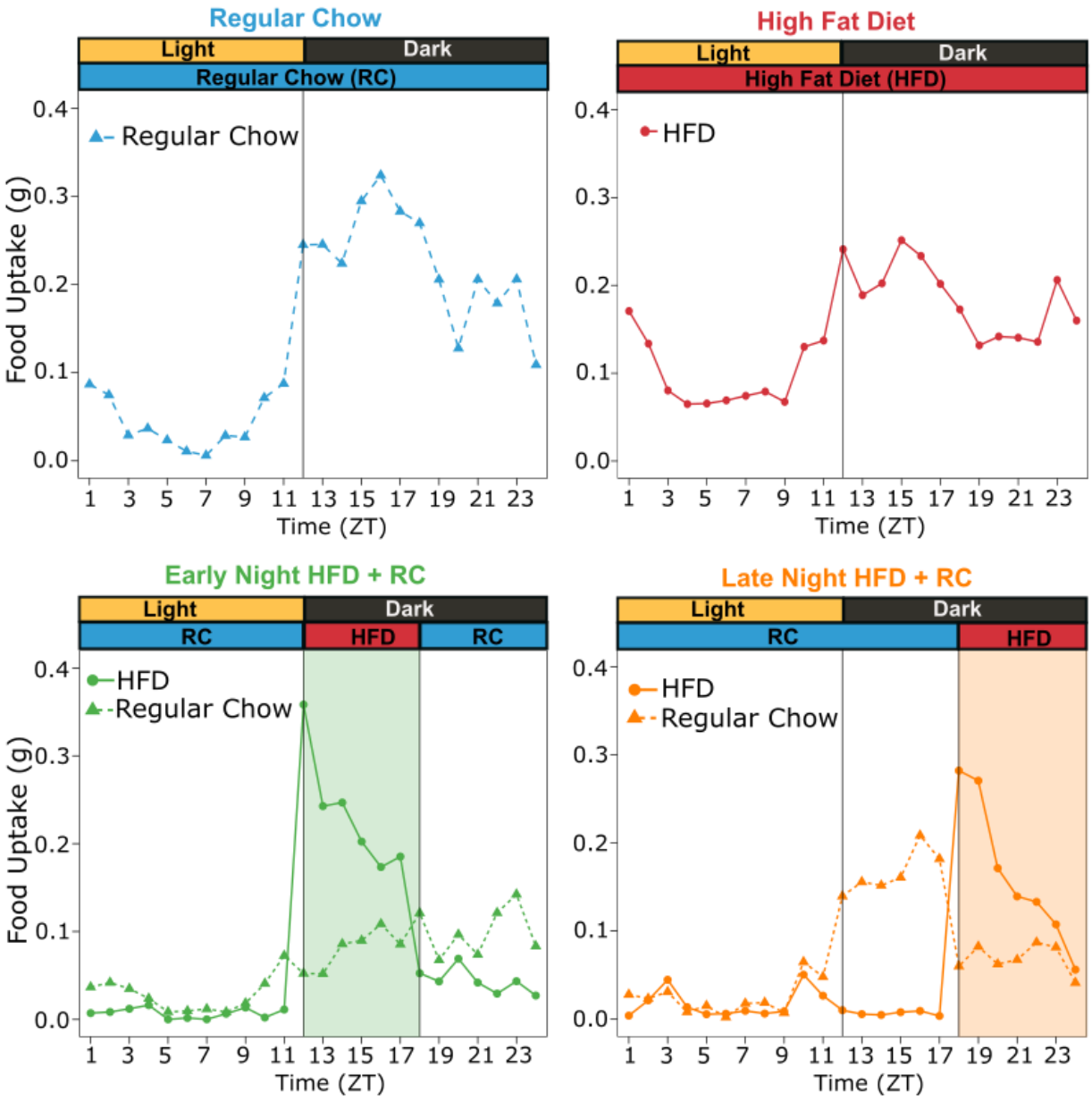

Figure 2.4. Mice with *ad libitum* access to regular chow eat throughout the active phase.
Individual feeding profiles for RC (A), HFD (B), Early HFD with RC *ad libitum* (C), and Late HFD with RC *ad libitum* (D) mice. Average hourly food intake for HFD (circles) and RC (triangles) was calculated for each feeding group based on the full 10 day feeding regime (n=4). For Early and Late HFD with RC *ad libitum*, shaded regions indicate where HFD was available for mice in that group.

The largest effect we observed was in the respiratory exchange ratio (RER), which is a measure that reports the ratio of carbohydrates versus fats being burned at a

given time based on the mouse's VO_2 inhalation and VCO_2 exhalation rate (Figure 2.5). A two-way repeated measures ANOVA conducted on RER data showed a significant interaction between days on feeding regime and group on RER (P-value=0.002). We performed another two-way repeated measures ANOVA on the initial 6 days when all mice were on RC *ad libitum* and found no significant difference, suggesting this effect did not come from an inherent bias between treatment groups prior to the change in their feeding regime (Table S2.5). When looking at the values over time, we see that this effect is largely caused by both a lowering of the RER in mice given HFD and a peak shift seen in the Early and Late groups consistent with the timing of their access to the HFD (Figure 2.5A). The effect of the feeding group on RER also appeared to be largest during the first few days on the new diets. By as soon as day 8, RER rapidly returned to levels comparable with the RC *ad libitum* group. While we did not see any difference in our post hoc analysis between the mouse groups that received HFD, there appears to be a difference in recovery of the RER when compared to the regular chow. Specifically, mice on the Early HFD regimen had daily average RER values similar to the RC group by day 8, whereas the Late HFD group still showed a difference by day 10.

Changes in RER indicate an altered oxidation rate of carbohydrates and lipids so we next investigated how carbohydrates were being oxidized. The same VO_2 inhalation and CO_2 exhalation values use to calculate RER can be used to determine the carbohydrate oxidation or lipid oxidation rates based on substrate oxidation rate equations by Frayn (1983) and Hall *et al.* (2016). Similar to the RER data, we found an interaction between Day and Feeding Group. There was also a notable peak shift

between the Early and Late HFD groups that occurred at the times that HFD was presented (Figure 2.6.). As with the RER, the largest effects seen on carbohydrate oxidation between groups occurred near the onset of the mice being put on their feeding groups and gradually recovered to levels comparable to the regular chow group by day eight and onward. With respect to carbohydrate oxidation, we do not see a variation in recovery between the Early and Late HFD with RC *ad libitum* groups as was the case for RER. Both groups still show differences after 10 days on the diet, although noticeably smaller than at the start of the study.

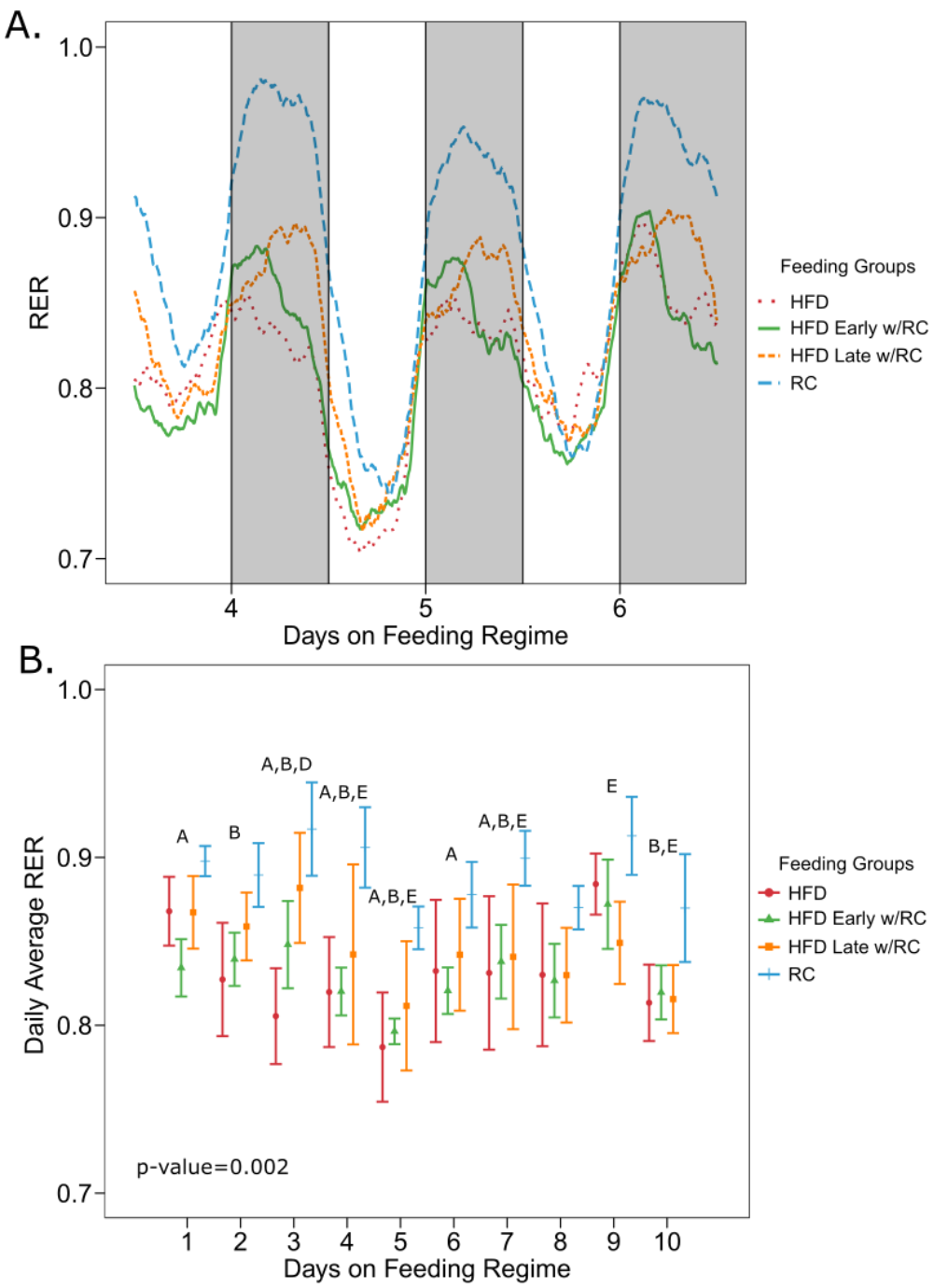

Figure 2.5. Respiratory exchange ratio (RER) peak shifts based on HFD meal timing with HFD decreasing daily RER values. (A) Average RER for each feeding group: HFD *ad libitum* (red), HFD Early with RC *ad libitum* (green), Late HFD with RC *ad libitum* (orange), and RC *ad libitum* (blue) during days 4-6 on feeding regimes. Shaded regions indicate the 12-hour lights off period. Data shows RER at every 5 minutes and was smoothed via a two-hour moving average. (B) Average daily RER (indicated by bars) for each group over the entire feeding regime. Error bars indicate standard deviation. P-value is the interaction of day and feeding group in a two-way

repeated measures ANOVA. Letters indicate significant differences between feeding groups at the indicated day: A=RC v. Early, B=RC v. HFD, C=HFD v. early, D=HFD v. late, E=RC v. late. (see supplementary table S2.5 for detailed analysis)

Finally, we investigated the effect of feeding regime on lipid oxidation. Once again, we found a significant interaction between feeding group and days on feeding regime (P-value<0.001, Figure 2.6B). Mice on the Early and Late night groups showed a dip in lipid oxidation during the HFD presentation (Figure 2.7.). Interestingly, both the Early and Late HFD with RC *ad libitum* groups began to increase lipid oxidation at the end of their respective HFD feeding access (Figure 2.7A shaded regions/lights-off period). However, given that the Early HFD group had their HFD presented at an earlier time than the Late HFD group, this led to higher lipid oxidation in the Early HFD group at the time of the transition to lights-on than in the Late night group (Figure 2.7A). This effect is observed until day 4, then disappears in the later days (Figure 2.7B). Peak lipid oxidation for all groups occurs at the middle of the lights-on period and does not vary between the feeding groups. On average, lipid oxidation was higher in the Early HFD with RC *ad libitum* group than in the Late HFD with RC *ad libitum*, though not significantly, and even significantly different from the HFD group by day 3 (Figure 2.7B). The Late Night HFD with RC *ad libitum* also appears to show differences for longer than the Early HFD with RC *ad libitum* group, although by day nine and ten the Late HFD with RC *ad libitum* group shows a slightly higher daily lipid oxidation than the Early HFD with RC *ad libitum* group. These data suggest that the timing of HFD presentation is affecting the rate of lipid oxidation, with mice feeding at the beginning of the active phase burning more lipids during the short-term period of adapting to the new feeding regime.

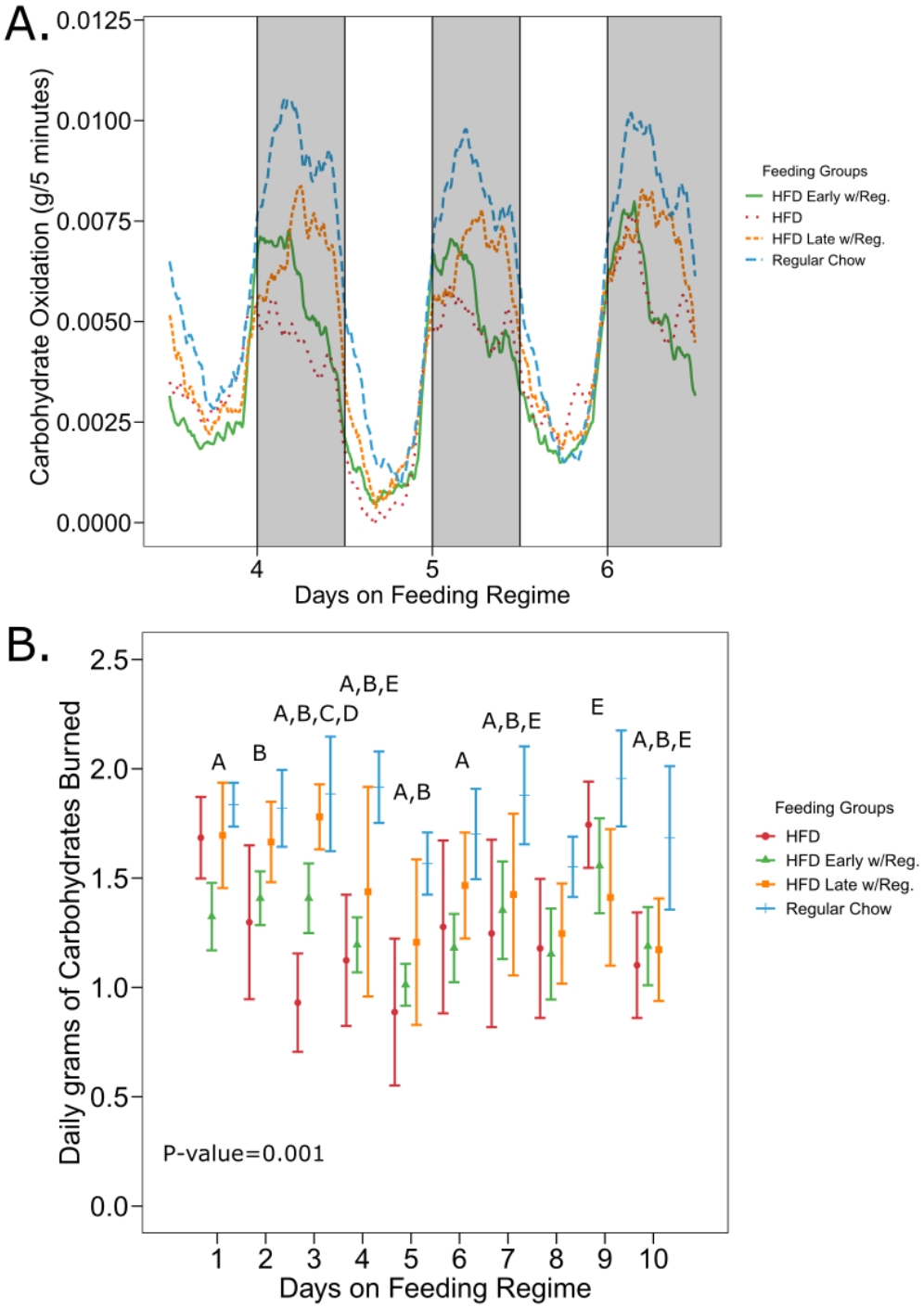

Figure 2.6. Carbohydrate oxidation is dependent on the phase of HFD presentation.

(A) Average carbohydrate oxidation in grams every 5 minutes for each feeding group: HFD *ad libitum* (red), HFD Early with RC *ad libitum* (green), Late HFD with RC *ad libitum* (orange), and RC *ad libitum* (blue) during days 4-6 of the feeding regimes. Shaded regions indicate the 12-hour lights off period. Data was smoothed using a two-hour moving average. (B) Average daily carbohydrates oxidized in grams for each

group over the entire feeding regime. Error bars indicate standard deviation. p-value is the interaction of day and feeding group in a two-way repeated measures ANOVA. Letters indicate significant differences between feeding groups at the indicated day:A=RC v. Early, B=RC v. HFD, C=HFD v. Early, D=HFD v. Late, E=RC v. Late (see supplementary table S2.6 for detailed analysis)

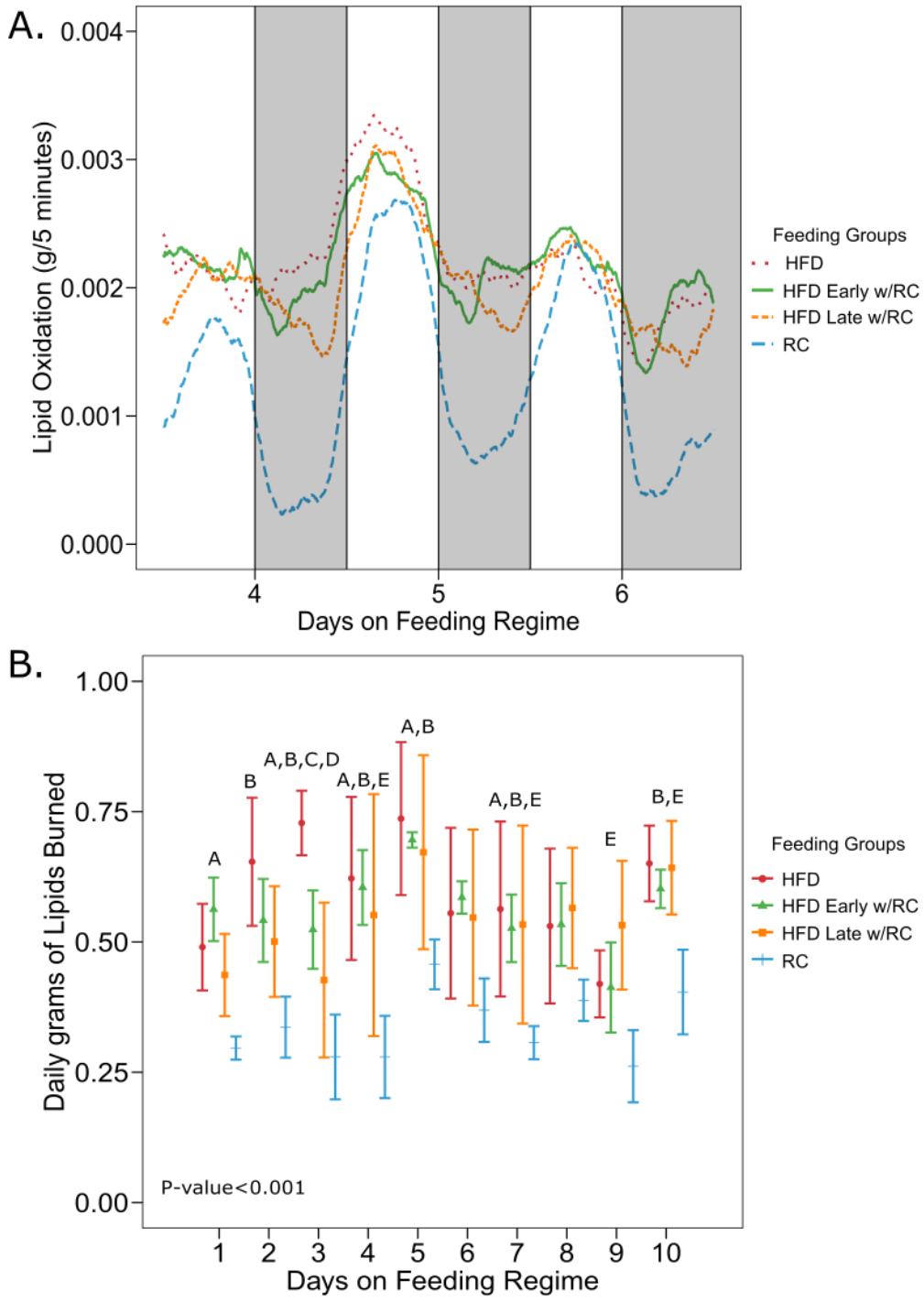

Figure 2.7. Lipid oxidation is altered based on the phase of HFD presentation. (A) Average lipid oxidation in grams per 5 minutes for each feeding group: HFD *ad libitum* (red), HFD Early with RC *ad libitum* (green), Late HFD with RC *ad libitum*

(orange), and RC *ad libitum* (blue) during days 4-6 on feeding regimes. Shaded regions indicate the 12-hour lights off period. Data was smoothed using a 2-hour moving average. (B) Average daily lipids oxidized in grams for each group over the entire feeding regime. Error bars indicate standard deviation. P-value is the interaction of day and feeding group in a two-way repeated measures ANOVA. Letters indicate significant differences between feeding groups at the indicated day: A=RC v. Early, B=RC v. HFD, C=HFD v. Early, D=HFD v. Late, E=RC v. Late. (see supplementary tables S2.7 for detailed analysis)

Discussion

The effects of daily meal timing is an active area of research in chronobiology, including both mouse and human studies. Despite the growing number of studies, the mechanism(s) behind these weight gain effects are still not well understood. In particular, it has been unclear whether these effects of circadian timing of feeding have been responsible due to onset/offset of feeding or from the inherent shift in fasting that is inadvertently created in many time restricted feeding protocols (Nas *et al.*, 2017, Hibi *et al.*, 2013, Sato *et al.*, 2011, Ravussin *et al.*, 2019). Research from other circadian groups have shown the importance of the food restriction on weight gain (Hatori *et al.*, 2012, Chaix *et al.*, 2014, Gill and Panda, 2015). From this research, the currently understood mechanism is that increasing the fasting duration, rather than the temporal location of the meals, is critical to the weight gain resistance phenotype seen in mice via an increase in overall energy expenditure in mice that fast for a longer period. However, this study did not look into altered timing of the fast.

We designed our study to determine if the temporal timing of food intake was enough to cause changes in metabolism with or without a fast. We find that under 18-hour fasting conditions we see a resistance to weight gain, consistent with other reports. We also do not observe a change in weight gain based on when the feeding occurred

when mice are placed under an 18-hour fast, also consistent with published literature (Figure 2.2A). However, in our novel feeding protocol in which mice eat around 70% of their caloric intake within a six-hour period but the fasting window is eliminated by allowing *ad libitum* RC, we still find a similar resistance in weight gain despite having the same level of kcals burned and consumed (Figure 2.2B) suggesting that timing of feeding is impacting weight gain. Furthermore, in this paradigm the timing of the meal has a significant effect on weight gain, with mice eating the majority of calories at the onset of activity having lower weight gain compared to mice that consumed most of their calories near the end of the active phase. Taken together, these data suggest that both the timing of the onset/offset of the majority of calories as well as the feeding duration affect metabolism and weight gain. These data also show that the effects on weight gain are protocol dependent. The effects on weight gain through timing of feeding are obscured when compounded with an 18-hour fast also imposed on mice. Future studies that want to isolate timing of feeding effects should use caution when designing their protocol and minimize the fasting duration as much as possible.

Looking further into mice under our altered timing of HFD access but limited fasting duration (i.e. Early HFD with RC and Late HFD with RC), we found that metabolism was affected differently than reported in studies strictly examining fasting duration. As in previous fasting duration studies, we found that caloric intake was not affected, but we also found little change to the calories burned which is thought to be the primary mechanism for the weight gain resistance in mice under increased fasting duration. Instead, we find that the effects on weight gain are due to switching between the preferred substrates for oxidation, with the Late night group preferentially burning

carbohydrates compared to lipids for several days compared with the Early night group, causing a difference in weight gain.

We also note that these effects on differential carbohydrate/lipid oxidation are relatively short lived. The largest differences seen in carbohydrate/lipid oxidation occur during the first 4 days on the feeding regimes. This is due to the initial shift in RER causing a preferential burning of carbohydrates in the Late night group that then extends into their inactive phase, leading to less lipids oxidized which we do not see in mice feeding in the Early night HFD with RC group. However, while the peak shift remains, mice in the Late night with RC group adapt to their new feeding cycle by day 8 when we no longer see extended periods of carbohydrate oxidation during the inactive phase (Figure 2.6 and 7, S2.6 and 7). This suggests that the long-term weight gain effects seen in our mice are due to short-term carbohydrate/lipid oxidation imbalance as the mice adapt to their new feeding times. In our study, the largest increases in weight gain occur during the first week followed by marginal weight gain rate seen in all groups that ultimately leads to a significant difference in weights after a period of several weeks. This seems to be true for other studies as well (Arble *et al.*, 2009, Hatori *et al.*, 2012, Chaix *et al.*, 2014).

In conclusion, our data suggest that both the timing of onset/offset of feeding as well as the feeding duration are critical to weight gain in mice. Our weight gain data suggests that the length of feeding duration can mask effects caused by onset/offset of the majority of feeding. Furthermore, restriction in the timing of consumption of the majority of calories leads to weight gain effects not by changing energy expenditure but

by short-term changes to the carbohydrate/lipid oxidation rate, which lead to long-term differences in weight gain.

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Chapter II Supplementary Information

Supplementary Table S2.1. Two-way repeated measures ANOVA of long-term weight gain of fasted mice (see Figure 2.2.A).

A. Overall interaction effects

Source of Variation	DF	SS	MS	F	P
group	3	1277.046	425.682	8.652	<0.001
mouse(group)	20	984.016	49.201		
Week	7	967.547	138.221	109.902	<0.001
group x week	21	405.603	19.314	15.357	<0.001

B. Week by Week interaction analysis

Comparisons for factor: group within Day 0

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.86	0.505	0.997	No
Reg. ad lib. vs. Early Night HFD	0.508	0.322	0.999	No
Reg. ad lib. vs. Late Night HFD	0.483	0.306	0.997	No
Late Night HFD vs. HFD ad lib.	0.377	0.239	0.993	No
Early Night HFD vs. HFD ad lib.	0.352	0.223	0.969	No
Late Night HFD vs. Early Night HFD	0.0257	0.0179	0.986	No

Comparisons for factor: group within Week 1

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Early Night HFD	3.86	2.448	0.119	No
HFD ad lib. vs. Reg. ad lib.	4.136	2.429	0.105	No
HFD ad lib. vs. Late Night HFD	2.293	1.454	0.495	No
Late Night HFD vs. Reg. ad lib.	1.843	1.169	0.582	No
Late Night HFD vs. Early Night HFD	1.567	1.089	0.49	No
Early Night HFD vs. Reg. ad lib.	0.276	0.175	0.862	No

Comparisons for factor: group within Week 2

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Reg. ad lib.	5.228	3.07	0.028	Yes
HFD ad lib. vs. Early Night HFD	4.559	2.891	0.036	Yes

HFD ad lib. vs. Late Night HFD	2.662	1.688	0.351	No
Late Night HFD vs. Reg. ad lib.	2.566	1.628	0.307	No
Late Night HFD vs. Early Night HFD	1.897	1.318	0.357	No
Early Night HFD vs. Reg. ad lib.	0.669	0.424	0.675	No

Comparisons for factor: group within Week 3

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Early Night HFD	6.446	4.089	0.002	Yes
HFD ad lib. vs. Reg. ad lib.	6.55	3.846	0.003	Yes
HFD ad lib. vs. Late Night HFD	4.445	2.819	0.035	Yes
Late Night HFD vs. Early Night HFD	2.001	1.391	0.439	No
Late Night HFD vs. Reg. ad lib.	2.105	1.335	0.348	No
Early Night HFD vs. Reg. ad lib.	0.104	0.0658	0.948	No

Comparisons for factor: group within Week 4

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Early Night HFD	6.988	4.432	<0.001	Yes
HFD ad lib. vs. Reg. ad lib.	7.144	4.195	0.001	Yes
HFD ad lib. vs. Late Night HFD	4.073	2.583	0.06	No
Late Night HFD vs. Early Night HFD	2.916	2.026	0.149	No
Late Night HFD vs. Reg. ad lib.	3.071	1.948	0.119	No
Early Night HFD vs. Reg. ad lib.	0.156	0.0988	0.922	No

Comparisons for factor: group within Week 5

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Early Night HFD	9.178	5.821	<0.001	Yes
HFD ad lib. vs. Reg. ad lib.	9.79	5.749	<0.001	Yes
HFD ad lib. vs. Late Night HFD	6.021	3.819	0.003	Yes
Late Night HFD vs. Reg. ad lib.	3.769	2.39	0.07	No
Late Night HFD vs. Early Night HFD	3.157	2.194	0.072	No
Early Night HFD vs. Reg. ad lib.	0.612	0.388	0.701	No

Comparisons for factor: group within Week 6

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Early Night HFD	10.874	6.897	<0.001	Yes
HFD ad lib. vs. Reg. ad lib.	10.57	6.207	<0.001	Yes
HFD ad lib. vs. Late Night HFD	7.317	4.641	<0.001	Yes
Late Night HFD vs. Early Night HFD	3.557	2.471	0.058	No
Late Night HFD vs. Reg. ad lib.	3.253	2.063	0.095	No
Reg. ad lib. vs. Early Night HFD	0.304	0.193	0.849	No

Comparisons for factor: group within Week 7

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Early Night HFD	11.724	7.436	<0.001	Yes
HFD ad lib. vs. Reg. ad lib.	11.656	6.844	<0.001	Yes
HFD ad lib. vs. Late Night HFD	8.333	5.285	<0.001	Yes
Late Night HFD vs. Early Night HFD	3.391	2.356	0.075	No
Late Night HFD vs. Reg. ad lib.	3.323	2.108	0.087	No
Reg. ad lib. vs. Early Night HFD	0.068	0.0431	0.966	No

Supplementary Table S2.2. Two-way repeated measures ANOVA of long-term weight gain of non-fasted mice.

