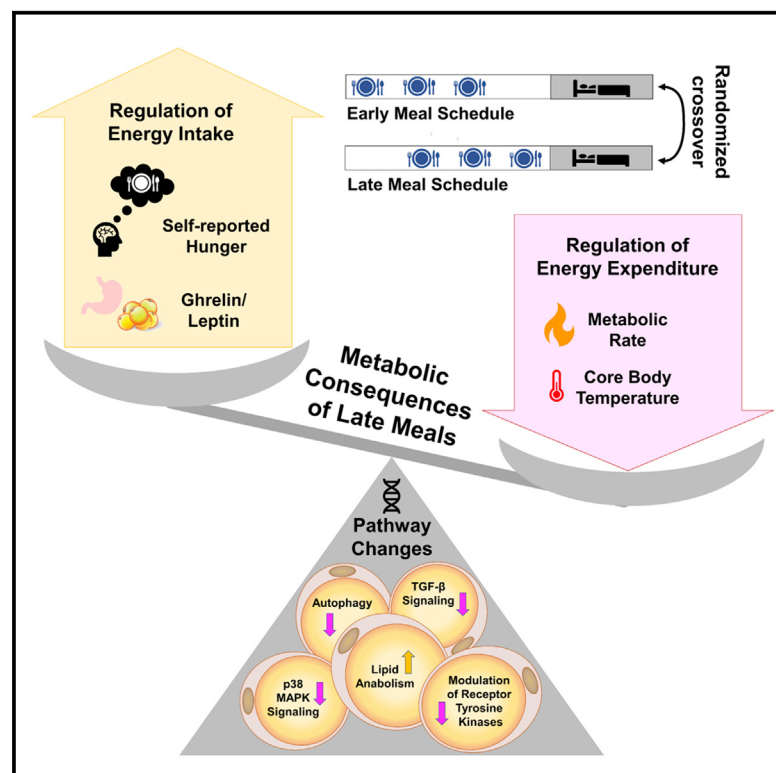


Late isocaloric eating increases hunger, decreases energy expenditure, and modifies metabolic pathways in adults with overweight and obesity

Graphical abstract



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In brief

In this randomized crossover trial that rigorously controlled for nutrient intake, physical activity, and sleep, Vujović et al. found that late eating increased hunger, modified appetite-regulating hormones, decreased daytime energy expenditure, and altered adipose gene expression consistent with increased adipogenesis/decreased lipolysis. Together, these findings may explain the increased obesity risk in late eaters.

Highlights

- Late eating increases waketime hunger and decreases 24-h serum leptin
- Late eating decreases waketime energy expenditure and 24-h core body temperature
- Late eating alters adipose tissue gene expression favoring increased lipid storage
- Combined, these changes upon late eating may increase obesity risk in humans



Clinical and Translational Report

Late isocaloric eating increases hunger, decreases energy expenditure, and modifies metabolic pathways in adults with overweight and obesity

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<https://doi.org/10.1016/j.cmet.2022.09.007>

SUMMARY

Late eating has been linked to obesity risk. It is unclear whether this is caused by changes in hunger and appetite, energy expenditure, or both, and whether molecular pathways in adipose tissues are involved. Therefore, we conducted a randomized, controlled, crossover trial (ClinicalTrials.gov NCT02298790) to determine the effects of late versus early eating while rigorously controlling for nutrient intake, physical activity, sleep, and light exposure. Late eating increased hunger ($p < 0.0001$) and altered appetite-regulating hormones, increasing waketime and 24-h ghrelin:leptin ratio ($p < 0.0001$ and $p = 0.006$, respectively). Furthermore, late eating decreased waketime energy expenditure ($p = 0.002$) and 24-h core body temperature ($p = 0.019$). Adipose tissue gene expression analyses showed that late eating altered pathways involved in lipid metabolism, e.g., p38 MAPK signaling, TGF- β signaling, modulation of receptor tyrosine kinases, and autophagy, in a direction consistent with decreased lipolysis/increased adipogenesis. These findings show converging mechanisms by which late eating may result in positive energy balance and increased obesity risk.