A. Overall interaction effects

Source of Variation	DF	SS	MS	F	P
Group	3	2255.143	751.714	11.308	<0.001
Mouse(Group)	18	1196.619	66.479		
Week	8	2056.239	257.03	174.526	<0.001
Group x Week	24	507.073	21.128	14.346	<0.001

B. Week by Week interaction analysis

Comparisons for factor: Group within day 0

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. Early Night HFD w/Reg.	0.69	0.386	0.999	No
Reg. ad lib. vs. HFD ad lib.	0.67	0.375	0.998	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.49	0.274	0.998	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.47	0.263	0.991	No
Reg. ad lib. vs. Late Night HFD w/Reg.	0.2	0.117	0.991	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.02	0.0107	0.992	No

Comparisons for factor: Group within week 1

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. HFD ad lib.	4.94	2.649	0.08	No
Reg. ad lib. vs. HFD ad lib.	3.363	1.884	0.309	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	3.193	1.788	0.302	No
Late Night HFD w/Reg. vs. HFD ad lib.	1.747	0.978	0.709	No

Reg. ad lib. vs. Late Night HFD w/Reg.	1.617	0.95	0.579	No
Early Night HFD w/Reg. vs. Reg. ad lib.	1.577	0.883	0.386	No

Comparisons for factor: Group within week 2

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. HFD ad lib.	7.92	4.247	0.002	Yes
Reg. ad lib. vs. HFD ad lib.	4.97	2.783	0.05	Yes
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	4.85	2.716	0.047	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	3.07	1.719	0.266	No
Early Night HFD w/Reg. vs. Reg. ad lib.	2.95	1.652	0.21	No
Reg. ad lib. vs. Late Night HFD w/Reg.	1.9	1.116	0.275	No

Comparisons for factor: Group within week 3

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. HFD ad lib.	9.04	4.847	<0.001	Yes
Reg. ad lib. vs. HFD ad lib.	5.833	3.267	0.016	Yes
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	5.69	3.187	0.015	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	3.35	1.876	0.202	No
Early Night HFD w/Reg. vs. Reg. ad lib.	3.207	1.796	0.162	No
Reg. ad lib. vs. Late Night HFD w/Reg.	2.483	1.459	0.157	No

Comparisons for factor: Group within week 4

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. HFD ad lib.	10.86	5.823	<0.001	Yes
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	7.65	4.284	0.001	Yes
Reg. ad lib. vs. HFD ad lib.	6.177	3.459	0.008	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	4.683	2.623	0.043	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	3.21	1.798	0.162	No
Reg. ad lib. vs. Late Night HFD w/Reg.	2.967	1.743	0.094	No

Comparisons for factor: Group within week 5

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. HFD ad lib.	11.96	6.413	<0.001	Yes

Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	8.623	4.829	<0.001	Yes
Reg. ad lib. vs. HFD ad lib.	6.503	3.642	0.005	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	5.457	3.056	0.016	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	3.337	1.869	0.142	No
Reg. ad lib. vs. Late Night HFD w/Reg.	3.167	1.86	0.075	No

Comparisons for factor: Group within week 6

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. HFD ad lib.	12.44	6.67	<0.001	Yes
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	8.757	4.904	<0.001	Yes
Reg. ad lib. vs. HFD ad lib.	7.017	3.93	0.002	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	5.423	3.037	0.017	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	3.683	2.063	0.097	No
Reg. ad lib. vs. Late Night HFD w/Reg.	3.333	1.958	0.062	No

Comparisons for factor: Group within week 7

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. HFD ad lib.	13.54	7.26	<0.001	Yes
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	9.043	5.065	<0.001	Yes
Reg. ad lib. vs. HFD ad lib.	8.78	4.917	<0.001	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	4.76	2.666	0.039	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	4.497	2.518	0.037	Yes
Reg. ad lib. vs. Late Night HFD w/Reg.	4.283	2.516	0.019	Yes

Comparisons for factor: Group within week 8

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. HFD ad lib.	15.32	8.214	<0.001	Yes
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	10.193	5.709	<0.001	Yes
Reg. ad lib. vs. HFD ad lib.	9.51	5.326	<0.001	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	5.81	3.254	0.01	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	5.127	2.871	0.016	Yes
Reg. ad lib. vs. Late Night HFD w/Reg.	4.383	2.575	0.016	Yes

Supplementary Table S2.3. Two-way repeated measures ANOVA kcals consumed

A. Overall interactions first six days (all groups on regular chow *ad libitum*)

Source of Variation	DF	SS	MS	F	P
ID	3	0.453	0.151	1.157	0.366
mouse(ID)	12	1.565	0.13		
days	4	0.035	0.00876	1.733	0.158
ID x days	12	0.0574	0.00478	0.946	0.511

B. Overall interactions after six days (all groups on their feeding regimes)

Source of Variation	DF	SS	MS	F	P
ID	3	2.519	0.84	2.395	0.119
mouse(ID)	12	4.207	0.351		
days	9	0.319	0.0355	1.382	0.205
ID x days	27	0.783	0.029	1.131	0.32

Supplementary Table S2.4. Two-way repeated measures ANOVA kcals burned

A. Overall interactions during first six days (all groups on regular chow *ad libitum*)

Source of Variation	DF	SS	MS	F	P
ID	3	0.00992	0.00331	1.124	0.378
mouse(ID)	12	0.0353	0.00294		
Days	5	0.00384	0.000767	8.522	<0.001
ID x days	15	0.000463	3.08E-05	0.342	0.988

B. Overall interactions after six days (all groups on experimental feeding regimes)

Source of Variation	DF	SS	MS	F	P
ID	3	0.0118	0.00392	0.822	0.507
mouse(ID)	12	0.0572	0.00477		
Days	9	0.0142	0.00158	11.742	<0.001
ID x days	27	0.00597	0.000221	1.643	0.039

Supplementary Table S2.5. Two-way repeated measures ANOVA RER

A. Overall interactions during first six days (all groups on regular chow *ad libitum*)

Source of Variation	DF	SS	MS	F	P
ID	3	0.00167	0.000557	1.26	0.332
mouse(ID)	12	0.0053	0.000442		

day	5	0.00614	0.00123	6.866	<0.001
ID x day	15	0.00451	0.000301	1.68	0.08

B. Overall interactions after day six (all groups on experimental feeding regimes)

Source of Variation	DF	SS	MS	F	P
ID	3	0.0946	0.0315	7.841	0.004
mouse(ID)	12	0.0483	0.00402		
day	9	0.0523	0.00582	14.977	<0.001
ID x day	27	0.0229	0.000849	2.187	0.002

C. Day by day interactions

Comparisons for factor: Group within day 7

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. Early Night HFD w/reg.	0.0636	3.279	0.013	Yes
HFD ad lib. vs. Early Night HFD w/reg.	0.0337	1.739	0.376	No
Late Night HFD w/reg. vs. Early Night HFD w/reg.	0.033	1.704	0.333	No
Reg. ad lib. vs. Late Night HFD w/reg.	0.0305	1.575	0.327	No
Reg. ad lib. vs. HFD ad lib.	0.0299	1.54	0.246	No
HFD ad lib. vs. Late Night HFD w/reg.	0.000672	0.0347	0.973	No

Comparisons for factor: Group within day 8

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.0622	3.21	0.016	Yes
Reg. ad lib. vs. Early Night HFD w/reg.	0.0502	2.589	0.066	No
Late Night HFD w/reg. vs. HFD ad lib.	0.0316	1.629	0.377	No
Reg. ad lib. vs. Late Night HFD w/reg.	0.0307	1.581	0.323	No
Late Night HFD w/reg. vs. Early Night HFD w/reg.	0.0195	1.008	0.537	No
Early Night HFD w/reg. vs. HFD ad lib.	0.012	0.621	0.538	No

Comparisons for factor: Group within day 9

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.111	5.748	<0.001	Yes
Late Night HFD w/reg. vs. HFD ad lib.	0.0764	3.94	0.002	Yes
Reg. ad lib. vs. Early Night HFD w/reg.	0.0688	3.551	0.004	Yes
Early Night HFD w/reg. vs. HFD ad lib.	0.0426	2.197	0.099	No
Reg. ad lib. vs. Late Night HFD w/reg.	0.0351	1.808	0.151	No

Late Night HFD w/reg. vs. Early Night HFD w/reg.	0.0338	1.743	0.089	No

Comparisons for factor: Group within day 10

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.0862	4.444	<0.001	Yes
Reg. ad lib. vs. Early Night HFD w/reg.	0.0858	4.426	<0.001	Yes
Reg. ad lib. vs. Late Night HFD w/reg.	0.0638	3.288	0.009	Yes
Late Night HFD w/reg. vs. HFD ad lib.	0.0224	1.156	0.586	No
Late Night HFD w/reg. vs. Early Night HFD w/reg.	0.0221	1.138	0.455	No
Early Night HFD w/reg. vs. HFD ad lib.	0.000345	0.0178	0.986	No

Comparisons for factor: Group within day 11

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.0711	3.665	0.004	Yes
Reg. ad lib. vs. Early Night HFD w/reg.	0.0617	3.181	0.014	Yes
Reg. ad lib. vs. Late Night HFD w/reg.	0.0465	2.397	0.083	No
Late Night HFD w/reg. vs. HFD ad lib.	0.0246	1.268	0.511	No
Late Night HFD w/reg. vs. Early Night HFD w/reg.	0.0152	0.784	0.684	No
Early Night HFD w/reg. vs. HFD ad lib.	0.00938	0.484	0.631	No

Comparisons for factor: Group within day 12

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. Early Night HFD w/reg.	0.0572	2.95	0.032	Yes
Reg. ad lib. vs. HFD ad lib.	0.0454	2.344	0.116	No
Reg. ad lib. vs. Late Night HFD w/reg.	0.0357	1.843	0.261	No
Late Night HFD w/reg. vs. Early Night HFD w/reg.	0.0215	1.106	0.62	No
HFD ad lib. vs. Early Night HFD w/reg.	0.0117	0.606	0.796	No
Late Night HFD w/reg. vs. HFD ad lib.	0.00971	0.501	0.619	No

Comparisons for factor: Group within day 13

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.0683	3.524	0.007	Yes
Reg. ad lib. vs. Early Night HFD w/reg.	0.0617	3.18	0.014	Yes
Reg. ad lib. vs. Late Night HFD w/reg.	0.0587	3.03	0.017	Yes
Late Night HFD w/reg. vs. HFD ad lib.	0.00959	0.495	0.947	No
Early Night HFD w/reg. vs. HFD ad lib.	0.00667	0.344	0.929	No

Late Night HFD w/reg. vs. Early Night HFD w/reg.	0.00292	0.151	0.881	No

Comparisons for factor: Group within day 14

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. Early Night HFD w/reg.	0.0435	2.242	0.171	No
Reg. ad lib. vs. Late Night HFD w/reg.	0.0402	2.073	0.205	No
Reg. ad lib. vs. HFD ad lib.	0.04	2.064	0.171	No
HFD ad lib. vs. Early Night HFD w/reg.	0.00346	0.178	0.997	No
Late Night HFD w/reg. vs. Early Night HFD w/reg.	0.00328	0.169	0.982	No
HFD ad lib. vs. Late Night HFD w/reg.	0.000182	0.00941	0.993	No

Comparisons for factor: Group within day 15

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. Late Night HFD w/reg.	0.0637	3.288	0.013	Yes
Reg. ad lib. vs. Early Night HFD w/reg.	0.0408	2.103	0.193	No
HFD ad lib. vs. Late Night HFD w/reg.	0.035	1.806	0.28	No
Reg. ad lib. vs. HFD ad lib.	0.0287	1.482	0.378	No
Early Night HFD w/reg. vs. Late Night HFD w/reg.	0.023	1.185	0.427	No
HFD ad lib. vs. Early Night HFD w/reg.	0.012	0.621	0.538	No

Comparisons for factor: Group within day 16

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.0564	2.91	0.035	Yes
Reg. ad lib. vs. Late Night HFD w/reg.	0.0542	2.796	0.039	Yes
Reg. ad lib. vs. Early Night HFD w/reg.	0.0502	2.591	0.053	No
Early Night HFD w/reg. vs. HFD ad lib.	0.00618	0.319	0.985	No
Early Night HFD w/reg. vs. Late Night HFD w/reg.	0.00399	0.206	0.974	No
Late Night HFD w/reg. vs. HFD ad lib.	0.0022	0.113	0.91	No

Supplementary Table S2.6. Two-way repeated measures ANOVA for carbohydrate oxidation.

A. Overall interactions during first six days (all groups on Reg. chow *ad libitum*)

Source of Variation	DF	SS	MS	F	P
ID	3	0.000356	0.000119	0.705	0.567
mouse(ID)	12	0.00202	0.000168		
days	5	0.000761	0.000152	5.602	<0.001
ID x days	15	0.000484	3.23E-05	1.188	0.306

B. Overall interactions after day six (all groups on experimental feeding regimes)

Source of Variation	DF	SS	MS	F	P
ID	3	0.011	0.00366	8.699	0.002
mouse(ID)	12	0.00505	0.000421		
days	9	0.0051	0.000566	10.138	<0.001
ID x days	27	0.00347	0.000128	2.299	0.001

C. Day by day interactions

Comparisons for factor: Group within day 7

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. Early Night HFD w/Reg.	0.0195	2.872	0.035	Yes
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.0142	2.092	0.191	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.012	1.761	0.297	No
Reg. ad lib. vs. HFD ad lib.	0.00755	1.111	0.614	No
Reg. ad lib. vs. Late Night HFD w/Reg.	0.0053	0.78	0.686	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.00225	0.331	0.742	No

Comparisons for factor: Group within day 8

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.0203	2.994	0.025	Yes
Reg. ad lib. vs. Early Night HFD w/Reg.	0.016	2.357	0.107	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.0137	2.013	0.184	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.00935	1.376	0.438	No
Reg. ad lib. vs. Late Night HFD w/Reg.	0.00666	0.981	0.553	No
Early Night HFD w/Reg. vs. HFD ad lib.	0.00433	0.637	0.527	No

Comparisons for factor: Group within day 9

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.037	5.444	<0.001	Yes

Late Night HFD w/Reg. vs. HFD ad lib.	0.0324	4.769	<0.001	Yes
Early Night HFD w/Reg. vs. HFD ad lib.	0.0186	2.74	0.034	Yes
Reg. ad lib. vs. Early Night HFD w/Reg.	0.0184	2.703	0.028	Yes
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.0138	2.029	0.093	No
Reg. ad lib. vs. Late Night HFD w/Reg.	0.00458	0.675	0.503	No

Comparisons for factor: Group within day 10

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Reg. ad lib. vs. HFD ad lib.	0.0311	4.576	<0.001	Yes
Reg. ad lib. vs. Early Night HFD w/Reg.	0.0281	4.137	<0.001	Yes
Reg. ad lib. vs. Late Night HFD w/Reg.	0.0191	2.805	0.028	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	0.012	1.771	0.228	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.00905	1.332	0.342	No
Early Night HFD w/Reg. vs. HFD ad lib.	0.00298	0.439	0.663	No

Comparisons for factor: Group within day 11

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Reg. ad lib. vs. HFD ad lib.	0.0265	3.893	0.002	Yes
Reg. ad lib. vs. Early Night HFD w/Reg.	0.0216	3.177	0.013	Yes
Reg. ad lib. vs. Late Night HFD w/Reg.	0.0142	2.084	0.159	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.0123	1.809	0.213	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.00742	1.092	0.482	No
Early Night HFD w/Reg. vs. HFD ad lib.	0.00487	0.716	0.477	No

Comparisons for factor: Group within day 12

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Reg. ad lib. vs. Early Night HFD w/Reg.	0.0204	3.003	0.025	Yes
Reg. ad lib. vs. HFD ad lib.	0.0166	2.45	0.086	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.0108	1.586	0.397	No
Reg. ad lib. vs. Late Night HFD w/Reg.	0.00963	1.417	0.413	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.00702	1.033	0.519	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.00376	0.553	0.583	No

Comparisons for factor: Group within day 13

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Reg. ad lib. vs. HFD ad lib.	0.0245	3.61	0.004	Yes

Reg. ad lib. vs. Early Night HFD w/Reg.	0.0206	3.034	0.019	Yes
Reg. ad lib. vs. Late Night HFD w/Reg.	0.0178	2.617	0.046	Yes
Late Night HFD w/Reg. vs. HFD ad lib.	0.00675	0.993	0.693	No
Early Night HFD w/Reg. vs. HFD ad lib.	0.00391	0.576	0.813	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.00283	0.417	0.679	No

Comparisons for factor: Group within day 14

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. Early Night HFD w/Reg.	0.0154	2.272	0.154	No
Reg. ad lib. vs. HFD ad lib.	0.0144	2.123	0.179	No
Reg. ad lib. vs. Late Night HFD w/Reg.	0.012	1.772	0.291	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.0034	0.5	0.945	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.00239	0.351	0.925	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.00101	0.149	0.883	No

Comparisons for factor: Group within day 15

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. Late Night HFD w/Reg.	0.0216	3.172	0.015	Yes
Reg. ad lib. vs. Early Night HFD w/Reg.	0.0157	2.305	0.121	No
HFD ad lib. vs. Late Night HFD w/Reg.	0.0128	1.877	0.24	No
Reg. ad lib. vs. HFD ad lib.	0.0088	1.295	0.49	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.00686	1.01	0.534	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	0.00589	0.867	0.39	No

Comparisons for factor: Group within day 16

Comparison	Diff of Means	t	P	P<0.050
Reg. ad lib. vs. HFD ad lib.	0.023	3.379	0.008	Yes
Reg. ad lib. vs. Late Night HFD w/Reg.	0.0203	2.993	0.021	Yes
Reg. ad lib. vs. Early Night HFD w/Reg.	0.0195	2.863	0.024	Yes
Early Night HFD w/Reg. vs. HFD ad lib.	0.00351	0.516	0.94	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.00262	0.386	0.911	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	0.000883	0.13	0.897	No

Supplementary Table S2.7. Two-way repeated measures ANOVA for lipid oxidation

A. Overall interactions during the first six days (all groups on Reg. *ad libitum*)

Source of Variation	DF	SS	MS	F	P
ID	3	4.34E-05	1.45E-05	2.027	0.164
mouse(ID)	12	8.56E-05	7.13E-06		
days	5	0.000123	2.46E-05	9.359	<0.001
ID x days	15	5.91E-05	3.94E-06	1.497	0.136

B. Overall interactions after day six (all groups on experimental feeding regimes)

Source of Variation	DF	SS	MS	F	P
ID	3	0.00236	0.000787	6.217	0.009
mouse(ID)	12	0.00152	0.000127		
days	9	0.000929	0.000103	15.891	<0.001
ID x days	27	0.000496	1.84E-05	2.831	<0.001

C. Day by day interactions

Comparisons for factor: Group within day 7

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. Reg. ad lib.	0.00996	3.274	0.018	Yes
HFD ad lib. vs. Reg. ad lib.	0.00674	2.217	0.167	No
Late Night HFD w/Reg. vs. Reg. ad lib.	0.00538	1.768	0.312	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	0.00458	1.507	0.374	No
Early Night HFD w/Reg. vs. HFD ad lib.	0.00322	1.057	0.511	No
HFD ad lib. vs. Late Night HFD w/Reg.	0.00137	0.45	0.657	No

Comparisons for factor: Group within day 8

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Reg. ad lib.	0.0122	4.005	0.003	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	0.00791	2.602	0.074	No
Late Night HFD w/Reg. vs. Reg. ad lib.	0.00625	2.056	0.187	No
HFD ad lib. vs. Late Night HFD w/Reg.	0.00593	1.949	0.176	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.00427	1.403	0.316	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	0.00166	0.546	0.59	No

Comparisons for factor: Group within day 9

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Reg. ad lib.	0.0172	5.664	<0.001	Yes
HFD ad lib. vs. Late Night HFD w/Reg.	0.0117	3.843	0.004	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	0.0094	3.09	0.019	Yes
HFD ad lib. vs. Early Night HFD w/Reg.	0.00783	2.574	0.048	Yes
Late Night HFD w/Reg. vs. Reg. ad lib.	0.00554	1.822	0.154	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	0.00386	1.269	0.216	No

Comparisons for factor: Group within day 10

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Reg. ad lib.	0.0133	4.366	0.001	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	0.0127	4.162	0.002	Yes
Late Night HFD w/Reg. vs. Reg. ad lib.	0.0104	3.421	0.009	Yes
HFD ad lib. vs. Late Night HFD w/Reg.	0.00287	0.945	0.73	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	0.00225	0.74	0.715	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.000621	0.204	0.84	No

Comparisons for factor: Group within day 11

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Reg. ad lib.	0.0107	3.534	0.01	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	0.00928	3.051	0.026	Yes
Late Night HFD w/Reg. vs. Reg. ad lib.	0.0081	2.664	0.052	No
HFD ad lib. vs. Late Night HFD w/Reg.	0.00265	0.87	0.776	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.00147	0.483	0.865	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	0.00118	0.387	0.702	No

Comparisons for factor: Group within day 12

Comparison	Diff of Means	t	P	P<0.050
Early Night HFD w/Reg. vs. Reg. ad lib.	0.00845	2.779	0.06	No
HFD ad lib. vs. Reg. ad lib.	0.00715	2.351	0.127	No
Late Night HFD w/Reg. vs. Reg. ad lib.	0.00675	2.22	0.135	No

Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	0.0017	0.559	0.927	No
Early Night HFD w/Reg. vs. HFD ad lib.	0.0013	0.427	0.893	No
HFD ad lib. vs. Late Night HFD w/Reg.	0.000399	0.131	0.897	No

Comparisons for factor: Group within day 13

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
HFD ad lib. vs. Reg. ad lib.	0.00983	3.232	0.02	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	0.00859	2.824	0.045	Yes
Late Night HFD w/Reg. vs. Reg. ad lib.	0.00855	2.81	0.037	Yes
HFD ad lib. vs. Late Night HFD w/Reg.	0.00128	0.422	0.966	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.00124	0.408	0.902	No
Early Night HFD w/Reg. vs. Late Night HFD w/Reg.	4.22E-05	0.0139	0.989	No

Comparisons for factor: Group within day 14

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Late Night HFD w/Reg. vs. Reg. ad lib.	0.00661	2.172	0.215	No
Early Night HFD w/Reg. vs. Reg. ad lib.	0.00564	1.853	0.325	No
HFD ad lib. vs. Reg. ad lib.	0.00539	1.771	0.311	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.00122	0.401	0.971	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.000969	0.319	0.939	No
Early Night HFD w/Reg. vs. HFD ad lib.	0.000251	0.0825	0.935	No

Comparisons for factor: Group within day 15

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Late Night HFD w/Reg. vs. Reg. ad lib.	0.0103	3.372	0.014	Yes
HFD ad lib. vs. Reg. ad lib.	0.00622	2.045	0.232	No
Early Night HFD w/Reg. vs. Reg. ad lib.	0.00596	1.961	0.223	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.00429	1.412	0.429	No
Late Night HFD w/Reg. vs. HFD ad lib.	0.00404	1.327	0.354	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.000257	0.0845	0.933	No

Comparisons for factor: Group within day
16

Comparison	Diff of Means	t	P	P<0.050
HFD ad lib. vs. Reg. ad lib.	0.00959	3.152	0.025	Yes
Late Night HFD w/Reg. vs. Reg. ad lib.	0.00901	2.962	0.033	Yes
Early Night HFD w/Reg. vs. Reg. ad lib.	0.00775	2.549	0.067	No
HFD ad lib. vs. Early Night HFD w/Reg.	0.00183	0.603	0.91	No
Late Night HFD w/Reg. vs. Early Night HFD w/Reg.	0.00126	0.413	0.9	No
HFD ad lib. vs. Late Night HFD w/Reg.	0.000576	0.189	0.851	No

CHAPTER III

Corticosterone response to shifting High Fat Diet in fasted and unfasted mice

Abstract

While alteration of metabolic circadian rhythms likely is a major contributor to differences in weight gain between mice on different time-restricted feeding schedules, there is a possibility that a corticosterone response also contributes to weight gain. Corticosterone could confound interpretation of the results as a clock effect as the corticosterone response has a known on weight gain/loss in mammals. I hypothesized that shifts in feeding time in mice subjected to time-restricted diet would cause a corticosterone response that leads to a short-term hormonal imbalance that can result in weight gain. Through the use of a commercially available ELISA kit, I was able to assess corticosterone levels in my C57/BL mice model under different feeding regimes. More specifically, I placed mice into different groups, each with a different feeding protocol, to assess corticosterone levels in feeding paradigms that have varying times of HFD access and either had no regular chow (RC) or *ad libitum* access to RC. I measured corticosterone levels of mice before and after their respective feeding paradigms. To also account for a potential shift in corticosterone rhythms, a 24-hour assessment of stress levels was carried out for all feeding groups. I hypothesized that the corticosterone/stress levels would be reduced in the mice with unrestricted access to food but increased in mice that were fasted for 18 hours. Also, HFD feeding shifted to an irregular time window might also elevate corticosterone levels. The corticosterone levels will serve as a marker

to determine if any of our food restriction or unrestricted food access with limited HFD models are able to elicit a corticosterone response which could confound weight gain results caused by corticosterone's effect on metabolism as opposed to the effects on weight gain brought on by the timing and duration of feeding/fasting. The results show that the daily corticosterone rhythm is not phase shifted in any of the feeding groups and that corticosterone levels show rhythmic expression in all feeding groups. Surprisingly, I also found that there is no significant change over time in corticosterone among the different feeding regimes. In conclusion, the corticosterone response does not appear to be responsible for the changes in weight gain, energy expenditure, or carbohydrate/lipid oxidation seen in circadian meal timing studies with and without fasting.

Introduction

Corticosterone is a critical hormone for both circadian rhythms and metabolism. Corticosterone is a glucocorticoid which signals glucocorticoid receptors to trigger release of glucose into the blood stream and decrease fatty acid oxidation (Rose *et al.*, 2010). Schibler's group originally suspected it to be involved in synchronization of peripheral tissues due to the fact that glucocorticoid receptors are homogenously expressed in mammalian tissues with the exception of the suprachiasmatic nucleus where they are not present (Damiola *et al.*, 2000). Schibler and others have also noted that corticosterone is rhythmic in mammals and is at peak levels during the onset of activity and roughs at onset of sleep (Le Minh *et al.*, 2001). Since corticosterone is rhythmically regulated, it is logical that it would be sensitive to time dependent metabolic perturbations.

Previous research has shown that food restriction (aka fasting) can affect corticosterone, though the literature on this response was done in rats rather than mice. Adrenalectomized rats (the adrenal gland is where corticosterone/cortisol is produced) show decreased food intake and weight gain (Yukimura and Bray, 1978). In mice, this effect is more variable. Studies involving obesity strain show adrenalectomy decreases weight gain but causes no weight change in C57 mouse strains (Makimura *et al.*, 2003, Wittmers and Haller, 1983). Namvar *et al.* (2017), showed that in rats under a four-hour food restriction (or 20-hour fast) have elevated corticosterone at the time the meal is anticipated compared to rats under *ad libitum* feeding conditions. Fasting in mice can also cause a corticosterone response, and a wide number of studies have associated a corticosterone or stress response with alteration of weight in mice (Jeong *et al.*, 2013, Luque *et al.*, 2007, Makimura *et al.*, 2003, Mishima *et al.*, 2015). In particular, work in mice related to determining the effects of the food entrainable oscillator showed that ghrelin levels in fasted mice causes an elevation of corticotrophin releasing hormone (upstream regulator of corticosterone in the HPA axis) and neuropeptide Y (linked with corticosterone response and activated by glucocorticoids such as corticosterone) (Luque *et al.*, 2007). Furthermore, elevation in corticosterone in mice has been associated with elevation in core body temperature using a social defeat stress model (Keeney *et al.*, 2001).

With this in mind, it is not a large stretch of the imagination to consider that effects seen in previous research such as in Arble *et al.* (2009) or Hatori *et al.* (2012) were due in large part to the result of a corticosterone response rather than a timing-of-feeding response. Taken together, an elevation of corticosterone response may cause a change

in energy expenditure that could explain the changes in weight gain in a feeding duration dependent model such as that used by Hatori *et al.* (2012). Furthermore, corticosterone's effect on glucose release could also explain changes seen in RER in circadian meal timing studies caused by an increase in carbohydrate oxidation due to increased glucose release in the blood stream and explain changes in insulin sensitivity and glucose tolerance seen in human meal timing studies (Rose *et al.*, 2010, van der Kooij *et al.*, 2018, Karatsoreos *et al.*, 2010). Despite the likely possibility that studies involving circadian meal timing may affect corticosterone, few circadian meal timing studies have attempted to measure corticosterone, corticotrophin releasing hormone, or neuropeptide Y levels (Wehrens *et al.*, 2017).

In this study, we measure corticosterone levels in mice given HFD at the onset of the active phase or near the end of the active phase, with or without an 18-hour fast. The purpose of this study was to determine if corticosterone is elevated in mice under protocols involving time restricted feeding or in protocols that alter the onset/offset of meal timing. We find that the daily rhythmicity of corticosterone was not changed in response to time restricted feeding or to onset/offset of meal timing. We also find no short-term or long-term corticosterone response over the course of 14 days, using either time restricted feeding protocols or onset/offset of meal timing protocols. Our data shows that differences in energy expenditure/weight gain between time restricted feeding protocols and onset/offset of meal timing protocols is not likely to be due to a corticosterone response.

Methods

Circadian corticosterone response

Eleven-week-old mice were entrained on a 12:12 Light:Dark (LD) cycle for one week. Following entrainment, mice were put on one of several different feeding protocols: Regular chow ad. libitum, HFD ad. libitum, HFD early, HFD late, HFD early with regular chow ad. libitum, or HFD late with regular chow ad. libitum (Figure 3.1. panel 2). Mice in the HFD early group were given HFD chow during the first six hours of the night phase. Mice in the HFD late group were given HFD chow during the last six hours of the night phase. Mice underwent this feeding protocol for 14 days. At the end of 14 days, mice were sacrificed at zeitgeber times 0, 6, 12, 18, and 24 (n=5 per zeitgeber time per feeding protocol) and had their trunk blood collected. To avoid artificial corticosterone elevation due to stress of the euthanasia, mice were handled and sacrificed in under two minutes. Mice were brought to the sacrifice room from the light control room by one "clean" individual, and the sacrificing and tissue collection was performed by another individual. All mice were sacrificed within the hour of the specified ZT time.

Short versus long-term corticosterone response protocol

Eleven-week-old mice were entrained on a 12:12 LD cycle for one week. Following entrainment, mice were put on one of several different feeding protocols: Regular chow ad. libitum, HFD ad. libitum, HFD early, HFD late, HFD early with regular chow ad. libitum, or HFD late with regular chow ad. libitum. Mice in the HFD early group were given HFD chow during the first six hours of the night phase. Mice in the HFD late group were given HFD chow during the last six hours of the night phase. Mice were sacrificed at days 0, 3, 7, and 14 of their respective diets via cervical dislocation and had their trunk blood

collected. Trunk blood was immediately centrifuged to isolate and collect serum. Mice were sacrificed in the same method as indicated above and all mice were sacrificed within the hour of ZT 12.

Corticosterone assay

Serum was collected from mouse trunk blood. Corticosterone levels in serum were measured using a commercially available Corticosterone ELISA assay provided by ENZO Life Sciences. Samples were compared with a standard curve of known corticosterone concentrations provided in the kit.

Statistical analysis

Statistical significance between groups was determined using two-way ANOVA through Sigmaplot 13.

Results

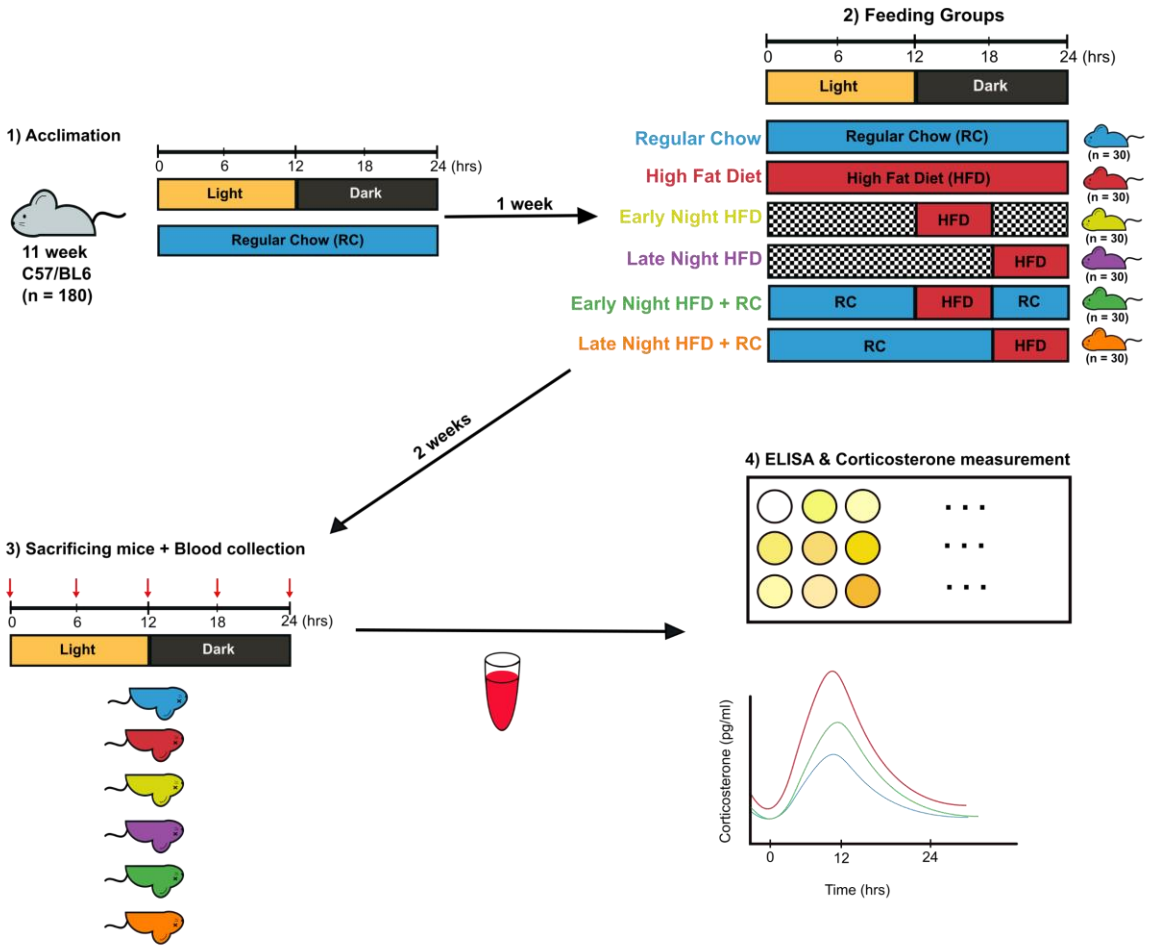

Figure 3.1. Schematic for analysis of corticosterone rhythms in feeding groups.

Diagram of method to analyze corticosterone over a 24-hour period for each feeding group. Briefly, 180 11 week old mice were first put on RC *ad libitum* for one week, then put on either RC *ad libitum* (blue), HFD *ad libitum* (red), HFD access for the first six hours of the night phase (referred to as Early, yellow), HFD access for the last six hours of the night (referred to as Late, purple), Early HFD with RC *ad libitum* (green), or Late HFD with RC *ad libitum* (orange). After14 days on the feeding protocol, mice were sacked every six hours over a 24-hour period and trunk blood was extracted for corticosterone analysis through an ELISA assay.

Because the results from the previous chapter were largely caused by short-term effects within the first 4 days on the experimental feeding protocol, I hypothesized

that weight gain effects could also be due to a short-term corticosterone response. Also, other previous literature that involved longer fasting times reported an increase in energy expenditure as the cause of the difference in weight gain (Keeney *et al.*, 2001, Poggioli *et al.*, 2013, Arlettaz *et al.*, 2008). Therefore, I further hypothesized that the cause of the weight gain differences in mice with RC *ad libitum* would be due to changes in timing of HFD presentation, while the weight gain effects for mice without RC *ad libitum* and a restricted HFD were caused largely by energy expenditure increase, possibly due to a change in corticosterone levels. To confirm this hypothesis, we first needed to confirm that the endogenous corticosterone rhythms were not altered by the feeding regime. To do this, we analyzed corticosterone over a 24-hour period for all feeding groups after 2 weeks on the experimental diets (Figure 3.1). Briefly, I acclimated 11-week-old mice to a 12:12 LD cycle on RC *ad libitum* for one week and then sorted them into one of the following feeding groups: RC *ad libitum*, HFD *ad libitum*, HFD during the first 6 hours of the lights off period (denoted as HFD Early), HFD during the last 6 hours of the lights off period (denoted as HFD Late), HFD Early with RC *ad libitum* supplemented (denoted as HFD Early with RC), and HFD Late with RC *ad libitum* supplemented (denoted as HFD Late with RC). Following 14 days of treatment, mice were then sacrificed for trunk blood collection at ZT times 0, 6, 12, 18, and 24 for each group (n=5).

Figure 3.2 shows the average and individual levels of corticosterone for each group over zeitgeber time (time relative to light cycle). We found that for each group, there was a consistent corticosterone rhythm over the 24-hour period, with corticosterone levels peaking on average for all groups at ZT12 and troughing at ZT0/24. Indeed, in a two-way ANOVA analysis of the data, we find that time is a very significant factor on corticosterone

levels (P-value<0.001, see table S3.1). This is consistent with previous literature that shows corticosterone under *ad libitum* feeding conditions peaks during the onset of activity in mice (ZT 12) and troughs during the wake to sleep transition (ZT0) (Le Minh *et al.*, 2001). However, no interaction effect related to time and group was observed (P-value=0.835, Table S3.1). Surprisingly, there was also no significant difference between treatments in this experiment. These data suggest that corticosterone expression does not shift with HFD access and that a longer fast does not affect corticosterone levels significantly compared to mice without a fast.

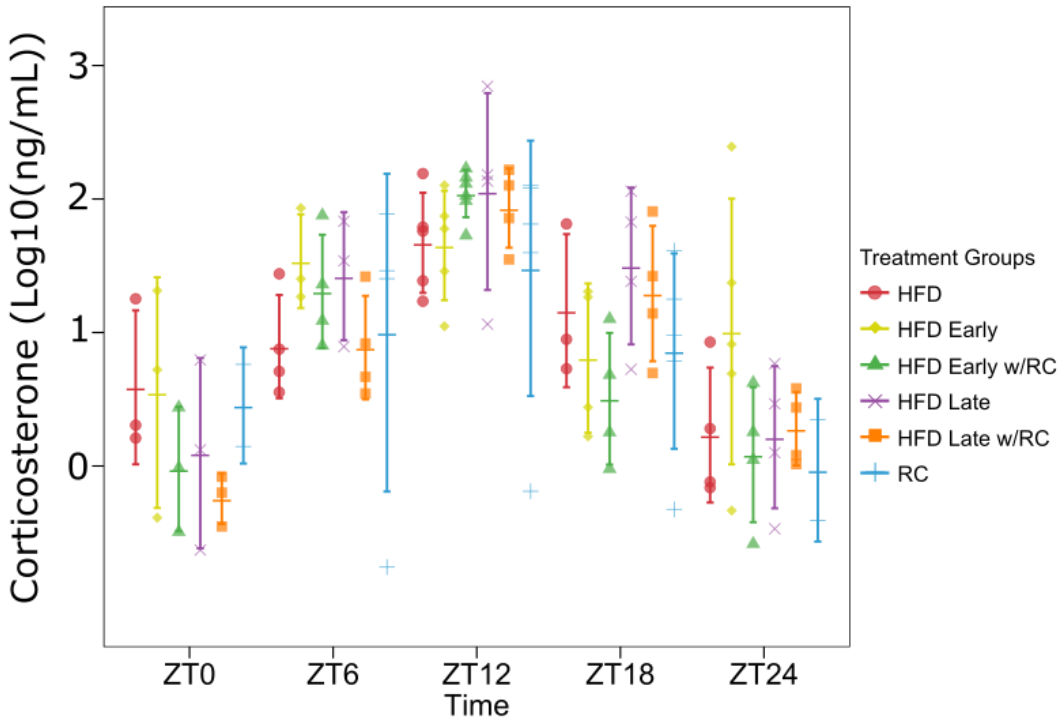

Figure 3.2. Corticosterone rhythms are maintained in feeding groups.
Corticosterone levels over 24 hours (listed as ZT or zeitgeber time, with ZT0 indicating the start of the lights on period in a 12:12 L:D cycle) for each feeding group on day 14. Feeding groups were HFD (red), HFD early (yellow), HFD Early with RC *ad libitum* (orange), HFD late (purple), HFD late with RC *ad libitum* (green), and RC *ad libitum* (blue). Bars indicated the average corticosterone levels for the feeding group. Circles indicate individual corticosterone levels and error bars indicate the standard deviation. Due to the large variation in corticosterone levels, values were log transformed. For reference Log10

0=1ng/mL, 1=10ng/mL, 2=100ng/mL, and 3=1000ng/mL of corticosterone respectively. See supplementary table S3.1 for detailed analysis.

Having found that the rhythm of corticosterone expression was not affected on Day 14 by the experimental feeding regimes, we then sought to test the hypothesis that corticosterone levels might be altered in the first few days after the transition to the various feeding regimens, but "adapts" so that there are no differences on Day 14. The experimental protocol for analyzing the effect of feeding regimes on corticosterone is detailed in Figure 3.3. Briefly, based on the values from the 24-hour experiment (Fig. 3.2), the largest variation in average corticosterone values between groups was observed at ZT12. We therefore assumed that if any effects were to be observed, they would be most obvious at this time point. I once again acclimated eleven-week-old mice to a 12:12 LD cycle for one week followed by two weeks on the various feeding regimes listed above. Over the two-week period, I sacrificed mice from each group at days 0 (day before mice were put on feeding regimes), 3, 7, and 14 for trunk blood to be used for ELISA analysis (n=5).

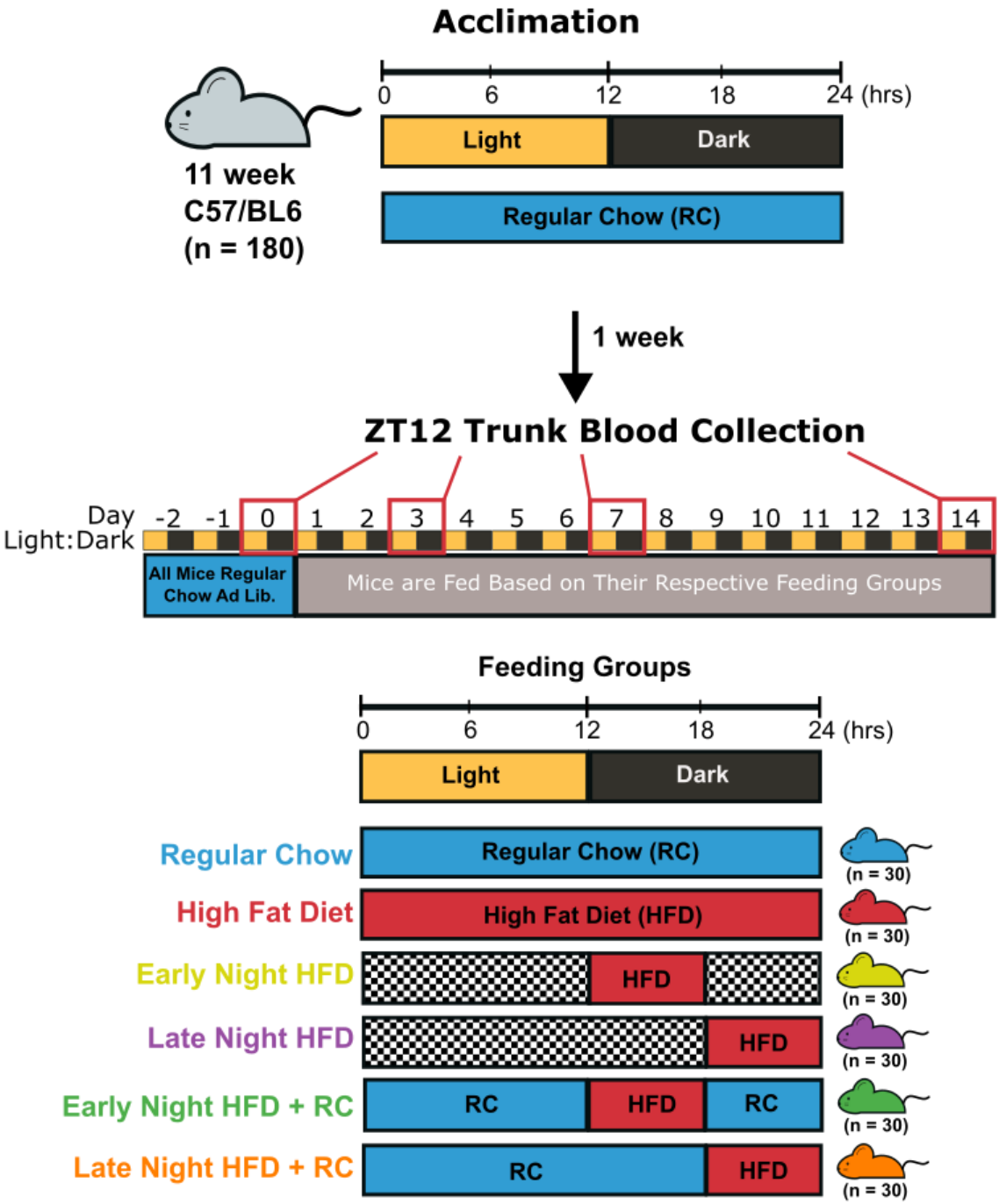

Figure 3.3. Schematic of serum collection for corticosterone analysis of feeding regimes over time. Diagram of method to analyze corticosterone over a 14-day period for each feeding group. Briefly, 180 11-week-old mice were first put on RC *ad libitum* for 1 week, then put on either RC *ad libitum* (blue), HFD *ad libitum* (red), HFD Early (yellow), HFD Late (purple), Early HFD with RC *ad libitum* (green), or Late HFD with RC *ad libitum* (orange). Five mice from each treatment group were sacrificed on days 0, 3, 7, and 14 at ZT12 for trunk blood collection. Trunk blood was extracted for corticosterone analysis through an ELISA assay.

I compared corticosterone levels to each other as well as day 0 (shown as a black line, Figure 3.4). Surprisingly, we once again found no interaction between day and feeding group with respect to corticosterone level (P-value= 0.989, Table S3.2). This was true even for Day 3, where the largest effects on metabolism were observed in the Early HFD and Late HFD with RC *ad libitum* as seen in the previous chapter. We did observe that the averages for the fasted groups (HFD Early and HFD Late) were higher than for the corresponding HFD Early and Late that had RC supplemented although this increase was not significantly different (Figure 3.4 and table S3.3). However, large variation between individual mice was apparent, as has been observed in other studies (Le Minh *et al.*, 2001). This experiment nonetheless shows that corticosterone is not affected either in the short-term or long-term by HFD access regardless of the fasting period length.

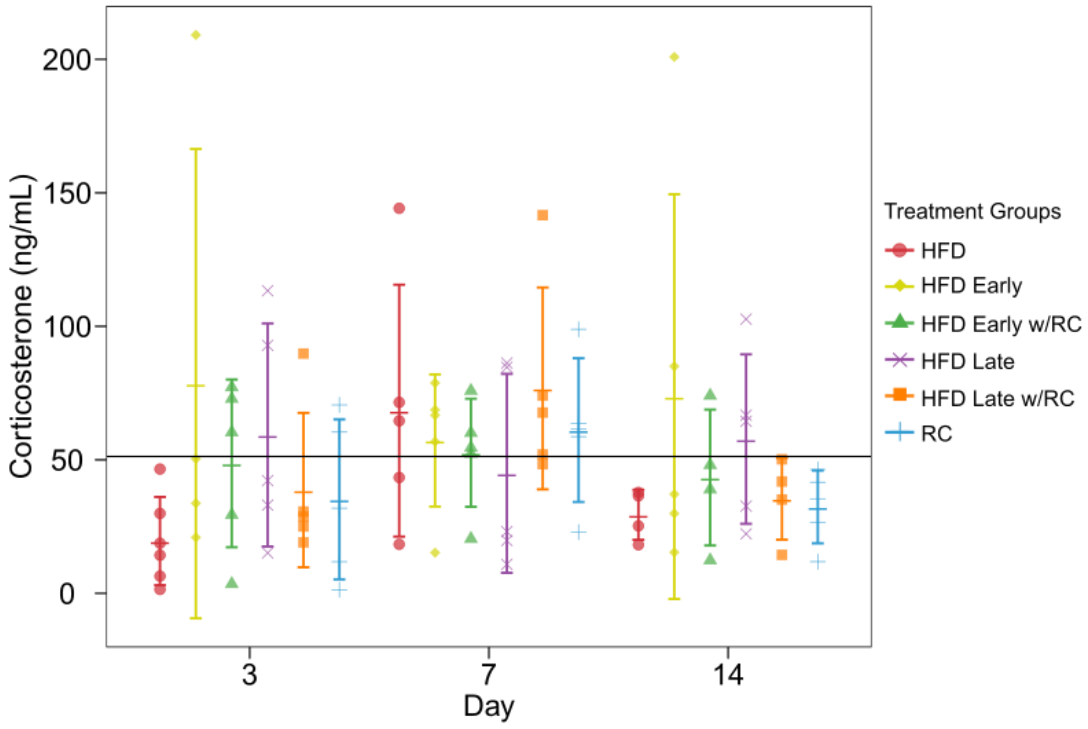

Figure 3.4. Corticosterone response is not changed over time in different feeding regimes. Average ZT12 corticosterone levels for all feeding groups on days 3, 7, and

14. Feeding groups are HFD (red), HFD Early (yellow), HFD Early with RC *ad libitum* (green), HFD Late (purple), HFD Late with RC *ad libitum* (orange), and RC (blue). Black line denotes the average corticosterone level of mice on D0 prior to mice being put on separate feeding groups. Bars indicate the average values for each feeding group, error bars denote the standard deviation, and circles indicate corticosterone levels of individual mice. See supplementary table S3.2 for detailed analysis.

Since there was no significant difference between the different days, I then combined the data to see if there was a significant variation overall between the feeding regimes (Figure 3.5). We found that on average, the Early HFD group showed the highest average corticosterone level compared to the other groups. However, in a one-way ANOVA analysis, none of the feeding groups showed a significant difference (P-value=0.448, S3. Table 3). Furthermore, with the exception of the Early HFD group, the remaining feeding groups were either the same or slightly lower than the day 0 average values (black line, Figure 3.5). From this, we can conclude that there was no meaningful variation in corticosterone levels among feeding groups.

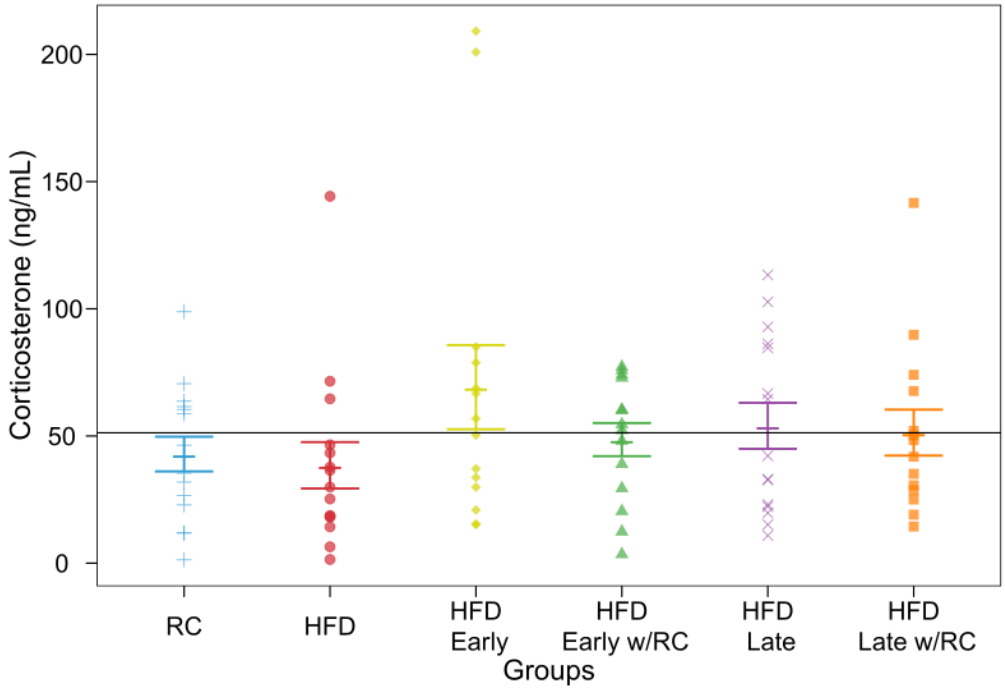

Figure 3.5. No effect of corticosterone on group. Corticosterone levels of mice based on feeding group at ZT12 (n=15). Feeding groups are HFD (red), HFD Early

(yellow), HFD Early with RC *ad libitum* (green), HFD Late (purple), HFD Late with RC *ad libitum* (orange), and RC (blue). Black line denotes the average corticosterone level of mice on D0 prior to mice being separated into feeding groups. Bars indicate average corticosterone level, error bars indicate the standard error of the mean, and circles indicate the corticosterone level of individual mice. See supplemental table S3.3 for detailed analysis.

Discussion

Recent studies observing the effect of circadian meal timing have reported that feeding duration is the primary cause of HFD weight gain resistance due to elevated energy expenditure in mice under a longer fast (Hatori *et al.*, 2012). However, several of these studies not only affect feeding duration but the onset and offset of feeding time (Hatori *et al.*, 2012, Gill and Panda, 2015, Nas *et al.*, 2017). Under these feeding paradigms, the effect on the corticosterone response has been largely unstudied despite the fact that corticosterone is a rhythmically regulated hormone, is known to respond to fasting in mice, and can regulate metabolism and weight gain in ways that are similar to what has been found in these feeding paradigms (Wright *et al.*, 2015, Luque *et al.*, 2007, Namvar *et al.*, 2016, Yukimura and Bray, 1978). Our study aimed to determine if there were any effects on corticosterone in circadian meal timing protocols that included a longer fasting duration that could explain the weight gain changes seen in other studies. We also compared these mice with the corticosterone response of mice under our circadian meal timing protocol that altered the onset/offset of the majority of feeding without affecting fasting duration to address whether fasting time was the only factor that influences the corticosterone response in these circadian meal timing protocols.

We found that corticosterone remained rhythmic and maintained the same peak and troughs in all feeding regimes, with or without an extended fast. These data suggest that corticosterone rhythms entrained to the LD cycle are maintained rather than being

influenced by the feeding cycle. It is worth mentioning however that in this study, we kept shifts in HFD presentation within the normal active period of mice. It remains to be seen if other more drastic protocols that restrict HFD feeding to the inactive phase cause a shift in corticosterone rhythms.

In our other experiments, we show that corticosterone levels remain similar to Day 0 levels across 14 days on the HFD feeding protocols, with or without an extended fasting period. This result was unexpected as corticosterone is known to elevate with fasting. We did observe on average higher levels of corticosterone between the extended fasting mice (Early HFD and Late HFD) compared to their non fasted counterparts (Early HFD with RC and Late HFD with RC), however this difference was not significant. This experiment also shows the effects observed in the previous chapter are not due to corticosterone altering metabolism as we would expect a large increase during day 3 followed by a decrease in corticosterone to day 0 levels by day 14.

From this study we cannot definitively conclude the involvement of corticosterone in the metabolic effects seen in circadian meal timing studies. This is critical to our understanding of the mechanism behind the weight gain effects seen in circadian meal timing studies, as corticosterone levels were a likely mechanism behind the metabolic effects seen in ours and others circadian meal timing studies. However, this also means that the effects on energy expenditure, carbohydrate/lipid oxidation, and weight gain caused by circadian meal timing still remain largely a mystery and further experimentation on other metabolic hormones and gene expression will be necessary to narrow down the metabolic pathways involved.

Acknowledgments

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Supplementary Information

Supplementary table S3.1. Two-way repeated measures ANOVA on 24-hour corticosterone rhythms after 14 days of treatment (figure 3.2).

Source of Variation	DF	SS	MS	F	P
Time	4	187333.5	46833.37	7.85	<0.001
Treatment	5	13576.85	2715.37	0.455	0.809
Time x Treatment	20	81254.13	4062.707	0.681	0.835

Supplementary table S3.2. Two-way repeated measures ANOVA of corticosterone response on feeding regimes for days 0, 3, 7, and 14.

Source of Variation	DF	SS	MS	F	P
Treatment	5	6698.264	1339.653	0.482	0.789
Time	3	1704.197	568.066	0.204	0.893
Treatment x Time	15	14415.93	961.062	0.346	0.989

Supplementary table S3.3. One-way ANOVA of cumulative corticosterone values of feeding regime at days 0, 3, 7, and 14.

Group	N	Missing	Median	25%	75%
Day 0 (baseline)	10	0	25.468	5.927	76.091
Reg. ad lib.	15	0	41.578	22.901	61.559
HFD ad lib.	15	0	29.922	18.17	46.529
Early Night HFD	14	0	53.585	27.644	80.355
Late Night HFD	15	0	42.206	22.276	86.252
Early Night HFD w/Reg.	14	0	53.427	27.156	73.162
Late Night HFD w/Reg.	14	0	45.124	27.79	69.268

H = 5.779 with 6 degrees of freedom.
(P = 0.448)

CHAPTER IV

Eating breakfast and avoiding the evening snack sustains lipid oxidation[1]

Abstract

Circadian (daily) regulation of metabolic pathways implies that food may be metabolized differentially over the daily cycle. To test that hypothesis, we monitored the metabolism of older subjects in a whole-room respiratory chamber over two separate 56-hour sessions, using a random crossover design. In one session, one of the three daily meals were presented as breakfast whereas in the other session, a nutritionally equivalent meal was presented as a late-evening snack. The duration of the overnight fast was the same for both sessions. Whereas the two sessions did not differ in overall energy expenditure, the respiratory exchange ratio (RER) was different during sleep between the two sessions. Unexpectedly, this difference in RER due to daily meal timing was not due to daily differences in physical activity, sleep disruption, or core body temperature. Rather, we found that the daily timing of nutrient availability coupled with daily/circadian control of metabolism drives a switch in substrate preference such that the late-evening snack session resulted in significantly lower lipid oxidation compared to the breakfast session. Therefore, the timing of meals during the day/night cycle affects how ingested food is oxidized or stored in humans and has important implications for optimal eating habits.