INTRODUCTION

Obesity is a serious epidemic afflicting an estimated 650 million adults worldwide (NCD Risk Factor Collaboration, 2017; Ezzati et al., 2018; Reilly et al., 2018), including 42% of the adult US population (Hales et al., 2020). Obesity is a major contributor to the global burden of chronic disease and disability, as it elevates the risk for a wide range of health issues including diabetes, cardiovascular disease, certain cancers, and COVID-19 mortality (Clemmensen et al., 2020; Pi-Sunyer, 2009). For this reason, prevention and treatment of obesity has been identified as a global imperative (GBD 2015 Obesity Collaborators et al., 2017).

Behavioral interventions targeting obesity have been mostly aimed at reducing dietary energy intake and/or increasing energy expenditure via increased physical activity (Hill et al., 2013). However, these behavioral interventions are often only transiently effective (Foright et al., 2018; Kim, 2021; Kraschnewski et al., 2010), as a complex array of factors beyond diet and

exercise influence energy balance and contribute to obesity risk (McAllister et al., 2009; Romieu et al., 2017). One such factor is the circadian timing system, which is tightly interwoven with energy metabolism (Bass and Takahashi, 2010). The influence of the circadian system and its interaction with time of eating on metabolism has been demonstrated at the whole-body physiology level (Chellappa et al., 2021; Coomans et al., 2013; Mason et al., 2020; Paschos et al., 2012; Turek et al., 2005) and at the molecular level, e.g., in adipocytes (Arredondo-Amador et al., 2020; Carrasco-Benso et al., 2016; Garaulet et al., 2011; Gomez-Abellan et al., 2010; Ramsey et al., 2009; Wehrens et al., 2017). Hence, the circadian timing of food intake has been proposed as a factor that may alter energy balance and act as a key modifiable risk factor for obesity (Allison and Goel, 2018; Mattson et al., 2014). Evidence for the importance of food timing for energy balance comes from animal models (Arble et al., 2009; Chaix et al., 2019; Fonken et al., 2010; Salgado-Delgado et al., 2010) as well as observational (Garaulet et al., 2013; McHill

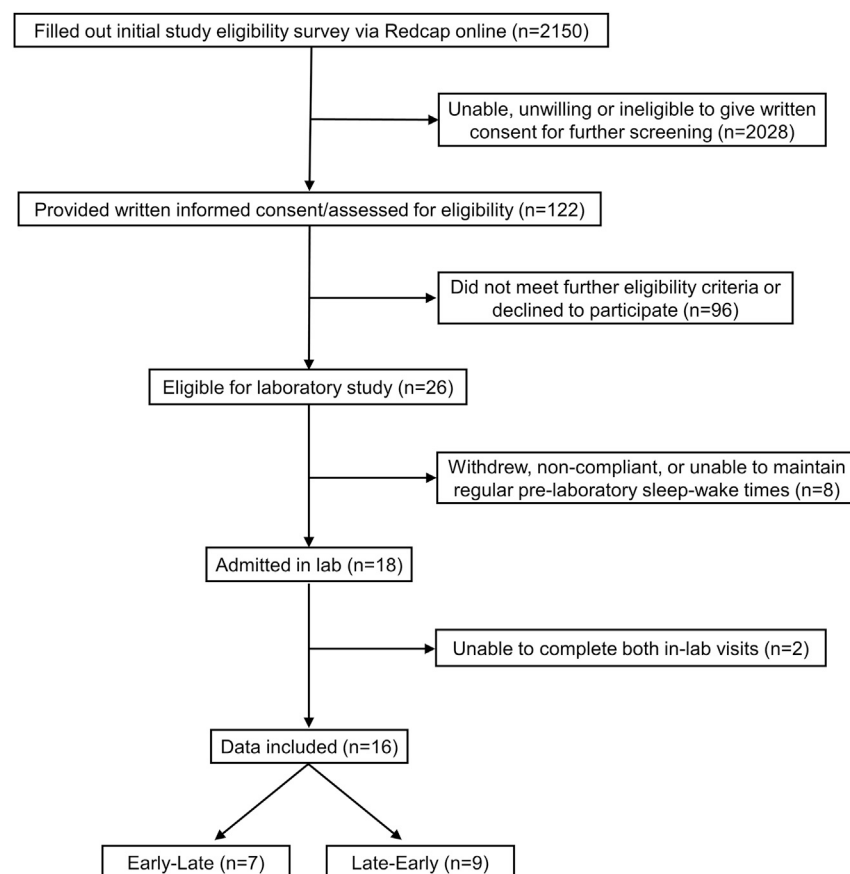


Figure 1. CONSORT flow diagram

under a late eating protocol (Allison et al., 2021; Kelly et al., 2020). Adipose tissue is a primary energy storage organ with an important role in energy balance control. This tissue expresses circadian rhythms in clock genes and genes regulating adipose tissue metabolism (Arredondo-Amador et al., 2020; Garaulet et al., 2011; Gomez-Abellan et al., 2010), and adipose-specific clock gene deletion increases obesity risk in rodents (Paschos et al., 2012). While clock gene expression in adipose tissue can be modified by late eating in humans (Wehrens et al., 2017), it is not known whether and how late eating may affect adipose tissue molecular pathways involved in energy storage and utilization.

Here we test three hypotheses regarding the mechanisms by which late eating may promote positive energy balance: (1) via increased drive for energy intake (using increased hunger, decreased leptin, and increased acylated ghrelin as primary end points); (2) via decreased energy expenditure (using decreased number of kilocalories burned and decreased core body temperature as primary end

points); and/or (3) via coordinated molecular changes favoring adipogenesis.

We therefore conducted an in-laboratory randomized crossover trial to simultaneously determine the effects of late meal timing on mechanisms involved in energy intake control, energy expenditure, and molecular regulation of adipose tissue metabolism. Because we aimed to investigate the direct effect of meal timing, without confounding by other behavioral and environmental factors, we rigorously controlled for timing, amount and type of food intake (total caloric intake and dietary composition), physical activity, posture, sleep, and light exposure. While this is not the first randomized crossover study to investigate the effect of late meal timing related to energy balance control, it may be the most well controlled and comprehensive.

RESULTS AND DISCUSSION

Sixteen participants with overweight or obesity (mean $\pm$ SD; age, 37.3 ± 2.8 years; 5 women; BMI, 28.7 ± 0.6 kg/m²) completed this crossover study (Figure 1) and were included in the analyses presented here. Prior to each of the two admissions to the laboratory, all participants completed an ambulatory segment with a fixed sleep/wake cycle during the last 2–3 weeks and consumed a calculated, timed, and pre-prepared diet during the last 3 days (Figure 2A). Each participant then completed two laboratory protocols: an early eating protocol (Figure 2B) and a late eating protocol for which meals were delayed by 250 min (Figure 2C).

et al., 2017) and experimental (Allison et al., 2021; Bandin et al., 2015; Jakubowicz et al., 2013; Keim et al., 1997) studies in humans, although not all studies are in agreement (Kelly et al., 2020; Nas et al., 2017; Sievert et al., 2019).

Despite this prior work, relatively little is understood about the physiological mechanisms by which the timing of food intake would influence energy balance. Previous observational human studies have linked late (circadian) eating with higher obesity risk and impaired dietary and surgical weight loss success that could not be explained by differences in reported caloric intake or physical activity (Allison et al., 2021; Garaulet et al., 2013; McHill et al., 2017; Ruiz-Lozano et al., 2016; Xiao et al., 2019). These observations suggest that meal timing per se might influence body weight without changes in energy intake and activity-related energy expenditure. However, experimental studies conjointly examining the underlying mechanisms of the effects of meal timing on energy regulation via changes in energy intake, energy expenditure, and molecular pathways, simultaneously and under controlled conditions, are still lacking. Existing literature suggests that diet-induced thermogenesis (also known as the thermic effect of food) is lower in the evening compared to the morning (Bo et al., 2015; Morris et al., 2015; Richter et al., 2020; Romon et al., 1993), and that this is under endogenous circadian control (Morris et al., 2015). However, not all papers are in agreement on this point (Ruddick-Collins et al., 2022) and it remains unclear how the endogenous circadian variations in diet-induced thermogenesis affect 24-h energy expenditure