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Introduction

Developed countries are experiencing an epidemic of obesity that leads to many serious health problems, foremost among which are increasing rates of Type 2 diabetes, metabolic syndrome, cardiovascular disease, and cancer. While weight gain and obesity are primarily determined by diet and exercise, there is tremendous interest in the possibility that the daily timing of eating might have a significant affect upon weight management [Panda, 2018, Dashti *et al.*, 2019, Challet, 2019]. Many physiological processes display day/night rhythms, including feeding behavior, lipid and carbohydrate metabolism, body temperature, and sleep. These daily oscillations are controlled by the circadian clock, which is composed of an autoregulatory biochemical mechanism that is expressed in tissues throughout the body and is coordinated by a master pacemaker located in the suprachiasmatic nuclei of the brain (aka the SCN [Panda, 2018, Reppert and Weaver, 2002]). The circadian system globally controls gene expression patterns so that metabolic pathways are differentially regulated over the day, including switching between carbohydrate and lipid catabolism [Panda, 2018, Challet, 2019, Panda *et al.*, 2002, Wefers *et al.*, 2018, Bonham *et al.*, 2019, Zitting *et al.*, 2018, Resuehr *et al.*, 2019]. Therefore, ingestion of the same food at different times of day could lead to differential metabolic outcomes, e.g., lipid oxidation vs. accumulation; however, whether this is true or not is unclear.

Non-optimal phasing of the endogenous circadian system with the environmental day/night cycle has adverse health consequences. Shiftworkers are a particularly

cogent example because their work schedule disrupts the optimal relationship between the internal biological clock and the environmental daily cycle, and this disruption leads to well-documented health decrements [Wefers *et al.*, 2018, Resuehr *et al.*, 2019, Scheer *et al.*, 2009, McHill *et al.*, 2014, McHill *et al.*, 2019]. A contributing culprit that is often implicated in shiftwork's temporal disruption is the disturbance of eating patterns and preferences. In non-human mammals, a persuasive literature demonstrates that manipulating the timing of feeding relative to biological clock phase effectively controls obesity [Panda, 2018, Arble *et al.*, 2009, Hatori *et al.*, 2012]. In particular, mice fed a high-fat diet on a restricted schedule maintain a healthy weight when fed only during their active phase, but become obese if the high-fat diet is present during the inactive phase, even though the long-term caloric intake and locomotor activity levels are comparable between day- vs. night-fed mice [Arble *et al.*, 2009, Hatori *et al.*, 2012].

Can the timing of eating relative to our circadian cycle of metabolism and sleep also help to regulate lipid metabolism and body weight in humans? Eating late in the day is correlated with weight gain [Mchill *et al.*, 2017], and there is an oft-discussed debate whether skipping breakfast versus dinner reaps weight-control benefits [Challet, 2019, Nas *et al.*, 2017]. While many factors can influence the timing of eating in everyday life [Dashti *et al.*, 2019, Challet, 2019, Gill and Panda, 2015, Ravussin *et al.*, 2019] we decided to take an experimental approach to test the metabolic consequences of a straightforward exchange of equivalent nutritional intake between early morning (8 am) and bedtime (10 pm). While it is not feasible to do the 12-h reversal of feeding time that was tested with mice [Arble *et al.*, 2009] because it would disrupt the consolidated sleep

episode of humans, we focused upon a 4.5-hour shift of feeding where human subjects ate either a ~700 kcal breakfast or an equivalent ~700 kcal late-evening snack. Not only are these two feeding schedules experimentally tractable for a human study, they are also commonly practiced by humans in everyday life (i.e., "skipping breakfast" and/or "late-evening snacking"). For each feeding schedule, we monitored the metabolism of our subjects for a 56-hour period in a whole-room calorimetry chamber to continuously measure their metabolic rate, respiratory exchange ratio (RER), carbohydrate oxidation, and lipid oxidation.

Previous studies of human metabolism for shorter monitoring periods (~24 hours) suggested that overall 24-h energy expenditure was not significantly affected by either breakfast skipping or a late dinner [Sato *et al.*, 2011, Kobayashi *et al.*, 2014]); however, those prior studies were performed on healthy Asian young adults of optimal BMI (18.5-25 kg/m2) for only ~24 h, which is inadequate to study a phenomenon based on circadian rhythmicity. Moreover, while differences in blood glucose levels were reported in those studies between breakfast-skipping or late-dinner sessions, lipid oxidation was either not affected (breakfast-skipping [Kobayashi *et al.*, 2014]) or counter-intuitively enhanced (late-dinner [Sato *et al.*, 2011]). In this investigation, we monitored older Caucasian adults (aged 50 or above) of varying BMI because we reasoned they are more representative of populations at-risk for metabolic disorders in many developed countries than are young and healthy adults. Each subject underwent two 56-h (2.5 d) sessions in a whole-room respiratory chamber. Using a randomized crossover design, we compared the energy expenditure (metabolic rate) and RER of each subject when

given a scheduled breakfast, lunch, and dinner (referred to as Breakfast Session) versus when they were given a lunch, dinner, and a late-evening snack (referred to as Snack Session).

While overall 24-hour energy expenditure was similar in this group of older subjects, RER was significantly different between the two sessions. We anticipated that daily differences in physical activity, sleep disruption, or core body temperature might lead to differential metabolism as reflected in the RER. Unexpectedly, however, our data demonstrated that even though the total daily energy and nutrient intake was equivalent between the sessions, switching the daily timing of a nutritionally equivalent 700-kcal meal from a "breakfast" to a "late-evening snack" had a significant effect upon carbohydrate and lipid metabolism such that nocturnal carbohydrate oxidation was favored at the expense of lipid oxidation when subjects ate the 700-kcal meal as a late-evening snack. Therefore, the daily cycle of metabolism and nutrient availability switches substrate preference so that the cumulative net lipid oxidation is altered by the timing of meals.

Methods

Subjects

Six Subjects (four Males and two Females) were first recruited through flyers and the Vanderbilt Kennedy Center. Subjects were between 51-63 (average age was 57) with Body Mass Indexes (BMIs) between 22.2 and 33.4 (Table 4.1.). Applicants had to be 50

years of age or older, have no serious health complications or medications that could affect metabolism. Female subjects were not required to be post-menopausal for inclusion in this study, but because of the age requirement, all females recruited to the study were post-menopausal. Subjects had no prior shiftwork experience. The study protocol was approved by the Institutional Review Board of Vanderbilt University's Human Research Protections Program (Approval number: 140536). The study was registered on clinicaltrials.gov as NCT04144426. Prior to the study, each subject signed an informed and written consent.

ID	Sex	Age [years]	Weight [kg]	Height [cm]	BMI	Self-Report Bedtime	Self-Report Wake time	1st Session Meal Plan
1	M	61	106.8	187.96	30.2	23:00	7:00	Breakfast
2	M	58	101.36	177.8	32.1	22:30	6:00	Snack
3	M	51	73.9	184	21.8	23:15	5:30	Snack
4	F	57	68.2	173	22.8	22:30	6:30	Breakfast
5	F	63	62.7	168	22.2	22:00	7:00	Breakfast
6	M	54	85	180	26.2	22:30	6:15	Snack

Table 4.1. Subjects Involved in this study

Subjects were requested to monitor their feeding and sleeping habits for the one week prior to each metabolic chamber visit, using a log that was provided. Subjects were asked to maintain their regular sleep and feeding schedule for one week prior to the chamber visits. Serendipitiously, all of the subjects maintained a typical sleeping

and eating schedule that was approximately in phase with the meal and sleep (lights-off) schedule of the chamber visit (Table 4.1.). Representative examples of the subjects' sleep schedule prior to chamber visits appear in Supplementary Figure S4.1. After the one-week period and a health assessment by a physician, subjects were admitted to the Center for Clinical Research at Vanderbilt University. Metabolism of the subjects was monitored in the Human Metabolic Chamber at Vanderbilt University, which is a whole-room calorimeter with CO_2 and O_2 detectors to monitor the rate of VO_2 and VCO_2 (see Supplementary Figure S4.6). The room had a set flow rate of O_2 and CO_2 that allowed the energy expenditure of each subject to be measured through indirect calorimetry. Subjects were maintained on an enforced daily light/dark schedule where lights-on occurred at 7:00 am and lights-off at 11:00 pm. The subjects ate and slept in the metabolic chamber, and were allowed only two brief 20 min episodes per day outside the chamber: once about 10:00 am to take a quick shower, and once about 3:00 pm to take a brief non-strenuous walk. While in the chamber, the subjects were instructed to do sedentary activities like reading, using a computer/internet/tablet, watching TV, etc.

During both visits, the Vitalsense® Intergrated Monitoring Physiological System was used to monitor core body temperature (CBT) over the course of the study. Subjects were given a Vitalsense telemetric core body temperature capsule that recorded the subject’s core body temperature and relayed information to a monitor attached to the subject’s waistband (or under the pillow during lights-off). Telemetric capsules were given every 24 hours to maintain consistent temperature readings independent of bowel movements when the sensor might be excreted. Meals and lights-off times were

scheduled regularly as shown in Figure 4.1.A. Subjects were admitted into the metabolic chamber for two and a half days starting at 5:30 pm and ending 7:00 am after the third night in the facility. Subjects were admitted for two separate sessions at the facility with a shifted meal schedule. For one session (the Breakfast Session), subjects were given a breakfast at 8:00 am, lunch at 12:30 pm, and dinner at 5:45 pm every day. For the other session (the Snack Session), subjects were given decaf coffee (without cream or sugar) at 8:00 am, lunch at 12:30 pm, dinner at 5:45 pm, and a late-evening snack at 10:00 pm. The breakfast on Day 2 was identical to the snack on Day 2, and the breakfast on Day 3 was identical to the snack on Day 3 (Supplemental Table S4.1A), and each were ~700 kcal. The menus of the meals are shown in Supplemental Table S4.1A. The order of the sessions (i.e., Breakfast Session first versus Snack Session first) was determined in a randomized design for each subject (Table 4.1.), and 4-12 days elapsed between sessions, depending upon the subject. Subjects were asked to eat all the meal provided but any leftover food was weighed and actual intake for each meal is shown in Supplemental Table S1B. The size of the meals was determined by nutritionist to account for calories burned for each individual (on average, a daily 2300 kcal diet). Calories were divided as follows: ~700 kcals for Breakfast/Snack, ~600 kcals for lunch, and ~1000 kcals for dinner.

Whole-room respiratory chamber

The room calorimeter at Vanderbilt University is an airtight room (17.9 m^3) providing an environment for daily living whose accuracy has been documented (Supplementary Figure S4.6 [Sun *et al.*, 1994]). The room has an entrance door, an air lock for passing

food and other items, and an outside window. The room is equipped with a TV/media system, toilet, sink, desk, chair, and rollaway bed allowing overnight stays. The calorimeter is located in the Clinical Research Center at Vanderbilt University and an intercom connects the chamber to a nearby station where nurses are on duty 24 hours/7 days per week. Temperature, barometric pressure, and humidity of the room are controlled and monitored. Minute-by-minute energy expenditure (kcal/min) are calculated from measured rates of O_2 consumption and CO_2 production using Weir's equation [Weir, 1949].

Quantification and statistical analyses

To quantify the differences between the Breakfast and Snack sessions, we applied a linear mixed model to the full 56-hour time-course for each of the following measurements: metabolic rate (MR), activity, carbohydrate oxidation (CO), lipid oxidation (LO), respiratory exchange ratio (RER), and core body temperature (CBT). Each measurement was averaged using hourly bins for each subject in each session. By using a mixed model, we were able to adjust for dependency of within-subject observations. The model included fixed effects for session (Breakfast vs. Snack), day (treated as a factor variable and defined as 3:00 pm on one day to 2:59 pm on the next day), hour (treated as a factor variable), an interaction between session and day, and an interaction between session and hour. If the p-value of an interaction was greater than 0.2, we removed that interaction from the model.

Calculations

Daily carbohydrate oxidation and daily lipid oxidation was calculated from indirect calorimetry measurements as described [Frayn, 1983, Hall *et al.*, 2016]. Nitrogen excretion rate was based on the amount of protein provided to subjects as well as previous research that monitored 24-hour nitrogen using similar parameters [Campbell *et al.*, 1994]. Because protein content was not altered between sessions, we assumed that 24-h nitrogen excretion rate was equivalent for Breakfast vs. Snack Sessions.

Results

We studied the metabolism of human subjects by indirect calorimetry under continuous monitoring in Vanderbilt University's Human Metabolic Chamber. During each visit, the minute-by minute oxygen consumption (VO_2), carbon dioxide production (VCO_2), actigraphy, and core body temperature (CBT) of the subjects were continuously measured, with subsequent calculation of RER (VCO_2/VO_2), metabolic rate, carbohydrate oxidation, and lipid oxidation. The subjects slept and ate in the metabolic chamber, and were allowed only two brief (20 min) episodes per day outside the chamber: once about 10:00 am to take a quick shower, and once about 3:00 pm to take a brief non-strenuous walk. Each subject was monitored for two full-duration 56-hours experiments that compared differences in the timing of their meals. In the “Breakfast Session” (Figure 4.1.A), the subjects had breakfast, lunch, and dinner, with a ~13.75-hour fast from 6:15 pm (end of dinner) to 8:00 am (breakfast). In the “Snack Session,” subjects only had a cup of tea or coffee (without sugar or creamer) at breakfast time, and their first meal was lunch (Figure 4.1.A). Then, a snack was served at 10:00 pm just before sleep (lights-off), and the subjects fasted ~14 hours until lunch (10:30 pm to

12:30 pm). The breakfasts and the snacks had equivalent nutritional and caloric values of ~700 kcal; the breakfast on Day 2 was identical to the snack on Day 2, and the breakfast on Day 3 was identical to the snack on Day 3 (Supplemental Table S4.1A). Therefore, the meals served to subjects during the Breakfast Session had equivalent energy and nutrient content as in the Snack Session over the 24-h day (see Supplementary Table 1 for detailed nutritional information). All subjects completed both sessions of this cross-over experiment, which allowed pairwise comparison of their data. Our study is distinguished from the earlier metabolic chamber studies of meal timing in humans [Sato *et al.*, 2011, Kobayashi *et al.*, 2014] by the cross-over design of our protocol and the fact that we studied older subjects of various BMI (51-63 years old, BMI 22.2 - 33.4, see Table 1) who may be less resilient to metabolic perturbations of energy expenditure than are younger subjects with BMIs of 20-25 [Roberts *et al.*, 1996, Melanson *et al.*, 1997]. Moreover, our Breakfast vs. Snack Sessions had essentially the same duration of daily fasting (13.75-14 hours) to avoid the confounding factor of differential fasting durations found in other studies.

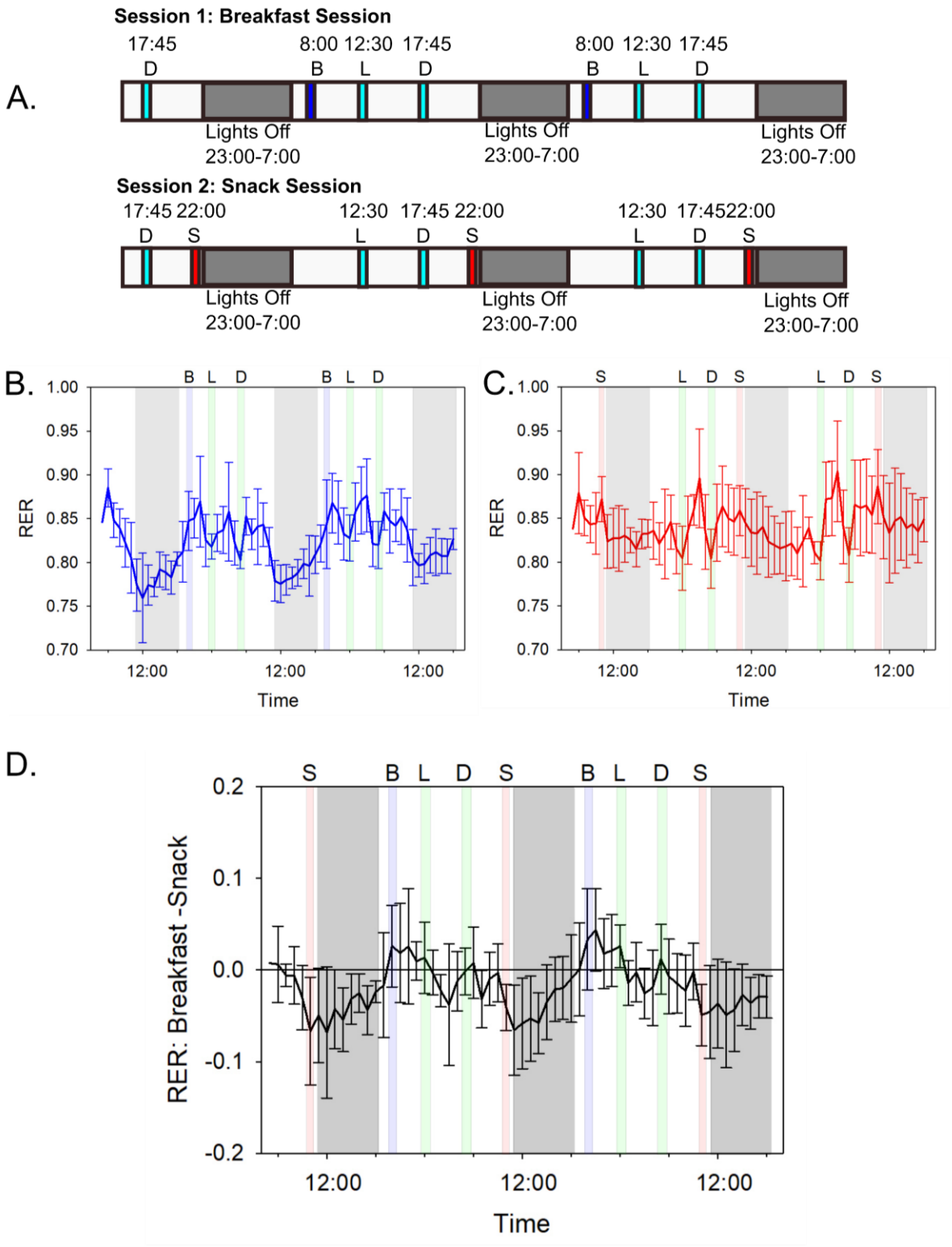

Figure 4.1. Chamber schedule and the effect of meal timing on subjects' respiratory exchange ratios (RERs). See also Supplementary Table S4.4A

A) Protocol for "Breakfast" Session vs. "Snack" Session. Subjects experienced two separate 56-hours continuous sessions with constant metabolic monitoring by indirect calorimetry, each session lasting 56 hours. The Breakfast session included a breakfast (B), lunch (L), and dinner (D) while the Snack session contained a lunch, dinner, and a late-evening snack (S). The late-evening snacks were of equivalent caloric and nutritional value to the breakfast meals (~700 kcal, see Supplementary Table S4.1A for details). Note from Supplementary Figure S1 that the daily phasing of sleep for the subjects prior to entry into the metabolic chamber was the same as the "lights-off" interval during the 56-hour time course, so the subjects did not experience a phase shift of their daily cycle when they entered the experimental conditions.

B) Breakfast Session: blue line indicates the average hourly RER over the entire 56-hour time course among all six subjects when a breakfast, lunch, and dinner were presented. Error bars are the standard deviation. Letters indicate time and type of meals and gray shaded areas indicate the lights off phases. Green shaded areas indicate meals that were given at the same time in both Breakfast and Snack Sessions (lunch and dinner). Blue shaded areas indicate when breakfast was given, and gray shading indicates the lights off period. See Supplementary Figure S4.2 for data of all subjects individually.

C) Snack Session: the red line indicates the average hourly RER over the entire 56-hour time course among all subjects when a lunch, dinner, and a late-evening snack were presented. Red shaded areas indicate when late-evening snacks were given. Breakfasts and late-evening snacks contained the same number of calories and the same lipid, carbohydrate, and protein content (Supplemental Table S4.1A/B). Error bars are the standard deviation (n=6).

D) Average difference in RER over the entire 56-hour time course for the Breakfast Session subtracted from the Snack Session. Deviation from zero (horizontal black line) indicates where differences in RER occurred between subjects. Error bars indicate standard deviation in the differences. In Panels B, C, & D, times of meals are indicated by letters (B = breakfast, L = lunch, D = dinner, S = late-evening snack), gray areas are lights-off (sleep) intervals. Meal times are shaded as in panels A-C; breakfasts and snacks occurred only in their respective sessions. All RER data were collected minute by minute, and in this figure the minute by minute data were binned and averaged for all 60 values within an hour. Abscissa are clock time.

As illustrated in Figure 4.1.B, subjects on the Breakfast Session that included breakfast and a fast throughout the time interval from dinner to the following breakfast (6:30 pm to 8:00 am), exhibited a strong daily rhythm of RER (aka Respiratory Quotient, calculated as VCO_2 / VO_2 [Frayn, 1983, Hall *et al.*, 2016]). RER values close to 0.7 indicate lipid oxidation while values of ~1.0 indicate almost exclusive carbohydrate

oxidation. The average RER of this diet is similar to that of typical diets in the USA (~0.85); it includes a mixture of lipids, protein, and carbohydrates [Frayn, 1983, Hall *et al.*, 2016]. RER of subjects on the Breakfast Session was low throughout the lights-off interval (indicating primarily lipid catabolism during sleep) and high during the active daytime (indicating primarily carbohydrate and protein catabolism). Therefore, humans share with other mammals a daily rhythm of substrate metabolism as assessed by indirect calorimetry [Shi *et al.*, 2010, Namvar *et al.*, 2016]. However, in the Snack Session, the metabolism of the same subjects was not as strongly rhythmic, and displayed a much lower amplitude rhythm of RER that did not drop into a largely lipid catabolic mode (Figure 4.1.C). When the difference between RER for the Breakfast and Snack Sessions is calculated as a function of daily time, the most significant difference was noted during the inactive sleep phase where lipid oxidation predominates in the Breakfast Session while RER remains high in the Snack Session (Figure 4.1.D).

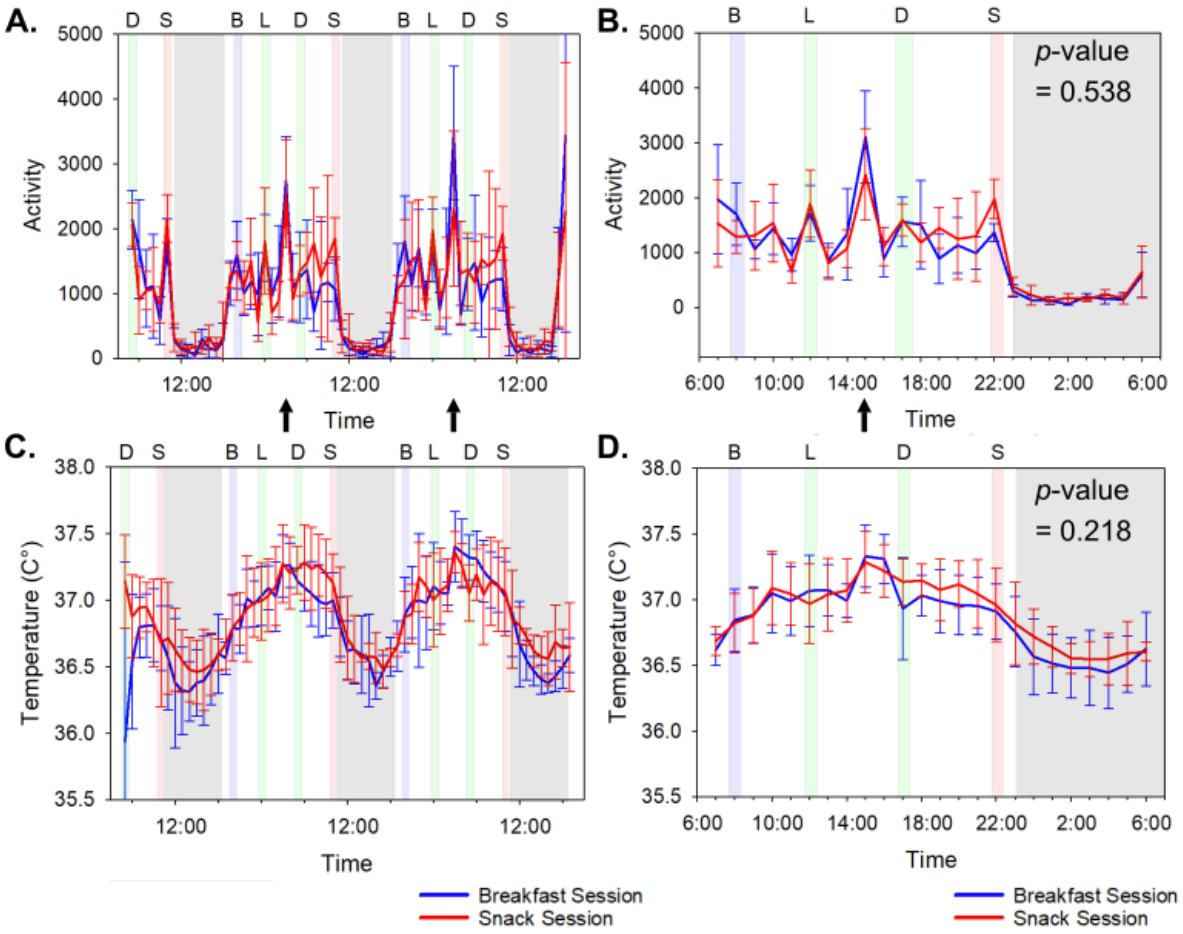

Figure 4.2. Activity and core body temperature patterns. See also Supplementary Table S4.4B and S4.4C

A) Average wrist locomotor activity (measured as vector of magnitude) of all subjects for the 56-hour time course. The blue line indicates values during the subjects' Breakfast sessions and the red line for the subjects' Snack sessions. Black arrows indicate the afternoon break where subjects were allowed to exit the chamber for a 30 minute break, during which the subjects were allowed a non-strenuous walk. (This 30-min interval was excluded in other measurements as calorimetric readings were not being taken during this break.)

B) Average wrist activity of all subjects plotted on a modulo-24 hours. Minute-by-minute activity data were averaged for all subjects into one-hour bins and aligned by clock time. The arrow denotes the 30 min break referred as noted in panel A. The p-value of 0.538 refers to a pairwise comparison of the average (breakfast – snack) values over the full 56 h time course for wrist activity. See Supplementary Table S4.4B for the hour-by-hour statistical comparison of the breakfast versus the snack sessions.

C) Average core body temperature (CBT) for all subjects over the 56-hour time course.

D) Average CBT of all subjects plotted on a modulo-24 hours. Minute-by-minute activity data were averaged for all subjects into one-hour bins and aligned by clock time. The p-value of 0.218 refers to a pairwise comparison of the average (breakfast –

snack values over the full 56-hour time course for CBT. See Supplementary Table 4.4C for the hour-by-hour statistical comparison of the breakfast versus the snack sessions.

All panels: The blue line indicates values during the subjects' Breakfast sessions and the red line for the subjects' Snack sessions. Shading indicates meals and lights-off as in Figure 4.1.B/C. Error bars indicate +/- standard deviation (n = 6).

We initially predicted that the session-dependent RER patterns were due to differences between the sessions in physical activity, sleep disruption, core body temperature, or the phasing/amplitude of the circadian clock. However, none of these parameters were different between the sessions. Actigraphy confirmed that the subjects' overall activity levels did not differ significantly between the two sessions (Figure 4.2.A/B, p = 0.538). Moreover, actigraphy can provide an assessment of restlessness during sleep [Winnebeck *et al.*, 2018] and by this criterion, the sleep quality was equivalent between the Breakfast and Snack Sessions (Figure 4.2.A/B). The daily rhythm of the core body temperature (CBT), that is frequently used as a marker of the central circadian clock in humans [Resuehr *et al.*, 2019, Czeisler *et al.*, 1986], did not show significant differences in the phasing or amplitude between the two sessions (Figure 4.2., p = 0.218). Moreover, the circadian rhythm of overall metabolic rate (MR, [Zitting *et al.*, 2018]) was not different in phase or amplitude between the two sessions (Figure 4.3.B, p = 0.11). Therefore, neither differences in core body temperature (Figure 4.2.) nor the thermic effect of food (TEF, see below) were responsible for the session-dependent RER differences. The phasing or amplitude of the daily rhythms of master clock markers (plasma melatonin and cortisol), insulin, or plasma triglycerides is also not responsible, as reported by Wehrens and co-workers who found no differences in those rhythms in a meal timing study using a very similar protocol to ours [Wehrens *et*

al., 2017]. Finally, our subjects kept a regularly timed sleep/wake cycle prior to the metabolic chamber experiments so that their internal rhythm was in phase with the light/dark cycle during the 56-hour experiment (Supplementary Figure S4.1, Table 4.1). These results suggest that the change in meal timing altered the RER rhythm (i.e., its amplitude) without changing overall activity, sleep quality, body temperature, or the phase relationship between circadian rhythms and the daily light/dark schedule.

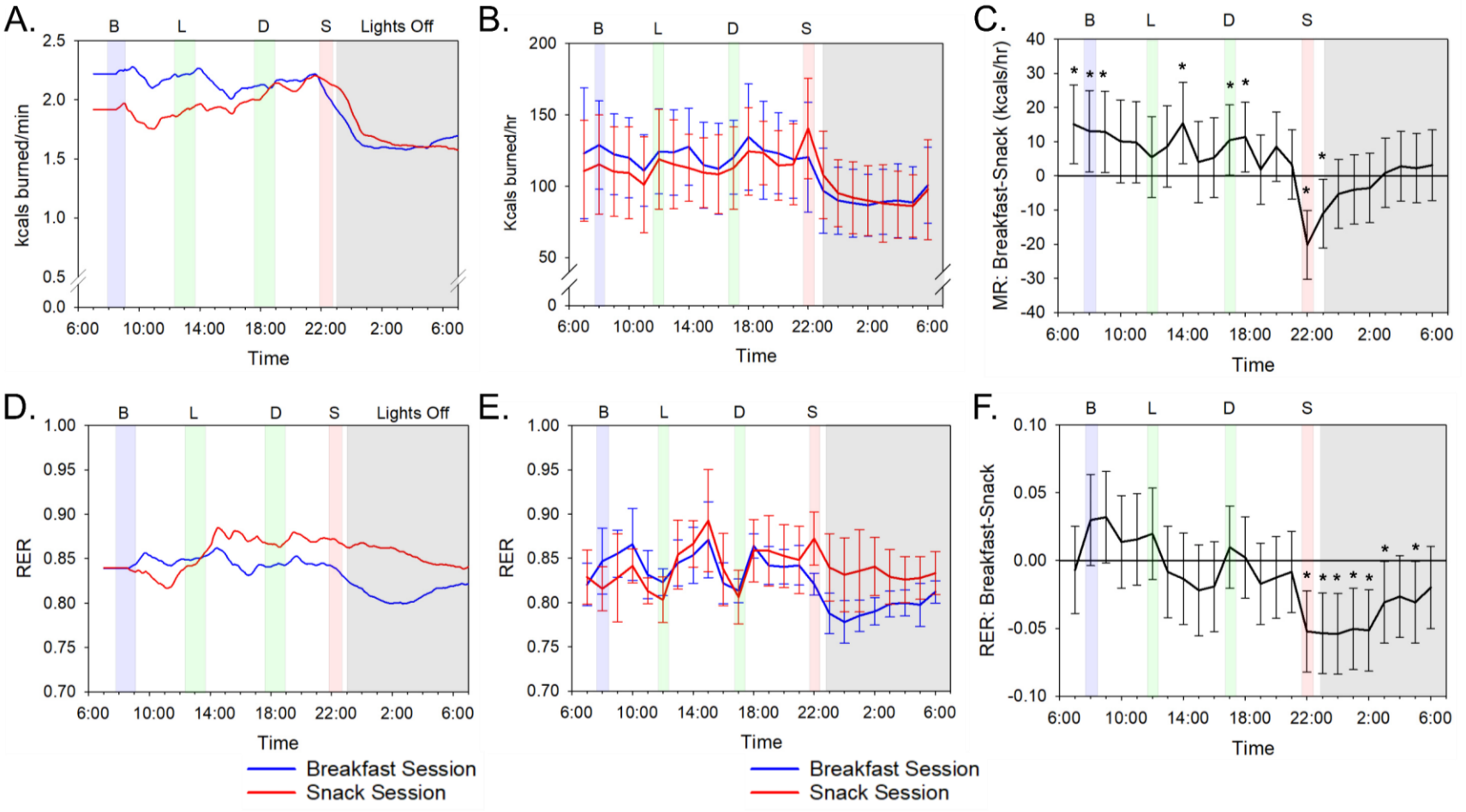

Figure 4.3. Metabolic rates and RER values. Refer to Supplementary Table S4.4A and S4.4D

A) Metabolic rate by indirect calorimetry for a representative participant (Subject #3). The data for Subject #3 are plotted as a moving average using 180 data points (for three hours) after aligning all time points to clock time and integrated on a 24-hour scale.

B) Average metabolic rate for all subjects plotted modulo-24 hours. Data were averaged into one-hour bins with error bars indicating standard deviation. See Supplementary Figure S4.3 for data of all subjects' metabolic rates individually plotted.

C) Average hourly pairwise comparison of (breakfast – snack) metabolic rate values for all subjects. Error bars indicate 95% confidence intervals and values are based on a mixed model analysis. Asterisks indicate significant differences (p-value<0.05) between breakfast and snack values for the indicated one-hour bins. See Supplementary Table S4.4D for the hour-by-hour statistical comparison of the breakfast versus the snack sessions.

D) RER (VCO_2/VO_2) by indirect calorimetry of a representative individual (Subject #3). The data for Subject #3 are plotted as a moving average using 180 data points (for three hours) after aligning all time points to clock time and integrated on a 24-h scale.

E) Average RER for all subjects plotted on a modulo-24 hours. Data were averaged into one-hour bins with error bars indicating standard deviation. See Supplementary Figure S4.2 for data of all subjects' RER individually plotted.

F) Average hourly pairwise comparison of (breakfast – snack) RER values for all subjects. Error bars indicate 95% confidence intervals and values are based on a mixed model analysis. Asterisks indicate significant differences (p-value<0.05) between breakfast and snack values for the indicated one-hour bins. See Supplementary Table S4.4A for the hour-by-hour statistical comparison of the breakfast versus the snack sessions.

All panels: The blue line indicates values during the subjects' Breakfast sessions and the red line for the subjects' Snack sessions. Shading indicates meals and lights-off as in Figure 4.1.B/C. Error bars indicate +/- standard deviation.

The differences in the RER patterns between the two sessions manifest primarily during the time of late-evening snacking and for at least several hours into the sleep episode (hours 22:00 – 03:00 (Figure 4.3.E, Supplementary Table S4.4.A). Apparently the late-evening snacking delays the clock-induced switching between primarily carbohydrate-catabolic mode (higher RER values) and primarily lipid-catabolic mode (lower RER values). Despite this change in the temporal pattern of RER, the values integrated over the entire 56-hour time courses indicate differences slightly above the $p = 0.05$ level between the two sessions in terms of overall RER or total energy expenditure (Figure 4.3.C/F; $p = 0.068$ for RER and $p = 0.11$ for MR). Moreover, while there was a significant thermic effect of food (TEF) for MR and RER at each meal, our calculations based on the method of McHill and coworkers [McHill *et al.*, 2014] indicated no differences in the TEF between the sessions for lunch and dinner–the two meals that were the same in both sessions ($p = 0.432$ for lunch and $p = 0.855$ for dinner). Moreover, the TEF for the breakfast as compared with the snack was also not different ($p = 0.284$). Therefore, differences in metabolic rates as assessed by TEF were not responsible for the substrate-switching preferences that are described below for breakfast-skipping versus late-evening snacking.

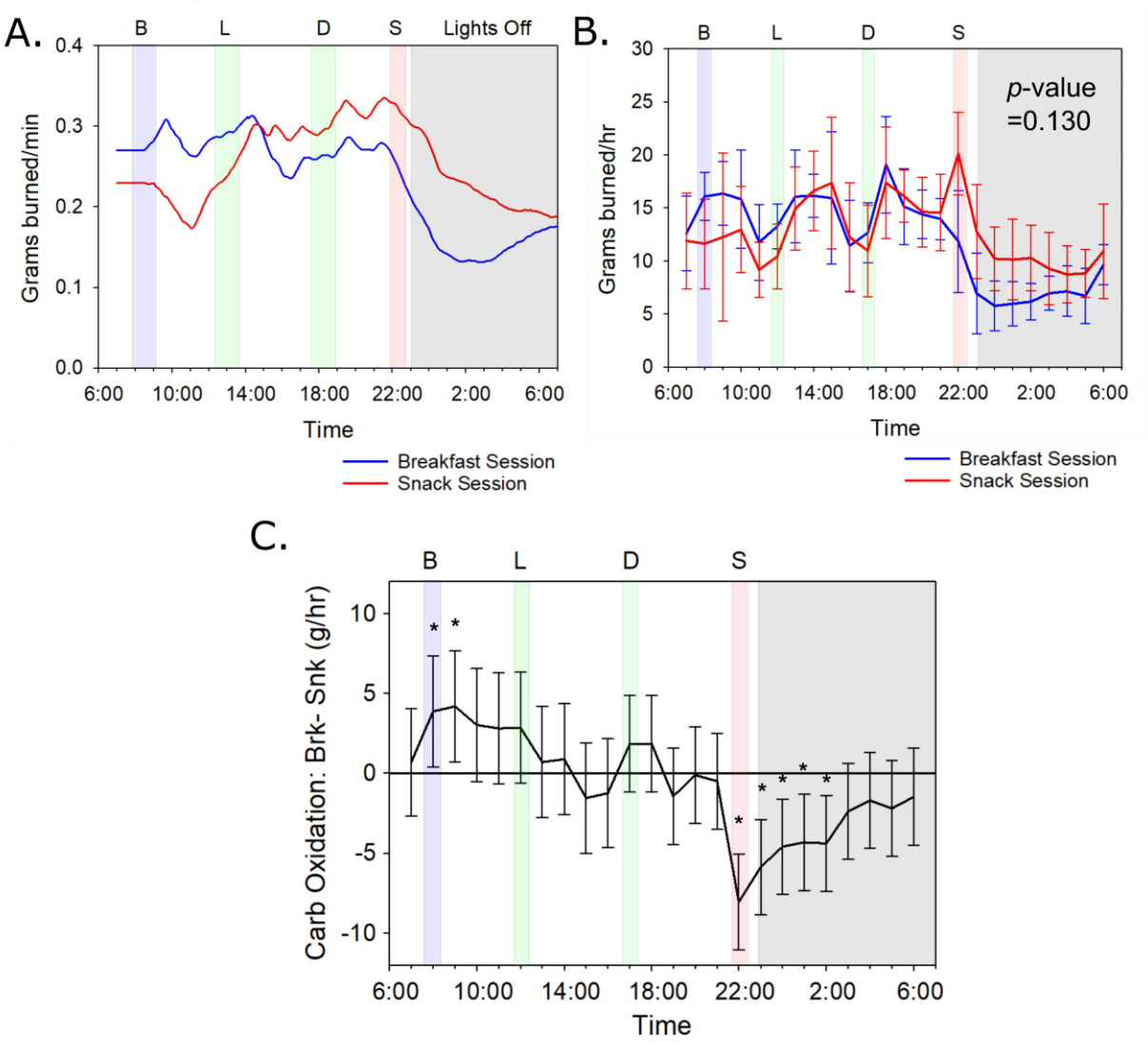

Figure 4.4. Carbohydrate oxidation patterns offset between the meal timing sessions; See also Supplementary Table S4.4E

A) Carbohydrate oxidation data of a representative subject (#3) calculated from indirect calorimetry measurements as described [Frayn, 1983, Hall *et al.*, 2016]. Data are plotted as a three-hour moving average (180-minute data points).

B) Average of all subjects for daily carbohydrate oxidation calculated from indirect calorimetry measurements as described [Frayn, 1983, Hall *et al.*, 2016] and the 56-hour time course data are plotted on a modulo-24 h scale. Averaged data for all subjects are organized in one-hour bins. The p-value of 0.130 refers to a pairwise comparison of the average (breakfast - snack) values over the full 56-hour time course for carbohydrate oxidation. See Supplementary Figure S4.4 for data of all subjects' carbohydrate oxidation rates plotted individually.

C) Average hourly pairwise comparison of (breakfast – snack) carbohydrate oxidation values for all subjects. Error bars indicate 95% confidence intervals and values are based on a mixed model analysis. Asterisks indicate significant differences (p-

value<0.05) between breakfast and snack values for the indicated one-hour bins. See Supplementary Table S4.4E for the hour-by-hour statistical comparison of the breakfast versus the snack sessions.
All panels: The blue line indicates values during the subjects' Breakfast sessions and the red line for the subjects' Snack sessions. Shading indicates meals and lights-off as in Figure 4.1.B/C. Error bars indicate +/- standard deviation.

The conclusion that altered meal timing delays the sleep-onset switching between carbohydrate and lipid catabolic modes can be more easily visualized by converting the RER values into carbohydrate vs. lipid oxidation rates [Hall *et al.*, 2016]. Carbohydrate oxidation during the Breakfast Session was high during the active day-phase with peaks just after each mealtime, but it dropped precipitously as the subjects entered their sleep episode after lights-out at 11:00 pm (Figure 4.4.A). The carbohydrate oxidation rate of subjects on the Breakfast Session who had not eaten since dinnertime began to fall before sleep onset and continued to be low through the first half of the nocturnal sleep episode (Supplementary Table 4E). On the other hand, in the Snack Session, the late-evening snack caused a peak carbohydrate catabolism just before going to bed, and while carbohydrate oxidation dropped thereafter, it remained higher throughout the sleep episode than when the same subjects were on the Breakfast Session (Figure 4.4.A). Overall, 24-hour carbohydrate oxidation did not differ between sessions because the increased oxidation after breakfast in the Breakfast Session was offset by less carbohydrate oxidation in the early night of the Breakfast Session (Figure 4.4.C; Supplementary Table S4.4E). Therefore, carbohydrate oxidation was not significantly

different between the sessions over the entire 56-hour time course (p = 0.130).

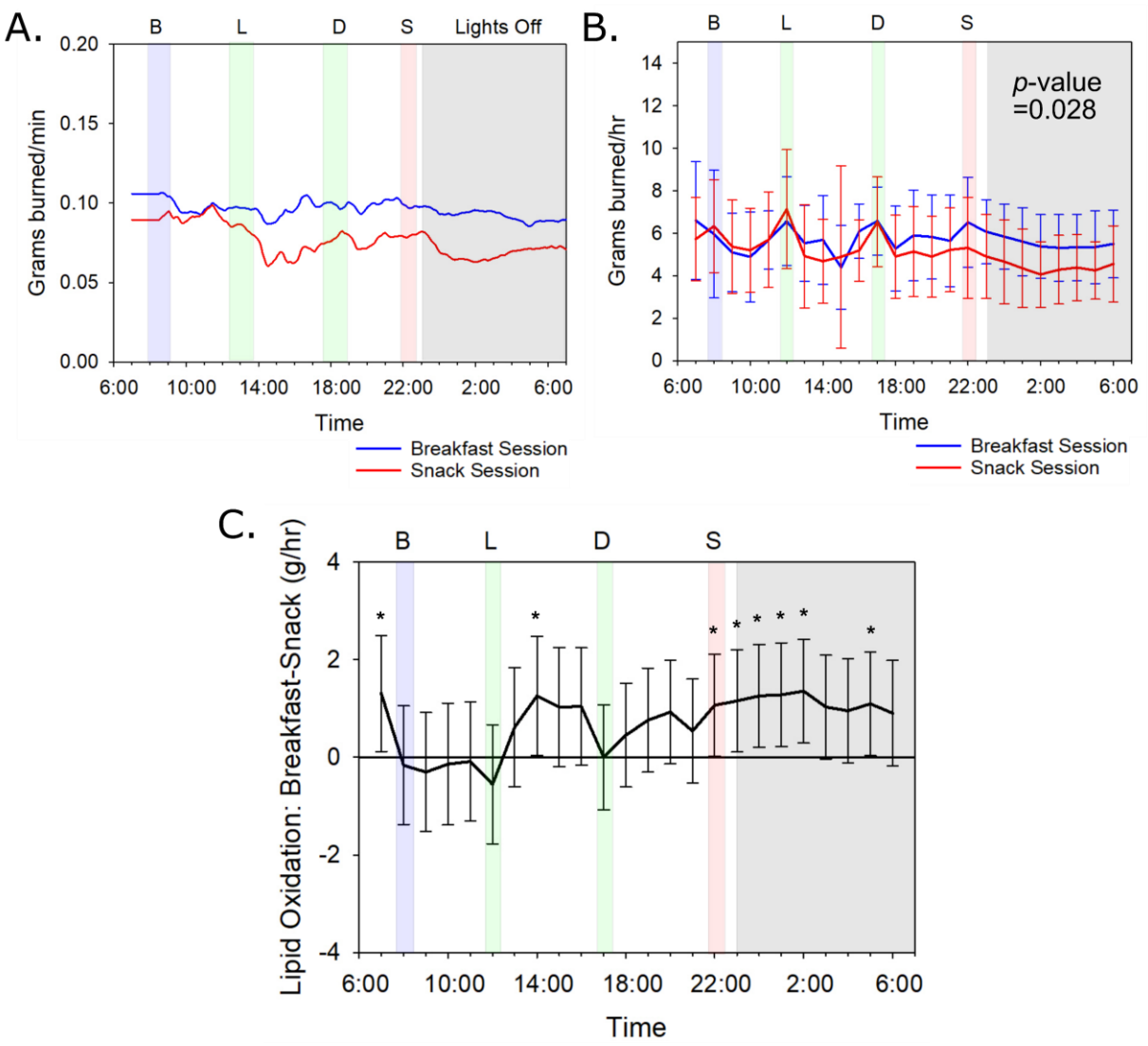

Figure 4.5. Meal timing alters overall lipid oxidation; Refer to Supplementary Table S4.4F

A) Lipid oxidation data of a representative Subject (#3) calculated from indirect calorimetry measurements as described [Frayn, 1983, Hall *et al.*, 2016]. Data are plotted as a three-hour moving average (180-minute data points).

B) Average of all subjects for daily lipid oxidation calculated from indirect calorimetry measurements as described [Frayn, 1983, Hall *et al.*, 2016] and the 56-hour time course data are plotted on a modulo-24-hour scale. Averaged data for all subjects are organized in one-hour bins. The p-value of 0.028 refers to a pairwise comparison of the average (breakfast – snack) values over the full 56-hour time course for lipid oxidation. See Supplementary Figure S4.5 for data of all subjects' lipid oxidation rates plotted individually.

C) Average hourly pairwise comparison of (breakfast – snack) lipid oxidation values for all subjects. Error bars indicate 95% confidence intervals and values are based on a mixed model analysis. Asterisks indicate significant differences (p-value<0.05) between breakfast and snack values for the indicated one-hour bins. See Supplementary Table S4.4F for the hour-by-hour statistical comparison of the breakfast versus the snack sessions.

All panels: The blue line indicates values during the subjects' Breakfast sessions and the red line for the subjects' Snack sessions. Shading indicates meals and lights-off as in Figure 4.1.B/C. Error bars indicate +/- standard deviation.

On the other hand, lipid oxidation was different between the sessions ($p = 0.028$). Subjects on the Breakfast Session experienced a relatively constant rate of lipid oxidation throughout the 24-hour cycle (Figure 4.5.B). Because the overall metabolic rate declined during the night (Figure 4.3.A), this means that carbohydrate catabolism was "switched off" and lipid oxidation was sustained metabolism during the nightly fast. However, on the Snack Session, the availability of carbohydrates that was enabled by the late-evening snack supported metabolism during the night by carbohydrate oxidation; since the nocturnal metabolic rate is lower than the diurnal metabolic rate and carbohydrate catabolism is maintaining nocturnal metabolism, the meal timing of the Snack Session inhibited lipid oxidation at night (Figure 4.4.B/C). On average, 15 more grams of lipid were burned over the 24-h cycle by subjects on the Breakfast Session as compared with the Snack Session (Figure 4.4.D; $p = 0.028$).

Using a mixed model pairwise hour-by-hour analysis, we found significant differences in both carbohydrate and lipid oxidation for hours 22:00-02:00 of the snack/night interval (Supplementary Table S4.4E/F), with carbohydrates being utilized at higher rates for a longer time over this temporal window in the Snack Session than in the Breakfast Session (Figure 4.4.B/C). Conversely, the Breakfast Session showed

significantly more lipids burned in the snack/night interval than did the Snack session (Figure 4.5.B/C). During the breakfast interval, we also found a significant difference in carbohydrate oxidation with more carbohydrates burned during the Breakfast Session than during the Snack Session at hours 08:00-09:00 (Supplementary Table S4.4E). Nevertheless, over the entire 24-hour span there is not a net difference in carbohydrate oxidation between sessions because the oxidation difference in the breakfast window is offset by opposite oxidation rates in the snack/night window (Figure 4.4.C). However, the enhanced lipid oxidation in the snack/night window of subjects in the Breakfast Session is not offset by an opposite effect in another window (Figure 4.5.C). These results indicate that the time of meal placement can cause variation in the amount of lipids oxidized regardless of the nutritional or caloric content of the meal; changing the daily timing of a nutritionally equivalent meal of 700 kcal has a significant effect upon carbohydrate and lipid metabolism.

Discussion

The major finding of this study is that the timing of feeding over the day leads to significant differences in the metabolism of an equivalent 24-hour nutritional intake. Daily timing of nutrient availability coupled with daily/circadian control of metabolism drives a switch in substrate preference such that the late-evening snack session resulted in significantly lower lipid oxidation compared to the breakfast session. When the subjects started bedrest after having just eaten the late-evening snack (Snack Session), they catabolized less lipid during their sleep episode than they did when they fasted from dinner to breakfast (Breakfast Session). This significant ($p = 0.028$, Figure

4.5.B) effect was measurable over only three sleep episodes in our experiments so that an average of 15 fewer grams of lipid were burned over the 24-h cycle by subjects on the Snack Session. The effect of a regular late-evening snack persisting over a longer time would progressively lead to substantially lower lipid oxidation (and therefore, more lipid accumulation) as compared with fasting during this interval of the day. As schematized in Figure 6, the daily patterns of substrate oxidation (Figure 4.6.A,6.B) are roughly following the daily eating patterns (Figure 4.6.C). However, a late-evening snack likely sustains liver glycogen stores (carbohydrate oxidation, Figure 4.6.A) so that metabolism does not transition as rapidly or as fully into lipid oxidation during the nocturnal fast (Figure 4.6.B).

Our interpretation of these data is based on the circadian clock orchestrating a switch between primarily carbohydrate oxidation to primarily lipid oxidation between the last meal of the day and the onset of circadian-timed sleep [Panda, 2018, Challet, 2019, Wefers *et al.*, 2018, Bonham *et al.*, 2019]. Instead of fasting between dinnertime and breakfast, if a person eats during the late evening, carbohydrates will be preferentially metabolized as sleep initiates, delaying the timing of the switch to primarily lipid oxidation. Over the 24-hour cycle, cumulative carbohydrate oxidation as compared with total carbohydrate intake was not dramatically different between the two sessions (Figure 4.6.D), so the net 24-hour carbohydrate storage is similar (Figure 4.6.F). On the other hand, the cumulative 24-hour lipid oxidation rate as compared with total lipid intake is substantially less when late-evening snacking detains the transition to lipid catabolism (Figure 4.6.E), thereby lessening the mobilization of lipid stores (i.e., the extent of lipids being oxidized, Figure 4.6.F). There is a clear trade-off between lipid and

carbohydrate oxidation during the night; the Breakfast Session clearly favors lipid oxidation at the expense of carbohydrate oxidation (Figures. 4.5. and 4.6.).

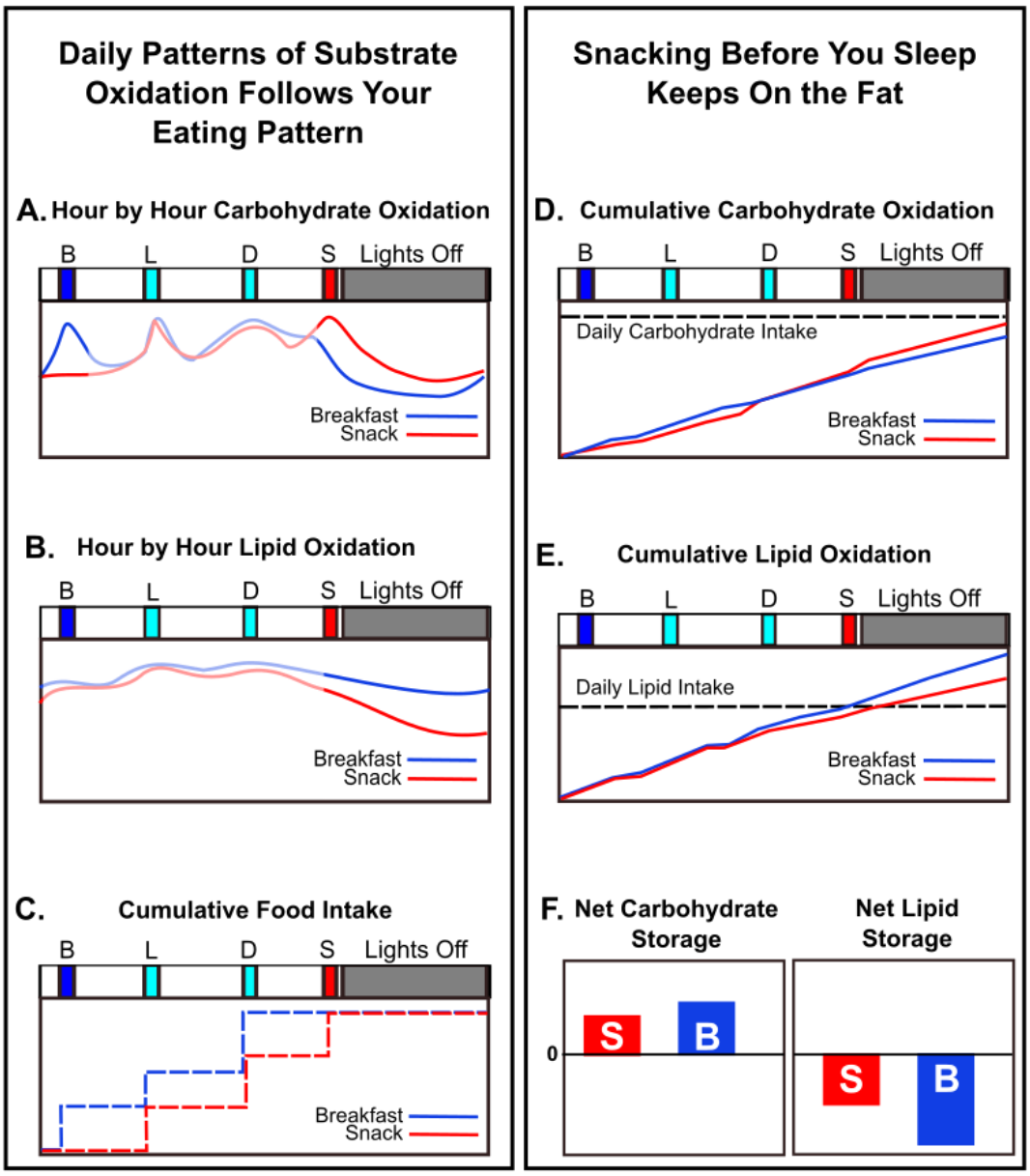

Figure 4.6. Schematic: late-evening snacking interacts with the circadian rhythm of metabolism to inhibit lipid oxidation.

A & B) Hour by hour oxidation rates for carbohydrates (panel A) and lipids (panel B) in the two sessions. These curves are smoothed versions of the experimental data in Figures 4.4. & 4.5.

C) Cumulative food intake on the Breakfast vs. Snack sessions.
D & E) Cumulative oxidation rates over the 24-hour cycle derived from the curves in panels A and B, and the experimental data of Figures 4.4.& 4.5. Panel D shows cumulative carbohydrate oxidation, while panel E show cumulative lipid oxidation. The horizontal dashed lines indicate the daily total intake of carbohydrates (D) and lipids (E) for comparison with the cumulative respective oxidations.
F) Approximate net relative daily storage of carbohydrates and lipids inferred from the data of Figures 4.4. & 4.5. and the analyses depicted in the other panels of this figure. Positive values indicate the extent of substrate accumulation/storage, negative values indicate the extent of substrate oxidation ("burning").

There was a small but significant increase in carbohydrate oxidation in the morning after eating breakfast (Figures 4.1.D & 4.5.A, hours 08:00-09:00 in Supplementary Table S4.4E) but not on lipid oxidation (Figure 4.5.B, hours 08:00-09:00 in Supplementary Table S4.4F). However, the effect of eating vs. skipping breakfast is not as significant as the effects of eating after dinner on both carbohydrate and lipid catabolism during the sleep episode (Figure 4.5.). In this study, there were no obvious differences among subjects based on BMI or gender (Table 1, Supplemental Figs. S4.2, S4.3, S4.4, S4.5). Unlike the conclusions of a previous investigation comparing morning versus evening carbohydrate-rich meals [Kräuchi *et al.*, 2002], in our investigation the different phasing of the meals between the two sessions did not change the phasing of the daily metabolic pattern. The phasing of sleep during the 56-hour time courses matched that of the subjects' sleep patterns for the prior week (compare Table 1 with Supplemental Figure S4.1.), and the phasing of the daily rhythms of activity, metabolic rate, and CBT were in phase between the two sessions (Figures 4.2. and 4.3.B). Therefore, in our protocol, the wake/sleep cycle appears to be locked in the same phase relationship to the lights-on/lights-off cycle in both sessions, and the altered meal

timing of the Snack Session has delayed the metabolic switching between primarily carbo-catabolism mode and primarily lipid-catabolism modes in relationship to either the circadian system and/or the timing of sleep (Figure 4.6.).

Consistent with the findings of other investigations of altered meal timing, breakfast skipping, early time restricted feeding, etc. [Sato *et al.*, 2011, Kobayashi *et al.*, 2014, Ravussin *et al.*, 2019], we found no significant differences in total energy expenditure between sessions (Figure 4.3.C). Nevertheless, metabolism was significantly affected. In particular, the average daily RER maintained a higher value in the Snack Session (Figure 4.3.E/F), which can be attributed to a delayed entry into primarily lipid oxidation mode (Figures 4.3.E, 4.5., 4.6.). The end result of the reduced lipid oxidation will be enhanced lipid storage, which over time will lead to increased adiposity. Therefore, in older adults who are potentially at-risk for metabolic disorders, avoiding snacking after the evening meal can sustain lipid oxidation and potentially improve metabolic outcomes.