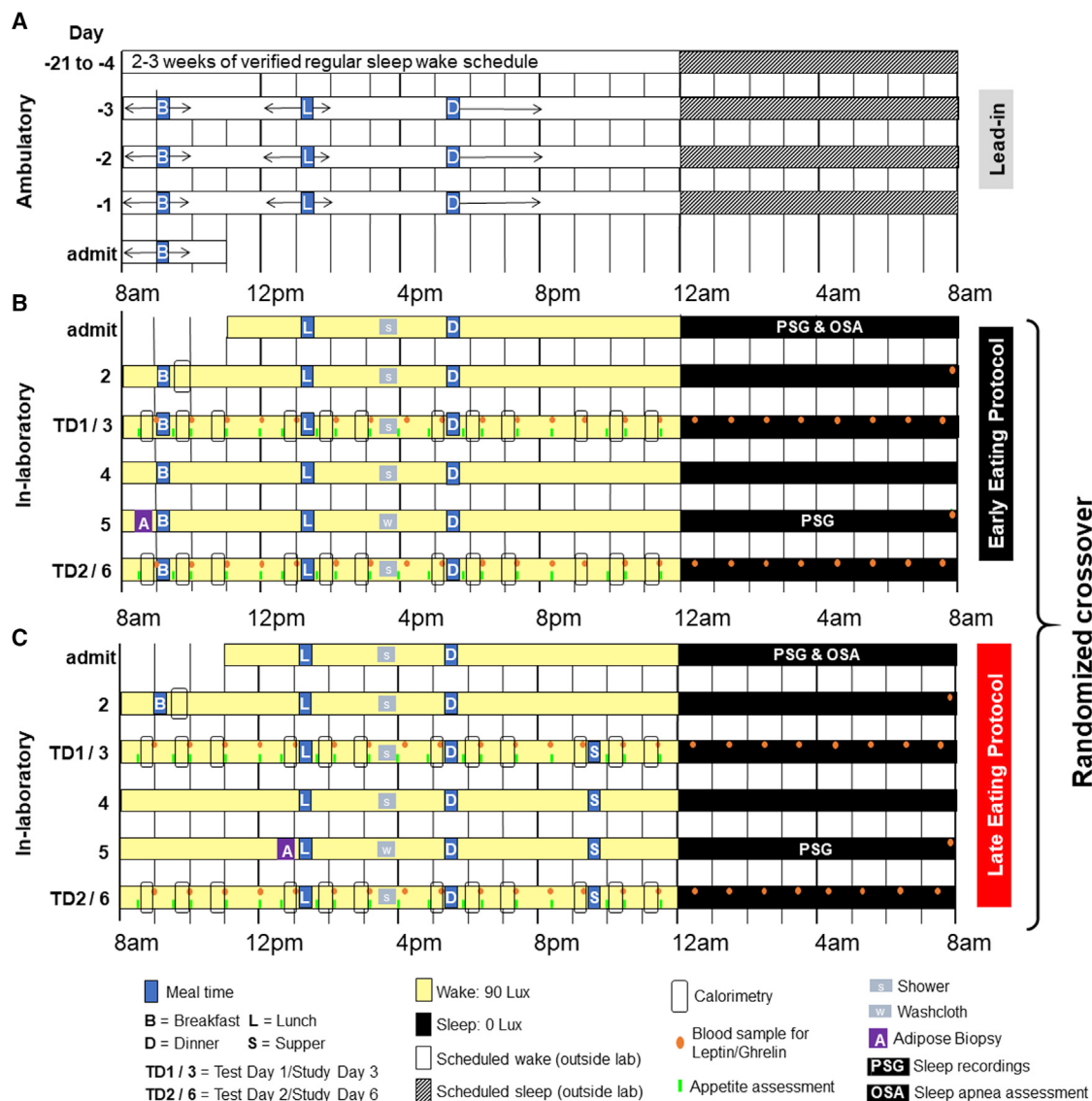


Figure 2. Experimental design

(A) Each of the two laboratory stays was preceded by a pre-laboratory lead-in with a regular sleep/wake cycle for 2–3 weeks and consumption of eucaloric meals (prepared and supplied by the dietary office) during the indicated time intervals (arrows indicate the time ranges) during the last 3 days. (B and C) Laboratory visits for the (B) early and (C) late meal schedule protocol began with two adaptation days. Beginning on day 3, late and early eating protocols were established. Study days 3 and 6 served as test days 1 and 2, respectively. Subcutaneous adipose tissue was biopsied (in a subset of participants) on study day 5.

The order of the protocols was randomized, and visits were separated by a washout period of 3–12 weeks (see [STAR Methods](#) for further details). There was no significant randomization order effect for any of the primary end points ($p > 0.05$ for all), and our randomization stratification approach ensured that there were no significant differences between the participants who went in early-late versus late-early order in terms of normoglycemic versus prediabetic status, sex, age, or BMI ([Table 1](#)). There were also no statistically significant effects of days into protocol (i.e., test day 1 versus 2) or a significant interaction of day into the protocol and the meal schedule (late versus early eating) for any of the primary outcome measures. Therefore, no post hoc pair-

wise comparisons were performed, and we present the results combining the two test days in each laboratory protocol, with p values corresponding to the main effect of late eating.

Late eating increased hunger