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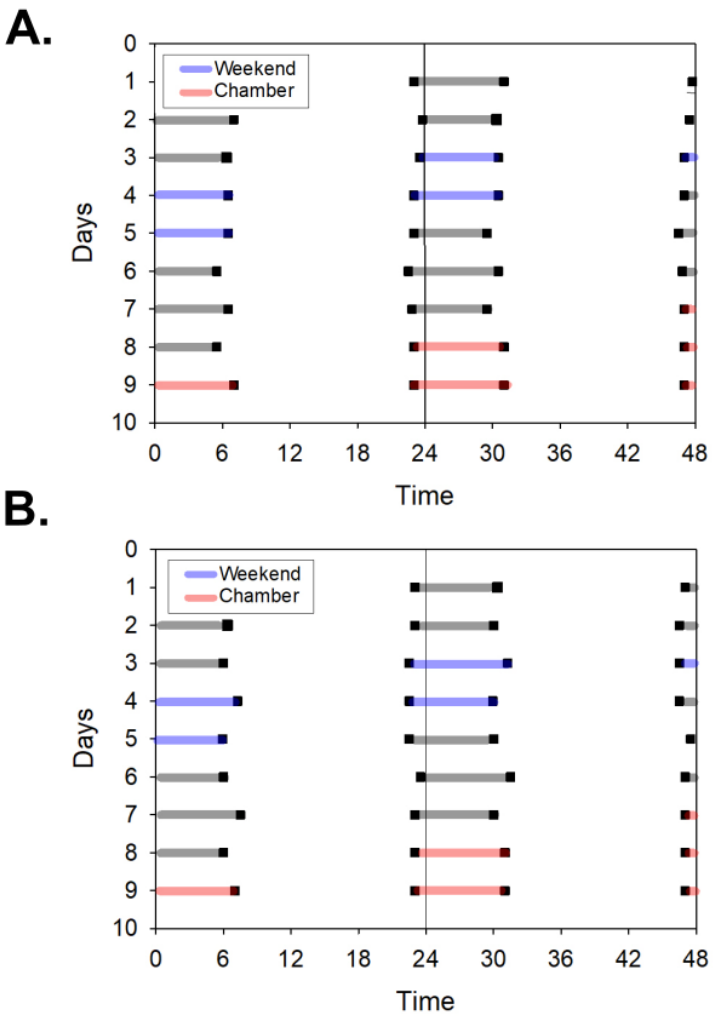
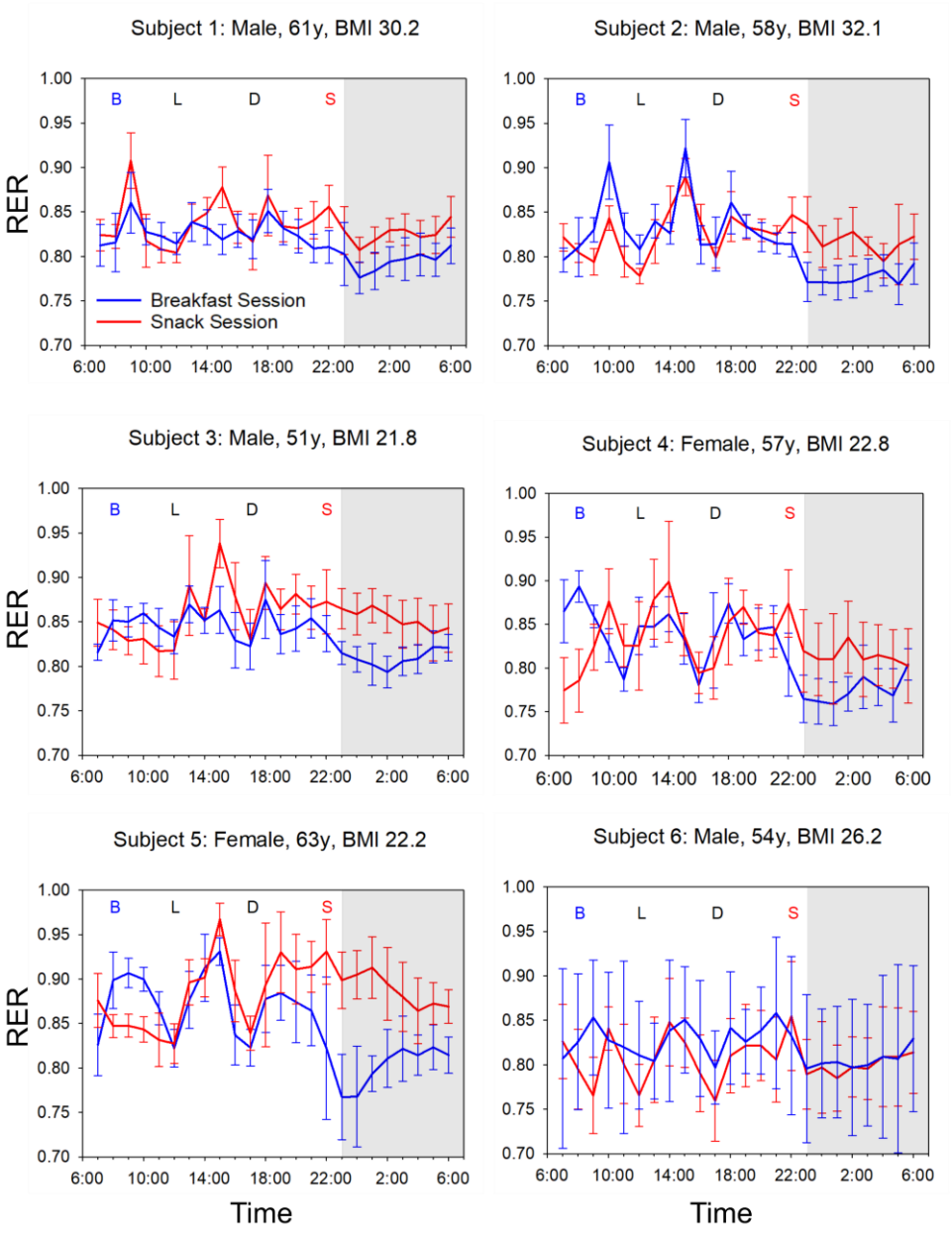
Supplementary Information

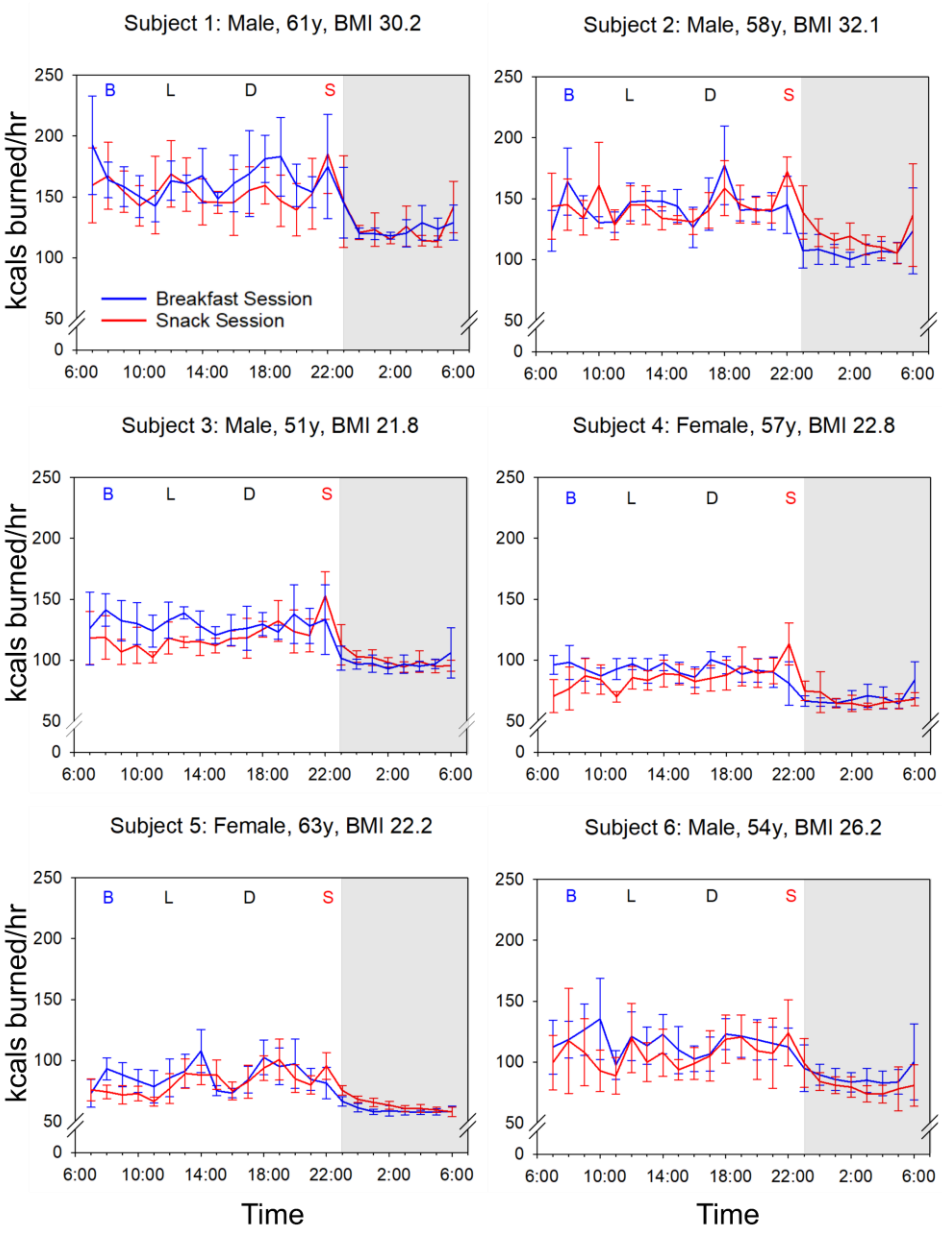
Supplemental Figures



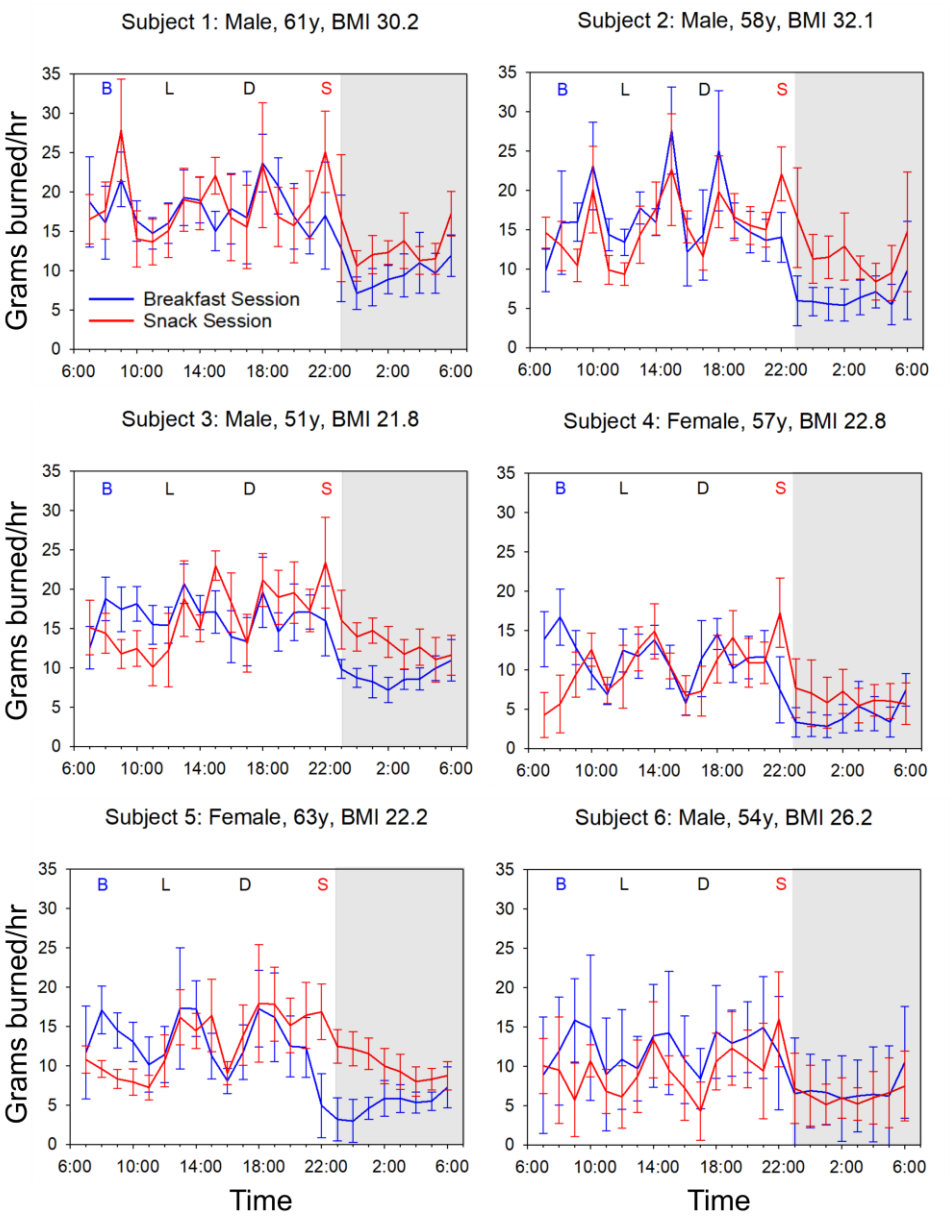
Supplementary Figure S4.1. Representative Subject's self-reported sleep schedules prior to entry in the experiment for Breakfast (A) and Snack (B) Sessions.



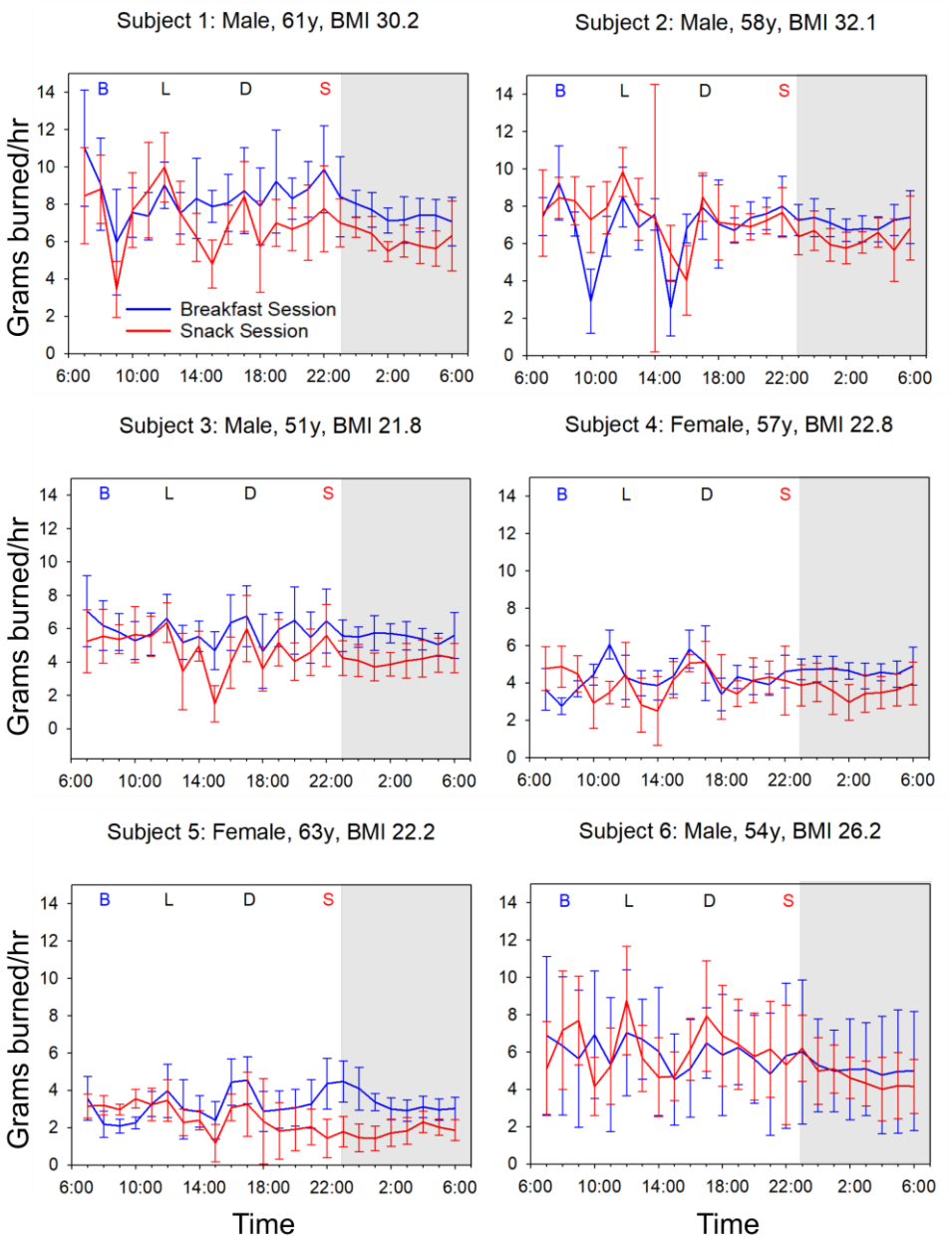
Supplemental Figure S4.2. Daily Respiratory Quotient data for all subjects.



Supplemental Figure S4.3. Daily Metabolic Rate data for all subjects.

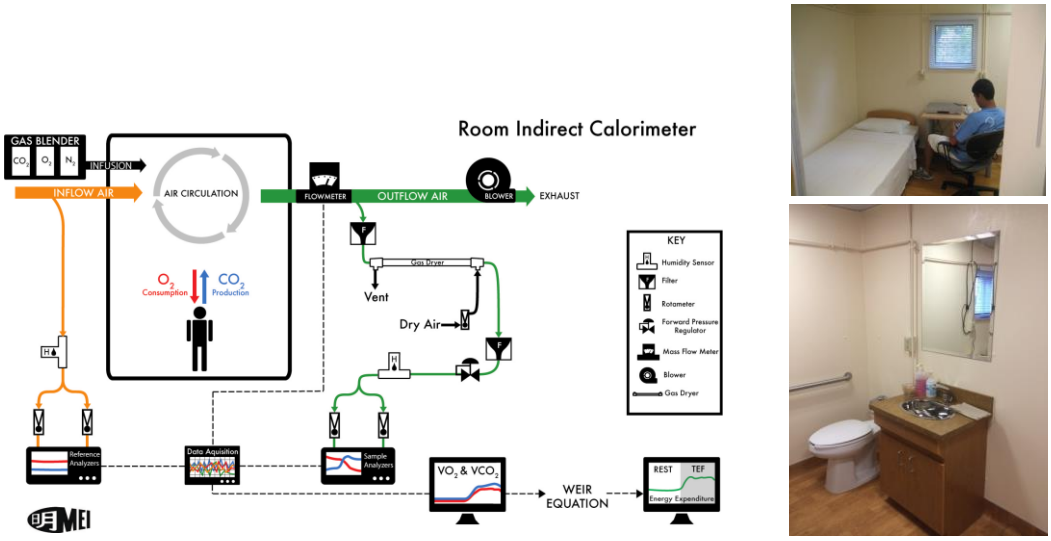


Supplemental Figure S4.4. Daily Carbohydrate Oxidation data for all subjects.



Supplemental Figure S4.5. Daily Lipid Oxidation data for all subjects.

Supplementary Figure S6



Supplemental Figure S4.6. Human Metabolic Chamber at Vanderbilt University.

Supplemental Tables

Supplementary Table S4.1. Meals and Nutritional Information

A. Representative Meals

Breakfast Session			
Admit Day 1	**Day 2**	**Day 3**	**Day 4**
Breakfast	***Breakfast 8AM***	***Breakfast 8AM***	***Breakfast 8AM***
not applicable	English muffin / Margarine	Bagel / Cream cheese	Regular breakfast meal after
	Fresh pork patty	Boiled egg	study is completed.
	Honey nut cheerios	Apple	
	Mandarin orange	String cheese	
	Cranberry juice / 2% Milk	Orange juice	

	Decaf coffee or decaf tea	Decaf coffee or decaf tea	
Lunch	***Lunch 12:30PM***	***Lunch 12:30PM***	***Lunch***
not applicable	Turkey Sandwich	Hamburger	not applicable
	Salad / Ranch dressing	Steak house fries	
	Pears	Salad / Fat free Italian dressing	
	Potato chips	Peaches	
	Graham crackers	Grape juice	
Dinner 5:45PM	***Dinner 5:45PM***	***Dinner 5:45PM***	***Dinner***
Italian chicken	Beef Roast / Gravy	Roast turkey / Gravy	not applicable
Mashed potatoes	Scalloped potatoes	Brown rice	
Green beans / Margarine	Peas & carrots	Broccoli	
Grapes	Dinner roll / Margarine	Dinner roll / Butter	
Snackwell cookies	Cantaloupe	Grapes	
Sprite	Yogurt	Raspberry bar	
	Peanut butter cookie	Apple juice	
	Grape juice		

Snack Session			
Admit Day 1	**Day 2**	**Day 3**	**Day 4**
Breakfast	***Breakfast 8AM***	***Breakfast 8AM***	***Breakfast 8AM***
not applicable	Decaf coffee or decaf tea	Decaf coffee or decaf tea	Regular breakfast meal after
			study is completed.

Lunch	***Lunch 12:30PM***	***Lunch 12:30PM***	***Lunch***
not applicable	Turkey Sandwich	Hamburger	not applicable
	Salad / Ranch dressing	Steak house fries	
	Pears	Salad / Fat free Italian dressing	
	Potato chips	Peaches	
	Graham crackers	Grape juice	
Dinner 5:45PM	***Dinner 5:45PM***	***Dinner 5:45PM***	***Dinner***
Italian chicken	Beef Roast / Gravy	Roast turkey / Gravy	not applicable
Mashed potatoes	Scalloped potatoes	Brown rice	
Green beans / Margarine	Peas & carrots	Broccoli	
Grapes	Dinner roll / Margarine	Dinner roll / Butter	
Snackwell cookies	Cantaloupe	Grapes	
Sprite	Yogurt	Raspberry bar	
	Peanut butter cookie	Apple juice	
	Grape juice		
Snack 11:00PM	***Snack 11:00PM***	***Snack 11:00PM***	***Snack***
Roast Beef Sandwich	English muffin / Margarine	Bagel / Cream cheese	not applicable
Orange	Fresh pork patty	Boiled egg	
Potato chips	Honey nut cheerios	Apple	
Graham crackers	Mandarin orange	String cheese	
Apple juice	Cranberry juice / 2% Milk	Orange juice	

B. Nutritional Summary

2500 kcal Diet

Meal Name	Avg. Total Grams	Avg. Energy (kcal)	Avg. Total Fat (g)	Avg. Total Carbohydrate (g)	Avg. Total Protein (g)
Breakfast	824.1 +/- 107.5	750.0 +/- 22.6	24.9 +/- 1.7	103.6 +/- 4.8	28.6 +/- 2.7
Lunch	624.1 +/- 121.3	691.9 +/- 29.6	20.6 +/- 29.6	105.3 +/- 7.5	26.5 +/- 7.7
Dinner	883.9 +/- 116.0	937.6 +/- 53.2	26.5 +/- 4.3	139.0 +/- 12.3	43.8 +/- 6.0
Snack	608.4 +/- 122.0	746.2 +/- 65.9	25.1 +/- 2.5	103.9 +/- 9.9	28.1 +/- 3.2
Beverage Only	236.3 +/- 7.8	3.1 +/- 0.1	0 +/- 0	0.7 +/- 0	0 +/- 0

2000 kcal Diet

Meal Name	Avg. Total Grams	Avg. Energy (kcal)	Avg. Total Fat (g)	Avg. Total Carbohydrate (g)	Avg. Total Protein (g)
Breakfast	632.2 +/- 157.9	624.8 +/- 72.8	22.9 +/- 4.7	77.0 +/- 4.6	26.9 +/- 6.4
Lunch	520.1 +/- 72.3	554.0 +/- 31.4	16.0 +/- 3.2	82.2 +/- 9.7	22.7 +/- 4.8
Dinner	715.2 +/- 50.8	736.7 +/- 49.2	19.5 +/- 2.1	106.7 +/- 10.2	38.0 +/- 5.0
Snack	506.1 +/- 72.4	579.5 +/- 37.5	19.3 +/- 4.1	79.5 +/- 8.3	24.0 +/- 1.5
Beverage Only	197.5 +/- 93.7	2.6 +/- 1.3	0 +/- 0	0.657 +/- 0.3	0 +/- 0

C. Nutritional Information for Each Subject

Subject	Date of Intake	Meal Name	Total Grams	Energy (kcal)	Total Fat (g)	Total Carbohydrate (g)	Total Protein (g)
1	11/5/15	Dinner/Supper	999.0	946.1	26.9	140.1	45.2
1	11/6/15	Breakfast	879.4	716.4	23.7	95.1	30.3
1	11/6/15	Lunch	509.2	691.5	18.2	108.5	33.1
1	11/6/15	Dinner/Supper	921.2	995.5	32.4	128.2	51.7
1	11/7/15	Breakfast	691.3	739.7	26.7	101.0	26.5
1	11/7/15	Lunch	544.2	688.4	23.7	100.2	19.7

1	11/7/15	Dinner/Supper	763.6	910.6	22.8	142.9	39.6
1	11/19/15	Dinner/Supper	1011.4	957.1	27.1	138.3	45.3
1	11/19/15	Snack	608.8	712.7	23.5	102.6	26.7
1	11/20/15	Beverage Only	241.2	3.2	0.0	0.8	0.0
1	11/20/15	Lunch	544.6	737.6	19.0	116.5	35.0
1	11/20/15	Dinner/Supper	919.1	1002.4	31.9	130.7	52.1
1	11/20/15	Snack	792.1	797.1	25.8	108.0	32.9
1	11/21/15	Beverage Only	240.9	3.2	0.0	0.8	0.0
1	11/21/15	Lunch	773.3	684.4	23.4	100.0	19.7
1	11/21/15	Dinner/Supper	760.5	910.6	22.8	142.9	39.6
1	11/21/15	Snack	445.9	724.4	26.9	96.3	26.7
2	1/6/16	Dinner/Supper	1072.6	989.2	28.3	146.1	47.1
2	1/6/16	Snack	499.3	803.3	26.5	116.7	29.0
2	1/7/16	Beverage Only	220.7	2.8	0.0	0.7	0.0
2	1/7/16	Lunch	509.6	684.0	17.9	107.6	32.4
2	1/7/16	Dinner/Supper	922.4	1011.5	32.7	131.1	52.2
2	1/7/16	Snack	714.1	805.4	25.4	110.4	33.3
2	1/8/16	Beverage Only	241.0	3.2	0.0	0.8	0.0
2	1/8/16	Lunch	762.3	681.4	23.4	99.3	19.6
2	1/8/16	Dinner/Supper	721.1	839.7	19.1	136.0	36.3
2	1/8/16	Snack	757.0	773.4	27.8	105.5	27.7
2	1/12/16	Dinner/Supper	972.2	923.3	25.9	140.5	41.5
2	1/13/16	Breakfast	943.0	783.9	24.3	107.4	33.2
2	1/13/16	Lunch	523.3	711.6	18.3	111.9	34.3
2	1/13/16	Dinner/Supper	912.7	970.6	29.9	130.2	49.0
2	1/14/16	Breakfast	748.7	761.8	26.5	106.0	27.4
2	1/14/16	Lunch	765.6	681.1	23.4	99.2	19.6
2	1/14/16	Dinner/Supper	757.1	877.4	19.7	141.4	39.3

Subject	Date of Intake	Meal Name	Total Grams	Energy (kcal)	Total Fat (g)	Total Carbohydrate (g)	Total Protein (g)
3	3/18/16	Dinner/Supper	975.4	925.9	26.4	143.2	38.5
3	3/18/16	Snack	607.3	744.3	23.8	108.6	27.7
3	3/19/16	Beverage Only	236.8	3.2	0.0	0.8	0.0
3	3/19/16	Lunch	524.9	719.6	18.5	113.6	34.3
3	3/19/16	Dinner/Supper	849.7	926.9	29.9	124.9	43.3
3	3/19/16	Snack	520.1	594.8	19.5	82.1	23.0
3	3/20/16	Beverage Only	237.2	3.2	0.0	0.8	0.0
3	3/20/16	Lunch	725.7	619.7	20.5	93.3	16.8

3	3/20/16	Dinner/Supper	671.2	828.8	21.3	132.9	32.0
3	3/20/16	Snack	531.1	760.4	26.9	105.8	26.4
3	4/1/16	Dinner/Supper	990.0	952.1	27.0	181.2	45.3
3	4/2/16	Breakfast	932.6	745.0	22.4	106.9	28.7
3	4/2/16	Lunch	532.3	719.6	18.4	113.6	34.6
3	4/2/16	Dinner/Supper	916.2	994.9	31.8	129.2	51.9
3	4/3/16	Breakfast	749.8	753.4	26.3	105.7	26.0
3	4/3/16	Lunch	774.9	684.3	23.4	100.0	19.7
3	4/3/16	Dinner/Supper	774.9	914.5	22.9	143.5	39.6
4	7/9/16	Dinner/Supper	707.1	734.4	21.1	110.5	32.6
4	7/10/16	Breakfast	761.9	579.9	18.3	80.0	23.5
4	7/10/16	Lunch	465.3	562.5	13.1	88.7	26.7
4	7/10/16	Dinner/Supper	729.9	724.6	20.0	96.1	41.7
4	7/11/16	Breakfast	674.8	586.3	22.1	74.0	23.2
4	7/11/16	Lunch	600.5	563.0	21.1	76.6	19.6
4	7/11/16	Dinner/Supper	764.1	844.2	20.8	130.7	39.5
4	7/21/16	Dinner/Supper	688.1	710.2	19.7	107.7	32.0
4	7/21/16	Snack	564.2	567.9	18.6	84.1	22.4
4	7/22/16	Beverage Only	233.2	3.2	0.0	0.8	0.0
4	7/22/16	Lunch	469.4	583.7	13.3	92.2	28.1
4	7/22/16	Dinner/Supper	734.2	757.0	22.1	98.0	43.6
4	7/22/16	Snack	496.8	625.0	20.7	82.4	26.8
4	7/23/16	Beverage Only	236.0	3.2	0.0	0.8	0.0
4	7/23/16	Lunch	553.0	489.7	17.7	68.7	16.5
4	7/23/16	Dinner/Supper	629.1	683.1	15.5	105.7	35.6
4	7/23/16	Snack	450.5	603.5	22.5	76.5	24.4

Subject	Date of Intake	Meal Name	Total Grams	Energy (kcal)	Total Fat (g)	Total Carbohydrate (g)	Total Protein (g)
5	6/21/16	Dinner/Supper	632.6	673.4	18.9	102.0	30.4
5	6/21/16	Breakfast	761.1	767.2	32.2	78.1	39.8
5	6/22/16	Lunch	411.0	561.9	13.0	88.7	27.0
5	6/22/16	Dinner/Supper	767.2	777.4	21.8	103.0	44.4
5	6/23/16	Breakfast	728.3	613.5	21.7	79.9	25.0
5	6/23/16	Lunch	559.8	501.5	19.1	68.3	17.8
5	6/23/16	Dinner/Supper	711.0	777.5	16.9	125.2	36.3
5	6/28/16	Dinner/Supper	690.3	721.8	20.5	110.1	31.0
5	6/28/16	Snack	561.1	523.7	17.7	74.6	22.4

5	6/29/16	Beverage Only	236.0	3.2	0.0	0.8	0.0
5	6/29/16	Lunch	424.4	575.1	13.3	91.2	26.8
5	6/29/16	Dinner/Supper	703.4	692.8	19.0	95.3	36.7
5	6/29/16	Snack	455.5	549.7	17.0	76.6	22.3
5	6/30/16	Beverage Only	236.1	3.2	0.0	0.8	0.0
5	6/30/16	Lunch	593.3	548.6	15.9	75.4	16.1
5	6/30/16	Dinner/Supper	717.2	733.6	17.5	111.8	38.1
5	6/30/16	Snack	421.5	632.4	24.1	78.9	25.2
6	2/3/17	Dinner/Supper	625.6	711.4	20.7	101.1	36.1
6	2/3/17	Snack	644.2	550.8	10.4	98.5	23.5
6	2/4/17	Beverage Only	6.3	0.0	0.0	0.0	0.0
6	2/4/17	Lunch	483.2	592.0	13.5	94.1	28.1
6	2/4/17	Dinner/Supper	758.3	768.0	22.1	98.5	45.7
6	2/4/17	Snack	514.8	561.2	20.7	69.0	24.7
6	2/5/17	Beverage Only	237.9	3.2	0.0	0.8	0.0
6	2/5/17	Lunch	596.9	553.6	20.7	75.4	19.6
6	2/5/17	Dinner/Supper	751.6	776.4	18.3	119.7	38.9
6	2/5/17	Snack	446.7	602.1	22.7	75.2	24.4
6	2/17/17	Dinner/Supper	719.5	652.9	18.3	93.2	35.2
6	2/18/17	Breakfast	461.0	628.4	21.3	81.0	26.9
6	2/18/17	Lunch	478.0	580.3	13.4	92.4	26.8
6	2/18/17	Dinner/Supper	820.6	809.2	23.1	104.4	48.0
6	2/19/17	Breakfast	406.6	573.6	22.5	69.0	23.3
6	2/19/17	Lunch	606.4	536.7	18.7	75.4	19.6
6	2/19/17	Dinner/Supper	725.0	713.4	15.9	109.6	38.3

Supplementary Table S4.2. Inclusion/Exclusion Criteria

Inclusion/Exclusion criteria

Inclusion Criteria --subject must

- Be able to understand the study, provide written informed consent (in English), and be able to fill out the questionnaire
- Be male or female older than 18 years of age
- Have a normal BMI (20-25) or be obese (BMI more than 30)
- Have a normal basal glucose level (70-100 mg/dL)
- If female of childbearing potential, have a negative pregnancy test on study day

Exclusion Criteria-- subject must not

- Be pregnant or lactating
- Have known sleep, metabolic (e.g., diabetes), or gastro-intestinal disorders except obesity
- Had alcohol less than 24 hours before admission
- Require assistance with activities of daily living
- Have difficulty swallowing
- Be unable to complete a food and sleep diary
- Be smokers

Supplementary Table S4.3. Subject Recruitment Questionnaire

1. When do you sleep on a typical day?
   a) Bedtime:_____(e.g., 10:00-11:00 PM)
   b) Wake-up time_____(e.g., 06:00-7:00 AM)

2. The following questions concern your typical meal times and food intake:

   a) Do you normally eat breakfast? ___Yes ___No
   If you answered Yes, what time do you usually eat breakfast? _____
   Please give an example of what you might eat for a typical breakfast (e.g., piece of toast, bowl of cereal, or bacon, eggs, & toast, etc).
   _____

   b) Do you normally eat lunch? ___Yes ___No
   If you answered Yes, what time do you usually eat lunch? _____
   Please give an example of what you might eat for a typical lunch
   _____

c) Do you normally eat dinner? ___Yes ___No
If you answered Yes, what time do you usually eat dinner? _____
Is dinner generally your largest meal of the day? ___Yes ___No

d) Do you frequently eat snacks between meals or after dinner? ___Yes ___No
If you answered Yes, what time(s) do you usually have your snacks relative to:
your main meals (check all that apply) ?
_____In the morning between breakfast and lunch
_____In the afternoon between lunch and dinner
_____After dinner

e) How many caffeinated drinks do you drink daily? (Please answer in terms of the number of cups of coffee or tea or number of portions of caffeinated soft drinks per
day): _____

f) What is your average alcohol intake? (Please answer in terms of the number of alcohol-containing drinks per week): _____

g) Do you have any dietary restrictions? ___Yes ___No
If you answered Yes, please describe what they are. _____

_____

3. Do you routinely exercise ___Yes ___No
If you answered Yes, please answer the following questions:
a) What type(s) of exercise do you do (e.g., jogging, swimming, yoga, etc.)
_____

b) When (relative to your mealtimes) do you normally exercise?
_____

c) In a typical week, how many days do you exercise?
____1-2 ____3-4 ____5-7

4. Have you ever been diagnosed with any of the following diseases: ___Yes ___No
Esophageal stricture
Diverticulosis
Inflammatory bowel disease (IBD),
Peptic ulcer disease
Crohn's disease
Ulcerative colitis

5. Have you ever had gastrointestinal surgery: ___Yes ___No

6. The protocol for this study involves swallowing a capsule the size of a large vitamin. Do you have any difficulty swallowing: ___Yes ___No

7. Do you have any chronic medical problems not listed above (e.g., diabetes, high blood pressure, asthma, etc)
___Yes ___No

If you answered yes, please describe them._____

_____

8. Are you currently taking any medications? ___Yes ___No

If you answered yes, please describe them. _____

_____

9. Do you have any sleep disorders (e.g., sleep apnea, insomnia, sleep walking, restless leg syndrome, etc.)?
___Yes ___No

If you answered yes, please describe them. _____

_____

10. Please complete the following demographic information:
Gender: _____male _____female
Age: _____ years
Ethnic group: _____ African-American
_____ Asian
_____ Caucasian
_____ Hispanic
_____ Other

Supplementary Table S4.4. Hour by Hour Mixed Model Analysis
A. RER

RER Pairwise	Hour	Estimate	SE	df	t.ratio	p.value
Breakfast - Snack	15	-0.0219	0.0131	5	1.6723	0.1553
Breakfast - Snack	16	-0.0191	0.0129	5	1.4761	0.1999
Breakfast - Snack	17	0.0099	0.0118	5	-0.8392	0.4396

Breakfast - Snack	18	0.0023	0.0117	5	-0.1951	0.853
Breakfast - Snack	19	-0.0171	0.0117	5	1.4586	0.2045
Breakfast - Snack	20	-0.0125	0.0117	5	1.065	0.3356
Breakfast - Snack	21	-0.0083	0.0117	5	0.7098	0.5095
Breakfast - Snack	22	-0.0522	0.0116	5	4.4943	0.0064
Breakfast - Snack	23	-0.0536	0.0116	5	4.6134	0.0058
Breakfast - Snack	0	-0.054	0.0116	5	4.6455	0.0056
Breakfast - Snack	1	-0.0503	0.0117	5	4.2948	0.0078
Breakfast - Snack	2	-0.0515	0.0117	5	4.3987	0.007
Breakfast - Snack	3	-0.0308	0.0117	5	2.6279	0.0466
Breakfast - Snack	4	-0.0265	0.0117	5	2.2608	0.0733
Breakfast - Snack	5	-0.0308	0.0117	5	2.6299	0.0465
Breakfast - Snack	6	-0.0199	0.0118	5	1.6892	0.152
Breakfast - Snack	7	-0.0069	0.0125	5	0.5527	0.6043
Breakfast - Snack	8	0.0299	0.0131	5	-2.2847	0.0711
Breakfast - Snack	9	0.032	0.0131	5	-2.4491	0.058
Breakfast - Snack	10	0.0138	0.0133	5	-1.0425	0.3449
Breakfast - Snack	11	0.0157	0.0131	5	-1.2025	0.283
Breakfast - Snack	12	0.0198	0.0131	5	-1.5133	0.1906
Breakfast - Snack	13	-0.0083	0.0131	5	0.632	0.5551
Breakfast - Snack	14	-0.0134	0.0131	5	1.0277	0.3512

B. Activity

Activity Pairwise	Hour	Estimate	SE	df	t.ratio	p.value
Breakfast - Snack	15	1.2773	0.4966	4	-2.5722	0.0618
Breakfast - Snack	16	-0.7947	0.4966	4	1.6003	0.1848
Breakfast - Snack	17	-0.3094	0.4066	4	0.7609	0.4891
Breakfast - Snack	18	0.4559	0.4066	4	-1.1211	0.325
Breakfast - Snack	19	-1.0109	0.4066	4	2.4861	0.0678
Breakfast - Snack	20	-0.449	0.4066	4	1.1043	0.3314
Breakfast - Snack	21	-0.3579	0.4066	4	0.8801	0.4285
Breakfast - Snack	22	-1.3114	0.4066	4	3.2253	0.0321
Breakfast - Snack	23	-0.164	0.4066	4	0.4033	0.7074
Breakfast - Snack	0	-0.3138	0.4066	4	0.7717	0.4834
Breakfast - Snack	1	0.36	0.4066	4	-0.8854	0.426
Breakfast - Snack	2	-0.488	0.4066	4	1.2002	0.2963
Breakfast - Snack	3	-0.0864	0.4066	4	0.2126	0.842
Breakfast - Snack	4	-0.1766	0.4066	4	0.4343	0.6865
Breakfast - Snack	5	-0.1878	0.4066	4	0.462	0.6681
Breakfast - Snack	6	-0.2409	0.4066	4	0.5925	0.5854
Breakfast - Snack	7	0.4582	0.4066	4	-1.1269	0.3228
Breakfast - Snack	8	0.7877	0.4966	4	-1.5863	0.1879
Breakfast - Snack	9	-0.5758	0.4966	4	1.1596	0.3107

Breakfast - Snack	10	0.298	0.5102	4	-0.5841	0.5905
Breakfast - Snack	11	0.0051	0.4966	4	-0.0102	0.9923
Breakfast - Snack	12	-0.4765	0.4966	4	0.9595	0.3917
Breakfast - Snack	13	0.7597	0.4966	4	-1.53	0.2008
Breakfast - Snack	14	0.6023	0.4966	4	-1.2128	0.2919

C. Temperature

Temperature Pairwise	Hour	Estimate	SE	df	t.ratio	p.value
Breakfast - Snack	15	0.0525	0.143	5	-0.367	0.7286
Breakfast - Snack	16	0.5505	0.1357	5	-4.0568	0.0098
Breakfast - Snack	17	-0.1352	0.1197	5	1.1297	0.3099
Breakfast - Snack	18	-0.055	0.114	5	0.4824	0.6499
Breakfast - Snack	19	-0.0562	0.1106	5	0.5084	0.6328
Breakfast - Snack	20	-0.106	0.1106	5	0.9587	0.3817
Breakfast - Snack	21	-0.081	0.1106	5	0.7331	0.4964
Breakfast - Snack	22	-0.0436	0.1106	5	0.3939	0.7099
Breakfast - Snack	23	-0.073	0.1121	5	0.6511	0.5437
Breakfast - Snack	0	-0.1591	0.1106	5	1.4391	0.2096
Breakfast - Snack	1	-0.1283	0.1121	5	1.1442	0.3043
Breakfast - Snack	2	-0.0812	0.1106	5	0.7342	0.4958
Breakfast - Snack	3	-0.0887	0.1121	5	0.7912	0.4647
Breakfast - Snack	4	-0.1382	0.1139	5	1.2139	0.279
Breakfast - Snack	5	-0.0937	0.1139	5	0.8225	0.4482
Breakfast - Snack	6	-0.0243	0.1139	5	0.213	0.8398
Breakfast - Snack	7	-0.0651	0.1197	5	0.5442	0.6097
Breakfast - Snack	8	0.002	0.1365	5	-0.0148	0.9888
Breakfast - Snack	9	0.0344	0.1401	5	-0.2459	0.8155
Breakfast - Snack	10	0.092	0.1553	5	-0.5926	0.5792
Breakfast - Snack	11	-0.025	0.1592	5	0.1569	0.8814
Breakfast - Snack	12	0.0777	0.143	5	-0.5435	0.6102
Breakfast - Snack	13	0.0547	0.143	5	-0.3823	0.7179
Breakfast - Snack	14	0.036	0.143	5	-0.2516	0.8113

D. Metabolic Rate

Metabolic Rate Pairwise	Hour	Estimate	SE	df	t.ratio	p.value
Breakfast - Snack	15	0.068	0.0771	5	-0.8814	0.4185
Breakfast - Snack	16	0.0887	0.0759	5	-1.1692	0.295
Breakfast - Snack	17	0.1747	0.0667	5	-2.617	0.0473
Breakfast - Snack	18	0.1899	0.066	5	-2.8778	0.0347
Breakfast - Snack	19	0.031	0.066	5	-0.47	0.6582
Breakfast - Snack	20	0.1421	0.066	5	-2.1528	0.0839

Breakfast - Snack	21	0.0552	0.066	5	-0.836	0.4413
Breakfast - Snack	22	-0.3364	0.0652	5	5.1592	0.0036
Breakfast - Snack	23	-0.1839	0.0652	5	2.8196	0.0371
Breakfast - Snack	0	-0.0874	0.0652	5	1.3409	0.2377
Breakfast - Snack	1	-0.0669	0.066	5	1.0134	0.3574
Breakfast - Snack	2	-0.0592	0.066	5	0.8974	0.4107
Breakfast - Snack	3	0.0151	0.066	5	-0.2283	0.8285
Breakfast - Snack	4	0.0468	0.066	5	-0.709	0.51
Breakfast - Snack	5	0.0377	0.066	5	-0.5706	0.593
Breakfast - Snack	6	0.052	0.0668	5	-0.7787	0.4714
Breakfast - Snack	7	0.2516	0.0748	5	-3.3623	0.0201
Breakfast - Snack	8	0.218	0.0771	5	-2.8262	0.0368
Breakfast - Snack	9	0.2154	0.0771	5	-2.792	0.0384
Breakfast - Snack	10	0.1684	0.0787	5	-2.1405	0.0853
Breakfast - Snack	11	0.1639	0.0771	5	-2.1246	0.087
Breakfast - Snack	12	0.0911	0.0771	5	-1.1807	0.2908
Breakfast - Snack	13	0.143	0.0771	5	-1.8543	0.1229
Breakfast - Snack	14	0.2571	0.0771	5	-3.3332	0.0207

E. Carbohydrate Oxidation

Carbohydrate Oxidation Pairwise	hour	estimate	SE	df	t.ratio	p.value
Breakfast - Snack	15	-0.0258	0.0225	5	1.1495	0.3023
Breakfast - Snack	16	-0.0207	0.0221	5	0.9377	0.3915
Breakfast - Snack	17	0.0309	0.0197	5	-1.5704	0.1771
Breakfast - Snack	18	0.0309	0.0195	5	-1.5817	0.1746
Breakfast - Snack	19	-0.024	0.0195	5	1.2298	0.2735
Breakfast - Snack	20	-0.0018	0.0195	5	0.0947	0.9282
Breakfast - Snack	21	-0.0082	0.0195	5	0.4182	0.6931
Breakfast - Snack	22	-0.1342	0.0193	5	6.9532	9.00E-04
Breakfast - Snack	23	-0.0978	0.0193	5	5.0662	0.0039
Breakfast - Snack	0	-0.0764	0.0193	5	3.9563	0.0108
Breakfast - Snack	1	-0.0721	0.0195	5	3.6973	0.014
Breakfast - Snack	2	-0.0732	0.0195	5	3.752	0.0133
Breakfast - Snack	3	-0.0398	0.0195	5	2.0397	0.0969
Breakfast - Snack	4	-0.0282	0.0195	5	1.4434	0.2085
Breakfast - Snack	5	-0.0367	0.0195	5	1.8788	0.1191
Breakfast - Snack	6	-0.0245	0.0197	5	1.2428	0.2691
Breakfast - Snack	7	0.0115	0.0218	5	-0.5267	0.6209
Breakfast - Snack	8	0.0646	0.0225	5	-2.8769	0.0347
Breakfast - Snack	9	0.0698	0.0225	5	-3.1083	0.0266
Breakfast - Snack	10	0.0504	0.0229	5	-2.2047	0.0786
Breakfast - Snack	11	0.0468	0.0225	5	-2.0845	0.0915
Breakfast - Snack	12	0.0476	0.0225	5	-2.1187	0.0877

Breakfast - Snack	13	0.0117	0.0225	5	-0.523	0.6233
Breakfast - Snack	14	0.0148	0.0225	5	-0.6571	0.5402

F. Lipid Oxidation

Lipid Oxidation Pairwise	Hour	Estimate	SE	df	t.ratio	p.value
Breakfast - Snack	15	0.0172	0.0079	5	-2.1771	0.0814
Breakfast - Snack	16	0.0174	0.0078	5	-2.2315	0.076
Breakfast - Snack	17	0	0.007	5	-7.00E-04	0.9994
Breakfast - Snack	18	0.0076	0.0069	5	-1.1047	0.3196
Breakfast - Snack	19	0.0127	0.0069	5	-1.8343	0.1261
Breakfast - Snack	20	0.0155	0.0069	5	-2.2461	0.0746
Breakfast - Snack	21	0.009	0.0069	5	-1.2967	0.2513
Breakfast - Snack	22	0.0178	0.0068	5	-2.6009	0.0482
Breakfast - Snack	23	0.0193	0.0068	5	-2.8271	0.0368
Breakfast - Snack	0	0.021	0.0068	5	-3.0619	0.028
Breakfast - Snack	1	0.0214	0.0069	5	-3.0991	0.0269
Breakfast - Snack	2	0.0226	0.0069	5	-3.2752	0.0221
Breakfast - Snack	3	0.0172	0.0069	5	-2.4921	0.055
Breakfast - Snack	4	0.0159	0.0069	5	-2.3075	0.0691
Breakfast - Snack	5	0.0183	0.0069	5	-2.6536	0.0452
Breakfast - Snack	6	0.0151	0.007	5	-2.1623	0.0829
Breakfast - Snack	7	0.0218	0.0077	5	-2.8242	0.0369
Breakfast - Snack	8	-0.0027	0.0079	5	0.3448	0.7442
Breakfast - Snack	9	-0.005	0.0079	5	0.6378	0.5517
Breakfast - Snack	10	-0.0023	0.0081	5	0.2818	0.7894
Breakfast - Snack	11	-0.0014	0.0079	5	0.1719	0.8703
Breakfast - Snack	12	-0.0092	0.0079	5	1.1675	0.2957
Breakfast - Snack	13	0.0103	0.0079	5	-1.2969	0.2513
Breakfast - Snack	14	0.021	0.0079	5	-2.6474	0.0456

CHAPTER V

Temperature compensation of mammalian cells influenced by metabolic regulators

Abstract

A key function of the circadian clock is its ability to maintain a constant period at different ambient temperatures, a property known as “temperature compensation”. While the temperature compensation of circadian clocks has long been observed, the mechanisms behind temperature compensation are poorly understood. One hypothesis (Pittendrigh, 1954) is that the cell’s metabolic pathways that are involved in maintaining homeostasis also allow for the core clock complex to be temperature compensated. However, to date this hypothesis has never been proven. In this study, we screened chemical compounds known to affect metabolism using Rat1 and U2OS cell lines with a core clock reporter to properties of temperature compensation. We find that compounds that effect AMPK and mTOR affect the temperature compensation properties of both Rat1 and U2OS cell lines. These findings implicate metabolism in maintaining the core clock’s temperature compensation properties.

Introduction

One of the most incredible aspects of the circadian clock is its temperature compensation properties. Circadian clocks maintain their near 24-hour period across a large range of constant ambient temperatures. This resistance to temperature is known as temperature compensation and is unusual as most biological reactions change their

rate in a temperature-dependent manner (Reyes *et al.*, 2008). This effect has been noted since a seminal publication in 1954 (Pittendrigh, 1954). It is important for the core circadian clock to be temperature compensated in order to avoid changes to period timing due to fluctuations in temperature. Yet, it is still not clear how this compensation is performed or why it is present in organisms that can thermoregulate like mammals. Pittendrigh, after his original 1954 paper, later suggested that the temperature compensation effects seen in circadian rhythms were the result of a general homeostatic property in the cell (Pittendrigh and Caldarola, 1973); however, almost no data were presented to support that hypothesis.

There is evidence that an unknown metabolic regulator can influence circadian rhythms in mammals. Research has shown that giving mice HFD causes mice to go arrhythmic (Kohsaka *et al.*, 2007). Furthermore, meal timing is also a known zeitgeber, or time giver, to which the circadian clock can entrain (Damiola *et al.*, 2000). Further evidence that metabolism can influence entrainment is the food anticipatory activity found in studies that research the food entrainable oscillator (Landrey *et al.*, 2006, Pendergast *et al.*, 2012). In these studies, there is a clear effect of metabolism on entrainment but the mechanism behind it is still largely a mystery. In fact, the food entrainable oscillator is able to function without the suprachiasmatic nuclei or core clock genes.

There are many potential metabolic enzymes that could be involved in keeping clock period constant in the face of changing temperature and many metabolites that are known to affect period in mammalian cells. For our study, we focused on the AMP activated protein kinase (AMPK) that regulates energy homeostasis, which it senses

through the amount of adenosine monophosphate (AMP) in the cell (Crozet *et al.*, 2014). In a low energy environment, AMP levels in the cell are high and AMPK becomes activated. AMPK then regulates energy homeostasis by inhibiting the mTOR pathway, lipid homeostasis, glycolysis, and mitochondria homeostasis through biogenesis/mitophagy (Egan *et al.*, 2011). Furthermore, disruption of AMPK with AICAR, an AMPK agonist, has been shown to alter period in cells (at a single temperature) (Mosser *et al.*, 2019, Lamia *et al.*, 2009). The energy sensing ability of AMPK and its nodal power to affect myriad pathways would make AMPK a good candidate gene to modulate metabolism and test its effects on circadian period at different temperatures.

This project aimed to test the hypothesis that affecting metabolic processes can affect temperature compensation in mammalian cell lines. To test this hypothesis, we used compounds that are known to perturb metabolic pathways and tested the effect of these compounds on circadian period at various temperatures. We found compounds that affected AMPK and mTOR pathways, major regulators of a variety of metabolic pathways, affected the temperature compensation properties of mammalian cell cultures. My data show that AMPK modulates the ability of cells to compensate.

Methods

Cell culture

U2OS with a BMAL1:Luc reporter and Rat1 cells with a PER2:Luc reporter were used. Cell lines were confirmed to be U2OS (human osteosarcoma) and Rat1 (rat fibroblasts) cells through genotyping by the American Type Culture Collection (ATCC)

prior to experimentation. Cells were maintained in Gibco DMEM medium (4.5g/L glucose) supplemented with 10% fetal bovine serum (FBS), and 1% Penicillin/Streptavidin (P/S) antibiotics in 100mm cell culture dishes. Cells were kept in an 35°C incubator with a 5% CO_2 concentration. All transfers were performed in a biosafety cabinet to avoid potential contamination. Once cells had reached confluency, they were washed with PBS and detached from the dish with 1X trypsin-EDTA in PBS. The cells were either replated at 10% of the original cell concentration or used in Lumicycle experiments. To avoid changes in period due to mutation, cells were replaced with stock cells kept in liquid nitrogen storage after 8 splits.

Lumicycle experiments

3X10^5 cells were cultured on a 35mm cell culture dish for 5 days in the DMEM medium listed above to achieve confluency. Cells were then treated with 10µM Dexamethasone for two hours to synchronize the cells in the population to the same circadian phase. Dexamethasone-containing medium was then aspirated and the dishes washed with PBS. The PBS was then also aspirated and replaced with DMEM media without phenol red, 10% FBS, 1% P/S, 100nM Luciferin, and the compound being tested. The covers for the culture dishes were then replaced with 35mm glass coverslips and were sealed onto the culture dishes with vacuum grease. The sealed culture dishes were then transferred to an environmental chamber set at 32°C containing two 32 channel Lumicycles (luminometers designed by ActiMetrics). One Lumicycle was set up in the environmental chamber while the other Lumicycle was housed in an incubator at either 35°C or 38°C. The culture dishes for each treatment

were divided into the two separate Lumicycles and their luminescence was recorded for seven days. Therefore, for each experiment, either 32°C and 35°C or 32°C and 38°C were measured simultaneously.

Statistical analysis

Q10 was calculated using the formula shown in Reyes *et al.* (2008) using the 32°C and 38°C data from U2OS and Rat1 cell lines (referred to as $Q10_{32°/38°}$). Period values were determined using ActiMetrics ClockLab software using a cosinor fit model. Differences in period over temperatures were determined using two-way ANOVA.

Results

To determine if changes in metabolic rate could in turn affect the temperature compensation of the core clock machinery, we assessed a number of metabolic pathways using compounds that are known to either inhibit or activate key metabolic enzymes. I assessed these affecters for effects on period at three different temperatures using the metrics shown in figure 5.1. To assess effects on the core clock period, we used a human osteosarcoma cell line (U2OS) stably transfected with a luciferase reporter attached to the BMAL1 gene promoter, and a Rat1 fibroblast cell line that was stably transfected with the PER2 promoter/reporter. Both BMAL1 and PER2 are critical components of the core clock complex that are rhythmically regulated and thus in untreated cell lines, we observe a rhythmic oscillation of expression with a roughly 24-hour period in both cell lines (Figure 5.1). By adding the luciferin substrate to the cell culture media, the enzymatic activity of luciferase produces luminescence that

can be used as a metric for gene expression of BMAL1 and PER2 (in our case) (Yamazaki and Takahashi, 2005). Using these reporter cell lines, we then assessed period at various temperatures. We find that both cell lines showed the most robust period at 35°C (the temperature where our lab and other labs typically culture these cell lines) and 38°C. Cell lines incubated at 32°C overall showed a damped amplitude of their rhythms and showed fewer cycles than the other two temperatures but still had 2-3 full cycles for a period estimate using a Chi-squared test.