To study effects of meal timing on subjective drive for energy intake while controlling for caloric intake, we studied perceived hunger and appetite assessed by a series of computerized visual analog scales (VAS) 18 times for each test day ([Figure 3A](#), left panels). Late eating doubled the odds of being hungry (defined as a >50 response on the VAS scale) as compared to early eating (odds ratio 2.02, increasing hunger probability from $\sim 10\%$ to

Table 1. Demographic information did not meaningfully differ for early-late versus late-early randomization order cohorts

Randomization order	Early-late	Late-early
Total participants	n = 7	n = 9
Participants with obesity (BMI ≥ 30 kg/m ²)	n = 2	n = 2
Cohort BMI (mean $\pm$ SEM in kg/m ²)	29.2 $\pm$ 0.8	28.3 $\pm$ 0.9
Participants with prediabetes (hemoglobin A1c 5.7%–6.5%)	n = 2	n = 3
Cohort hemoglobin A1c % (mean $\pm$ SEM)	5.3 $\pm$ 0.2	5.5 $\pm$ 0.1
Participants of older age (≥ 40 years)	n = 1	n = 3
Cohort age (mean $\pm$ SEM in years)	34.0 $\pm$ 4.4	39.8 $\pm$ 3.7
Participants who are female	n = 1	n = 4
Participants of Black/African American race	n = 3	n = 2
Participants of Asian ancestry	n = 2	n = 1
Participants of Hispanic ancestry	n = 0	n = 1

$\sim 20\%$; $p < 0.0001$; Figure 3A, right panel; Table 2). For effects of late eating on specific appetite measures, please see Figure S1 and Table 2. Specifically, late eating significantly increased the odds of a >50 response on the VAS scale of how much a participant would like to eat ($p < 0.0001$), of reporting a desire to eat starchy foods ($p < 0.0001$) or meat ($p < 0.0001$), and of reporting a strong desire to eat ($p < 0.0007$). There were also increases in reported desire for salty foods ($p < 0.0051$), dairy ($p < 0.034$), and vegetables ($p < 0.036$).