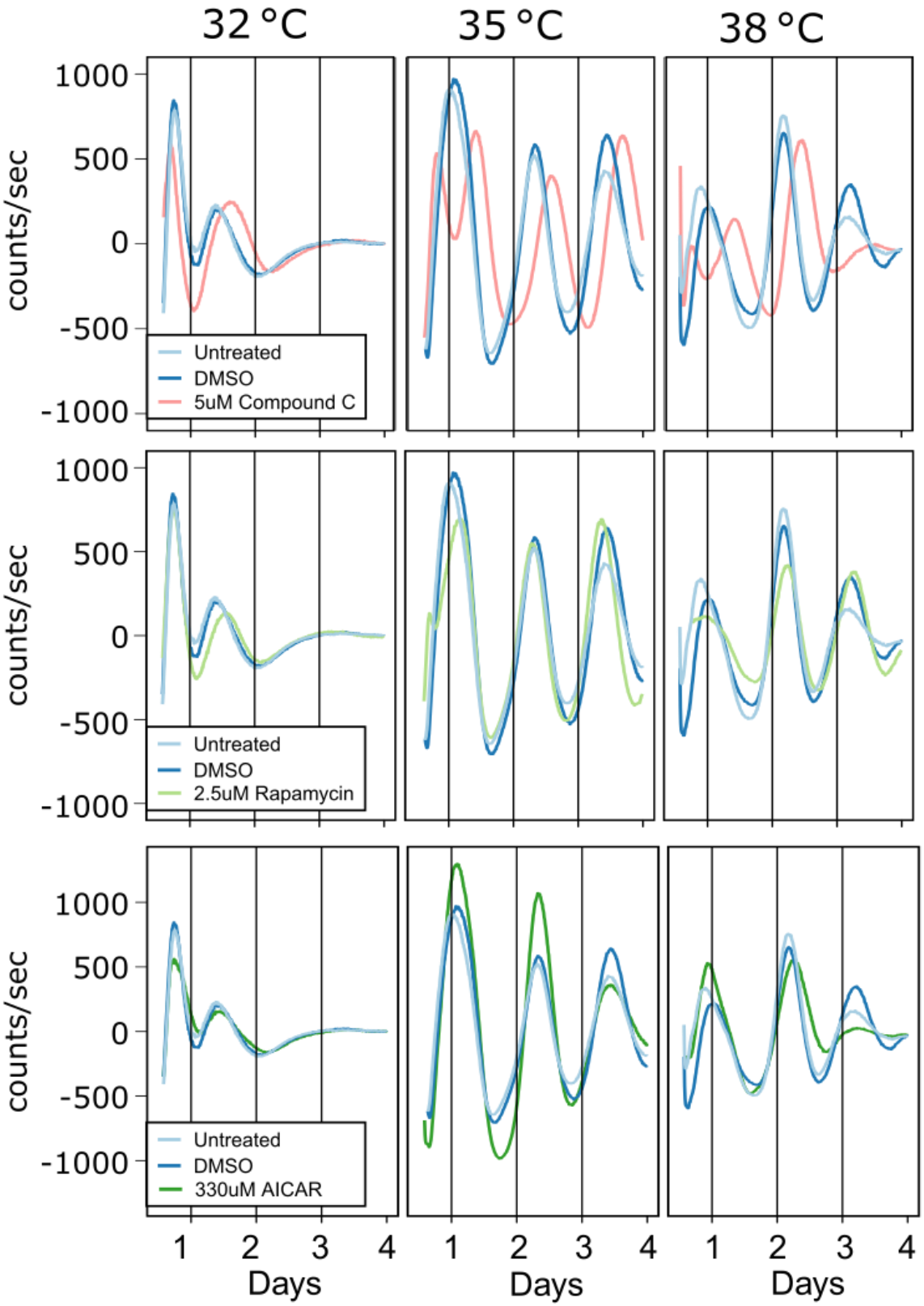


Figure 5.1. Detrended luminescence data over time in U2OS cells. Four days of the detrended counts/sec luminescence of representative traces of U2OS cells with a

BMAL1:Luc reporter. Vertical black lines denote 24 hours of time elapsed. Columns indicate luminescence rhythms taken at 32°, 35°, 38° degrees Celsius. Top row compares luminescence rhythms of untreated cells and cells treated with DMSO (vehicle control), or 5μM Compound C. Middle row compares luminescence rhythms of untreated, DMSO, and 2.5μM Rapamycin treated cells. Bottom row compares luminescence rhythms of Untreated, DMSO, and 330μM AICAR treated cells (n=6-12).

Before treating cells with compounds that perturb metabolism, we first analyzed the effect of different ambient temperatures on the period of both U2OS and Rat1 cell lines. We noted that the U2OS and Rat1 cell lines showed different Q10's and period values. In the case of U2OS, the untreated and DMSO controls have periods slightly above 24 hours at the highest temperature and slightly under 24 hours with a $Q10_{32°/38°}$ of 0.978 (Table 5.1.). Rat1 cells have a period of 24 hours at 38°C as do the U2OS cells, but show a decrease in period as the temperature decreases ending at period of 20 hours at 32°C and a $Q10_{32°/38°}$ of 0.752.

To assess how perturbing metabolism could affect temperature compensation of the clock, we assessed several compounds with known effects on metabolism and redox at different temperatures. To do this we calculated Q10, which is a coefficient that defines the rate of change of a biological or chemical system when temperature is increased by 10°C (Reyes *et al.*, 2008).Under normal conditions as shown in Figure 5.2., the period in both human and mouse cell lines is slightly altered over different temperatures. However, these periods have a Q10 near 1, which is indicative of temperature compensation as the vast majority of enzymatic activities have a Q10 of 2 or higher (Reyes *et al.*, 2008). We then compared the period of our DMSO (vehicle) control to a variety of different compounds that perturbed metabolism in various ways. Many compounds with a known effect on metabolism did not have an effect or showed similar period effects across all treatments such as Conoidin A (peroxiredoxin inhibitor),

PUGNAc (O-glycNAc inhibitor), Alloxan (glucose analogue), Carboxine (antihistamine), 16F16 (Protein Disulfide Isomerase (PDI) inhibitor), Alpha Ketoglutarate (key molecule in citric acid cycle), SR9009 (Rev-ERBA agonist), or N-Acetyl-Cysteine (glutathione precursor) (data not shown). However, we saw altered period effects depending on the temperature in the following compounds: Compound C (an AMPK antagonist (Liu *et al.*, 2014)), AICAR (an AMPK agonist (Kim *et al.*, 2016)), and Rapamycin (mTOR antagonist (Dumont and Su, 1995)).

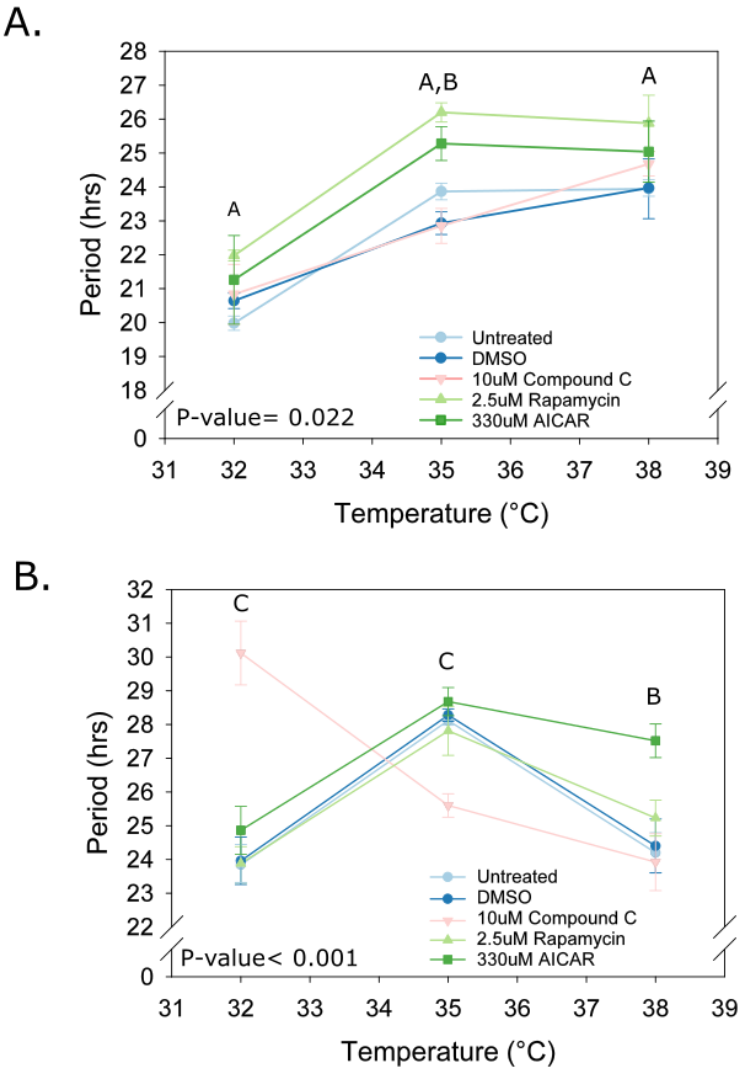


Figure 5.2. AMPK and mTOR target compounds alter period in a temperature dependent manner in U2OS and Rat1 cell lines. Average period values for Rat1 (A)

and U2OS (B) at various temperatures of untreated cells (light blue) or cells that have been treated with DMSO (dark blue), Compound C (pink), Rapamycin (light green), or AICAR (dark green). Error bars indicate standard deviation (n=6-9). Letters indicate P-value <0.05 between experimental treatments versus the DMSO vehicle control after a two-way ANOVA analysis of temperature and treatment on period followed by a Holms-Sidak post hoc test: A= Rapamycin v. DMSO, B=AICAR v. DMSO, and C=Compound C v. DMSO. All significant interactions can be found in the (Tables S5.1 and S5.2)

We also observed varying temperature compensation effects of Compound C, AICAR, and Rapamycin depending on the cell type. Figure 5.2. shows average period values of Rat1 and U2OS cell lines for Untreated, DMSO, Compound C, AICAR, and Rapamycin and significance was assessed between experimental compounds to DMSO using two-way ANOVA followed by a Holms-Sidak post hoc test. Compound C showed no change in period for Rat1 cells compared to the vehicle control. However, we see clear temperature effects of compound C in the U2OS cell line, with a large increase in period at 32°C, as compared with 35°C, and 38°C (Fig. 5.2). This caused the $Q10_{32°/38°}$ to increase in U2OS to 1.5 (Table 5.1.). Similarly, Rapamycin has no effect on period in the U2OS but elevated the period at all temperatures for Rat1 and decreased the $Q10_{32°/38°}$ compared to DMSO, although very slightly. Finally, AICAR affected temperature compensation in both U2OS and Rat1 cells. AICAR elevated the period at 35°C only for Rat1, and in U2OS cells it elevated the period only at 38°C, causing a decrease in the $Q10_{32°/38°}$ (Table 5.1). While the specific effect is variable between cell lines, it seems AMPK and mTOR generally have a temperature dependent effect on period.

Treatment	U2OS $Q10_{32°/38°}$	Rat1 $Q10_{32°/38°}$
Untreated	0.978	0.752
DMSO	0.973	0.783
Compound C	1.486	0.755
Rapamycin	0.919	0.775
AICAR	0.853	0.774

Table 5.1. $Q10_{32°/38°}$ values of cell lines based on treatment.

Discussion

The core clock mechanism has long been known to resist changes in period in response to different ambient temperature. This phenomenon is unusual as the rate of most biological processes is affected by temperature. Since this temperature compensation effect remains in mammals (Buhr *et al.* 2010, Pittendrigh C.S., 1954) that self-regulate their temperature, it is possible that the temperature compensation of the clock mechanism is not merely a response to changes in temperature (which are mostly absent in endotherms) but to metabolic changes. In this study, we assessed how temperature compensation may be a method cells use to respond to changes in metabolism resulting from the circadian meal timing effects discussed in previous chapters.

We first tested a number of potential compounds with known effects on metabolism and measured their effects on period at various temperatures. Compounds with known metabolic targets that affected temperature compensation were defined based on whether they significantly changed the period in a temperature-dependent manner. The first initial finding from this study is that between Rat1 and U2OS cell lines, the starting Q10 was not the same. In both cases, Rat1 and U2OS untreated period values were considered thermally independent with values close to 1, but the

Rat1 cells were more “over-compensated” (Q10<1). Therefore, Rat1 cells showed a much shorter period at the colder temperatures than did U2OS cells.

Surprisingly, we find that many of the compounds treated created non-linear affects on period at different ambient temperatures. This finding brings up a current issue in the field, namely that Q10 is a temperature coefficient based on a single enzyme activity model (Reyes et al., 2008). However, there are a number of studies that show temperature compensation using Q10 in a myriad of models in the circadian field (Barret et al., 1995, Izumo et al., 2003, Kusakina et al., 2004). However, it is also important to note that these studies also only look at two temperatures when measuring their Q10 values. In this study I find that Q10 varies in a non-linear fashion in almost all cases with the exception of DMSO or Compound C treatment in Rat1 cells when three temperature are compared (Figure 5.2). This is likely because assessing period as a proxy for enzymatic activity is an oversimplification as there are multiple proteins and enzymes involved in the creation of the circadian period. There have been some recent studies that suggest replacing Q10 in favor of more accurate mathematical models such as the temperature-amplitude coupling model performed by Kurosawa et al. (2017). However, this method has not been confirmed outside of their experiment on yeast and developing/testing new models to employ as a better assessment of temperature compensation was outside the scope of this project. However, my results indicate that Q10 may be an oversimplification of a more complex temperature compensation mechanism at play and future studies will need to create and validate a more robust model to analyze the temperature compensation properties of circadian period in model organisms/mammalian cell culture.

Despite the fact that the different cell lines had different initial Q10s, we still found compounds that affected temperature compensation in both cell lines. These were Compound C, AICAR, and Rapamycin. Compound C and AICAR are known AMPK antagonist and agonist respectively (Liu *et al.*, 2014, Kim *et al.*, 2016). AMPK is a major metabolic regulator, notably responsible for regulation of energy homeostasis and glucose uptake/fatty acid biosynthesis (Wu *et al.*, 2013, Carling *et al.*, 1987). These compounds had a drastic effect on temperature compensation of U2OS cells and interestingly showed opposite effects on Q10 as would be expected from compounds that have opposite effects on AMPK activity. On the other hand, in the Rat1 cell line, Compound C did not show a significant effect on period while AICAR at only the middle temperature (35° C) caused a slight decrease in the Q10 coefficient. The mTOR pathway, another large metabolic pathway that regulates a number of downstream processes based on cellular nutrient, oxygen, and energy levels (Saxton, R.A. and Sabitini, D.M. 2017) was also found to affect temperature compensation. In a similar fashion to the AMPK pathway, we saw a large effect of Rapamycin, a known mTOR antagonist, in Rat1 cells causing a lower Q10 but there was little effect in U2OS cells. Please note that none of these compounds altered temperature compensation to the point of being considered temperature dependent. An important caveat of these experiments is that AMPK and mTOR are both regulators of large metabolic pathways, and thus we have not yet isolated what specific enzyme(s) are causing these changes in Q10 or if the effects we are seeing are due to direct influence on the core clock complex or caused by transient effects. This will need to be further researched in future experiments. However, these data suggest that the changes in Q10 caused by AMPK

and mTOR would not remove the temperature independence of the clock proteins but may help modulate the period to make fine adjustments and changes in Q10 to the cells period in response to a metabolic challenge.

From this study, I found that AMPK and mTOR are metabolic pathways that can influence the cellular temperature compensation of the core clock mechanism. AMPK is an antagonist of the mTOR pathway, suggesting that the downstream pathway involving AMPK and mTOR could be responsible for the changes in temperature compensation observed in both cell lines. This work also shows a potential mechanism whereby metabolic pathways can alter period in individual cells independent of the core clock mechanism and SCN, thus providing a potential mechanism to explain the changes in period caused by metabolic changes resulting from the food entrainable oscillator or meal timing (Landry *et al.*, 2006, Pendergast *et al.*, 2013).

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Supplementary Information

Supplemental Table S5.1. Two-way ANOVA data of Rat1 Period data across temperature and pharmacological treatment.

A. Overall interactions

Source of Variation	DF	SS	MS	F	P
Temperature	2	181.539	90.769	209.059	<0.001
Treatment	4	35.226	8.806	20.283	<0.001
Temperature x Treatment	8	8.898	1.112	2.562	0.022

B. Interactions within temperatures

Comparisons for factor: Treatment within 32°C

Comparison	Diff of Means	t	P	P<0.050
Rapamycin vs. Untreated	2	5.257	<0.001	Yes
Rapamycin vs. DMSO	1.34	3.522	0.009	Yes
AICAR vs. Untreated	1.28	3.365	0.013	Yes
Rapamycin vs. Compound C	1.148	2.877	0.043	Yes
Compound C vs. Untreated	0.852	2.135	0.21	No
Rapamycin vs. AICAR	0.72	1.893	0.286	No
DMSO vs. Untreated	0.66	1.735	0.314	No
AICAR vs. DMSO	0.62	1.63	0.296	No
AICAR vs. Compound C	0.428	1.073	0.495	No
Compound C vs. DMSO	0.192	0.481	0.633	No

Comparisons for factor: Treatment within 35°C

Comparison	Diff of Means	t	P	P<0.050
Rapamycin vs. Compound C	3.347	5.564	<0.001	Yes
Rapamycin vs. DMSO	3.267	5.431	<0.001	Yes
AICAR vs. Compound C	2.427	4.51	<0.001	Yes
AICAR vs. DMSO	2.347	4.362	<0.001	Yes
Rapamycin vs. Untreated	2.333	3.879	0.002	Yes
AICAR vs. Untreated	1.413	2.627	0.058	No
Untreated vs. Compound C	1.013	1.883	0.24	No
Untreated vs. DMSO	0.933	1.735	0.246	No
Rapamycin vs. AICAR	0.92	1.529	0.249	No
DMSO vs. Compound C	0.08	0.149	0.882	No

Comparisons for factor: Treatment within 38°C

Comparison	Diff of Means	t	P	P<0.050
Rapamycin vs. Untreated	1.933	3.593	0.008	Yes

Rapamycin vs. DMSO	1.907	3.544	0.009	Yes
Rapamycin vs. Compound C	1.2	2.23	0.223	No
AICAR vs. Untreated	1.093	2.032	0.293	No
AICAR vs. DMSO	1.067	1.983	0.282	No
Rapamycin vs. AICAR	0.84	1.561	0.489	No
Compound C vs. Untreated	0.733	1.363	0.548	No
Compound C vs. DMSO	0.707	1.313	0.48	No
AICAR vs. Compound C	0.36	0.669	0.757	No
DMSO vs. Untreated	0.0267	0.0496	0.961	No

Supplemental Table S5.2. Two-way ANOVA data of U2OS Period data across temperature and pharmacological treatment.

A. Overall interactions

Source of Variation	DF	SS	MS	F	P
Temperature	2	68.487	34.244	82.718	<0.001
Treatment	4	21.719	5.43	13.116	<0.001
Temperature x Treatment	8	163.429	20.429	49.347	<0.001

B. Interactions within temperatures

Comparisons for factor: Treatment within 32°C

Comparison	Diff of Means	t	P	P<0.050
Compound C vs. Untreated	6.28	16.906	<0.001	Yes
Compound C vs. Rapamycin	6.24	16.798	<0.001	Yes
Compound C vs. DMSO	6.16	16.583	<0.001	Yes
Compound C vs. AICAR	5.256	13.491	<0.001	Yes
AICAR vs. Untreated	1.024	2.628	0.069	No
AICAR vs. Rapamycin	0.984	2.526	0.074	No
AICAR vs. DMSO	0.904	2.32	0.096	No
DMSO vs. Untreated	0.12	0.323	0.984	No
DMSO vs. Rapamycin	0.08	0.215	0.971	No
Rapamycin vs. Untreated	0.04	0.108	0.915	No

Comparisons for factor: Treatment within 35°C

Comparison	Diff of Means	t	P	P<0.050
AICAR vs. Compound C	3.08	5.863	<0.001	Yes
DMSO vs. Compound C	2.68	5.101	<0.001	Yes
Untreated vs. Compound C	2.52	4.797	<0.001	Yes
Rapamycin vs. Compound C	2.213	4.213	<0.001	Yes
AICAR vs. Rapamycin	0.867	1.65	0.49	No
AICAR vs. Untreated	0.56	1.066	0.822	No

DMSO vs. Rapamycin	0.467	0.888	0.851	No
AICAR vs. DMSO	0.4	0.761	0.834	No
Untreated vs. Rapamycin	0.307	0.584	0.808	No
DMSO vs. Untreated	0.16	0.305	0.762	No

Comparisons for factor: Treatment within 38°C

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
AICAR vs. Compound C	3.6	6.853	<0.001	Yes
AICAR vs. Untreated	3.32	6.32	<0.001	Yes
AICAR vs. DMSO	3.12	5.939	<0.001	Yes
AICAR vs. Rapamycin	2.293	4.365	<0.001	Yes
Rapamycin vs. Compound C	1.307	2.487	0.096	No
Rapamycin vs. Untreated	1.027	1.954	0.254	No
Rapamycin vs. DMSO	0.827	1.574	0.408	No
DMSO vs. Compound C	0.48	0.914	0.745	No
Untreated vs. Compound C	0.28	0.533	0.837	No
DMSO vs. Untreated	0.2	0.381	0.705	No

Chapter VI

Conclusion

Timing of meals alters weight gain through lipid oxidation

My work in chapters II and IV has shown that in mice and humans altering the meal timing without influencing the fasting duration can cause differential weight gain via changes in lipid oxidation. In particular, my study shows that shifting meal timing alters the timing of carbohydrate and lipid oxidation. Both the human and mice studies also agree that subjects getting the majority of their calories near the end of the active phase show lower initial lipid oxidation that is likely the cause of differential weight gain seen in the mice in chapter II. Importantly, the results from my dissertation suggest that having the majority of calories consumed near the onset of activity decreases the time necessary to switch from carbohydrate to lipid oxidation during the inactive period and ultimately leading to more fat being burned. In context with the current understanding of the circadian literature, my research shows that feeding duration is not the only important component to circadian meal timing but that onset/offset and meal dispersal play critical roles in lipid oxidation as well. This information could also be beneficial weight loss and shift workers to design meal plans having the onset of feeding in line with onset of activity may help reduce the risk of obesity.

Findings from chapter II also show that altering the onset/offset of feeding leads to a short-term change in metabolism that ultimately leads to differences in weight gain later on. This data suggests that the shift to a new meal time is more critical to weight

gain than when the meal timing normally occurs. A good test of this would be to have human subjects undergo our protocol in chapter IV but have the subjects maintain the evening snack schedule two weeks in advance before entering the chamber. I predict that difference in daily lipid oxidation we saw in chapter IV would be lost due to the subjects' metabolism adapting to the new meal schedule. There is evidence by Chaix *et al.* (2014) that changing mice under HFD *ad libitum* to a restricted feeding is able to decrease weight gain. However, this study was done using feed restriction and not changing onset/offset of meal timing. Another potential experiment to test in mice would be to put them on a meal timing schedule that shifts from Early night to Late night every 5 days. I predict that because these mice have a constantly shifting meal time, they will gain weight similar to the HFD *ad libitum* group.

Another interesting finding from the mouse work is that there are clear differences consistent with a shift in meal timing between the Early night with RC and the Late night with RC groups even though based on the food intake analysis from both groups have the same onset of feeding (Figure 4.4.). These results suggest that the peak shifts in RER seen in my mouse and human work may be more influenced by where the majority of the calories are temporally rather than the time of the first meal and the time of the last meal. An experiment to look into this would be to have a meal at the onset of activity and the offset of activity and change the proportion of the calories between these two meals without altering the total calories the individual intakes and monitor the RER peak shift via indirect calorimetry. One study by Kuroda *et al.* 2012 has tested the effects of meal dispersal and how this affects peripheral clocks by increasing the meal frequency. Interestingly, the study found that while dispersing

meals evenly throughout 24 hours did not affect mice, having a dinner shift to the inactive phase caused a phase advance in kidney and liver clocks. However, this effect was lost when the dinner was subsequently divided into two smaller meals, with the feeding offset unaffected. Nonetheless, this study did not look at metabolic effects, which would be interesting to see if carbohydrate or lipid oxidation is affected by the meal frequency. Understanding more about how the dispersal of kcals affects weight gain is necessary for our further understanding of circadian meal timing and how to develop diets to maximize weight loss for individuals as well as develop obesity resistance for shift workers.

This work also highlights a growing issue in the field about the importance of redefining circadian meal timing. In the current context, feeding paradigms that aim to affect circadian meal timing affect one or more of the following properties of feeding in mice or humans: onset/offset of the meal timing, feeding/fasting duration, or dispersal of the caloric content (Hatori *et al.*, 2012, Nas *et al.*, 2017, Ravussin et al, 2019, Hibi *et al.*, 2013, Kuroda *et al.*, 2012). One of the most critical findings from my work in chapters II and III is that onset and offset of meal timing alone produce a very similar but distinct effect on weight gain in mice and humans compared to feeding duration. In particular, studies that have had feeding duration as the main component report an increase in RER amplitude and a daily increase in energy expenditure that creates the weight gain effects reported in previous literature (Hatori *et al.* 2012, Chaix *et al.*, 2014). In both the mouse and human study, we do not change the feeding duration and we do not see an effect on energy expenditure, but a peak shift in RER that during the first days on the new feeding regime lead to altered lipid oxidation during the inactive phase for the

subjects on the feeding regime with the later onset of feeding. In the current literature, both of these feeding regimes are considered to test circadian meal timing but have given conflicting results. This has been a serious issue, especially in the human field as there are several clinical trials that have been performed on humans but give seemingly contradictory results (Nas *et al.*, 2017, Ravussin *et al.*, 2019, Sato *et al.*, 2011). More concerning is that based on my mouse work in chapter II, the metabolic effects of an extended fast can mask the metabolic effects caused by altering meal timing onset/offset. Future experiments involving circadian meal timing should be cautious when designing their feeding regimes and if possible, include controls for either fasting duration or meal timing in order to differentiate what metabolic response is occurring. It is also important to note that in the human study we serendipitously had all subjects with a very similar chronotype which coincidentally overlapped well with our sleep/wake schedule imposed on them. Another hypothesis regarding the differences seen in different human studies may be due to carbohydrate or lipid oxidation having different affects on meal timing dependent on the subject's chronotype. A interesting future experiment would be to perform the same human study on subjects that had a late chronotype. I would hypothesize that subjects with a late chronotype would have the largest changes in lipid oxidation at the early morning hours rather than during the evening as seen in our human study.

Corticosterone response is not involved in circadian meal timing

The work I performed in chapter III has sufficiently shown that neither the effects seen on fasting duration or onset/offset of meal timing were due to a corticosterone

response. This was critical to understand, because a simple explanation for the differences between the metabolic effects of fasting duration and the onset/offset of meal timing would be that mice or humans under an increased fasting duration are experiencing a change in energy expenditure due to elevated corticosterone during their adaptation period and mice with no change to fasting duration do not. This had not yet been assessed in previous circadian meal timing studies. With the known effects of corticosterone on weight gain and energy expenditure, corticosterone was a likely candidate for the metabolic effects seen in circadian meal timing studies with increased fasting (Jacobson L., 1999, Keeney *et al.*, 2001). It is also important to note that our study indicates that if the fasting duration is 18 hours or less then we do not expect a change in corticosterone but this could change in more extreme feeding paradigms.

While the fact that corticosterone is not involved in fasting duration, a question remains why do fasting restrictions show an effect on energy expenditure while studies on onset/offset of meal timing do not? A future experiment to answer this would be to analyze the gene expression profiles through microarray analysis of the liver, adipose tissue, and muscle of the mice used in chapter II on day 3 of their feeding regimes. By comparing the metabolic gene expression of the fasted Early/Late night mice to the non-fasted Early/Late night mice we may be able to determine what specific metabolic pathways are being affected by fasting duration versus onset/offset of meal timing or if the same metabolic pathway is affected in both. This would ultimately help our understanding of the genes involved in circadian meal timing and may provide genes that could be used as diagnostics for future circadian meal timing studies.

AMPK and mTOR have potential to alter cell period via altered temperature compensation

The results from my work in chapter V show that AMPK and mTOR alter the temperature compensation ability of human and rat cell lines. The temperature compensation effects of the core clock complex have long been known and observed but the reason for a temperature compensated clock in organisms that are able to control their core body temperature is largely unknown. Our data suggest that by disrupting major regulators of metabolic homeostasis (AMPK and mTOR) we in turn disrupt the temperature compensation of the core clock complex. This gives evidence to the theory the cell's pathways that maintain homeostasis also control the circadian clock's temperature compensation (Pittendrigh and Caldarola, 1973). One potential use for altering temperature compensation could be how specific cells can speed up or slow down their clock in response to conflicting metabolic responses (such as a change in meal time) that are out of phase with the current entrainment to the light cycle. There is already evidence that a mechanism is present as seen in studies on the food entrainable oscillator and how HFD *ad libitum* leads to arrhythmic feeding and organ specific period shifts against the SCN (the nucleus responsible for entraining the rest of the body to the light cycle) (Koshaka *et al.*, 2007, Pendergast *et al.*, 2013).

However, while my study shows that AMPK and mTOR has the ability to alter period of cells via temperature compensation, it does not prove that this mechanism is involved in circadian meal timing or the food entrainable oscillator. Future experiments first need to isolate particular pathways that are affecting the temperature compensation. One caveat of this study was that AMPK and mTOR are involved in the

regulation of a large cohort of metabolic genes so isolating what genes are causing the period effects will be challenging. It would be interesting to see if any of the genes found in the microarray experiment I proposed above would be affecting feeding duration or onset/offset metabolism and also regulated by AMPK or mTOR. If so, then one potential experiment would be to knockdown those genes in the U2OS and Rat1 cell lines and monitor their period at various temperatures. If these knockouts show altered temperature compensation to controls then that might provide a link between the results on cellular period we see in chapter V and the organismal effects of circadian meal timing we observed in chapters II and IV.

Summary

Collectively, my dissertation has focused on how onset/offset of meal timing influences metabolism in humans and mice. I have shown that altering onset/offset of meal timing produces effects distinct from circadian meal timing that involves increasing the fasting duration. I have shown that eating the majority of calories near the inactive phase leads to an overall decrease in lipid oxidation due to a delayed shift from carbohydrate oxidation to lipid oxidation in the inactive phase. This effect on lipid oxidation was found to not be due to a short-term increase in corticosterone as a response to the shift in meal timing. Finally, my work has shown a potential mechanism by which key metabolic regulators such as AMPK and mTOR can alter period via temperature compensation in a cell specific manner to adapt to metabolic challenges, one of which could be meal timing. Future research will be needed to determine if alteration of core clock temperature compensation is a method used by cells to respond

to metabolic signals that conflict with entrainment to the light:dark cycle. Another important direction of future research is to isolate the metabolic genes involved in fasting duration versus onset/offset of meal timing to further our understanding of how circadian meal timing alters weight gain in mammals.

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