Late eating affected appetite-regulating hormones consistent with increased hunger

We next assessed the effect of late eating on two appetite-regulating hormones: leptin (Figure 3B), which promotes satiety (Heini et al., 1998), and acylated (active) ghrelin (Figure 3C), which promotes hunger (Levin et al., 2006). We also calculated the (acylated) ghrelin:leptin ratio (Figure 3D), which has been correlated with hunger (Adamska-Patrino et al., 2018; Hanlon and Van Cauter, 2011; Spiegel et al., 2004). These hormones were assessed hourly across 24 h throughout each test day. Our results (Table 2) show that late eating decreased 24-h leptin levels by 6% ($p = 0.171$) and increased the 24-h ghrelin:leptin ratio by 12% ($p = 0.0063$) without a significant change in 24-h acylated ghrelin. During the 16-h wake episode, late eating decreased average leptin by 16% ($p < 0.0001$) and increased the ghrelin:leptin ratio by 34% ($p < 0.0001$), consistent with the increased hunger probability during that time, while during the 8-h sleep episode, late eating increased average leptin by 10% ($p = 0.0028$), decreased ghrelin by 13% ($p = 0.0002$), and decreased the ghrelin:leptin ratio by 18% ($p < 0.0001$).

Late eating decreased wake episode energy expenditure

To investigate the influence of meal timing on energy expenditure and substrate (carbohydrate and lipid) oxidation, these outcomes were measured by indirect calorimetry 12 times over the 16-h wake episode on each test day (Figure 2). Energy expenditure was significantly lower in the late eating condition than in the early eating condition (Figure 4A), with participants expending 59.4 ± 13.9 fewer kcal per waking day (5.03% less)

as compared to in the early eating condition ($p < 0.0001$). Regarding substrate utilization, late eating did not significantly affect either carbohydrate or lipid oxidation (Figures 4B and 4C; Table 2).

Late eating decreased 24-h core body temperature

Because energy expenditure was assessed only during the wake episodes and not across the full 24 h, we measured core body temperature (CBT) continuously across the full 24-h sleep/wake cycle for each test day as a proxy of energy expenditure (Du Bois, 1921). Late eating significantly reduced average 24-h CBT ($p = 0.019$). Subsequent analysis showed that CBT was also significantly lower during the 16-h wake episodes ($p = 0.01$), without significant effect during the 8-h sleep episodes (Figure 4D; Table 2). Importantly, when comparing results of CBT with energy expenditure data, we found similar results during the 16-h wake episodes, showing lower values with both measures (Figure 4D versus Figure 4A). Of interest, upon inspection of the 24-h CBT profile, we noted that late eating significantly decreased CBT during the last 4 h of the sleep episode ($p = 0.0095$), when any acute diet-induced thermogenesis from the late meal (i.e., supper) in this protocol would be expected to have worn off (Table 2; Figure S2H). In addition, because behavioral and environmental factors, such as body posture, physical activity, and room temperature, remained constant during the sleep episode, there were no apparent extrinsic drivers of increased heat dissipation. This suggests that the decrease in CBT during the second half of sleep following late eating days may have been due to a decrease in thermogenesis and energy expenditure (Chen et al., 2020).

Therefore, although we could not measure energy expenditure during the sleep episode with our methods, the decrease in CBT during the wake episode, the end of the sleep episode, and across the full 24-h sleep/wake cycle indicates that there was no compensatory increase in energy expenditure during the night. Taken together, our results suggest that late eating caused a decrease in energy expenditure across the 24-h cycle, although future studies are needed to verify this.

Testing *a priori* hypothesized effects of late eating on primary physiological end points

Because we tested multiple *a priori* hypotheses, family-wise error rate correction was applied as described in the STAR Methods (Table 2). These results are consistent with unadjusted results in showing that late eating significantly increased wake-time hunger, decreased 24-h leptin, had no significant effect on 24-h ghrelin, decreased waketime energy expenditure, and decreased 24-h CBT.

Late eating delayed temporal profile of energy intake- and expenditure-related measures

As expected, the 250 min delay in meal timing was accompanied by a similar delay in the diurnal patterns of hunger (Figure 3A), appetite (Figures S1A–S1L), appetite-regulating hormones leptin (Figure 3B) and ghrelin (Figure 3C), energy expenditure (Figure 4A), carbohydrate oxidation (Figure 4B), and lipid oxidation (Figure 4C), and these effects were already observed on the first day after the delay of the meal schedule (test day 1). This

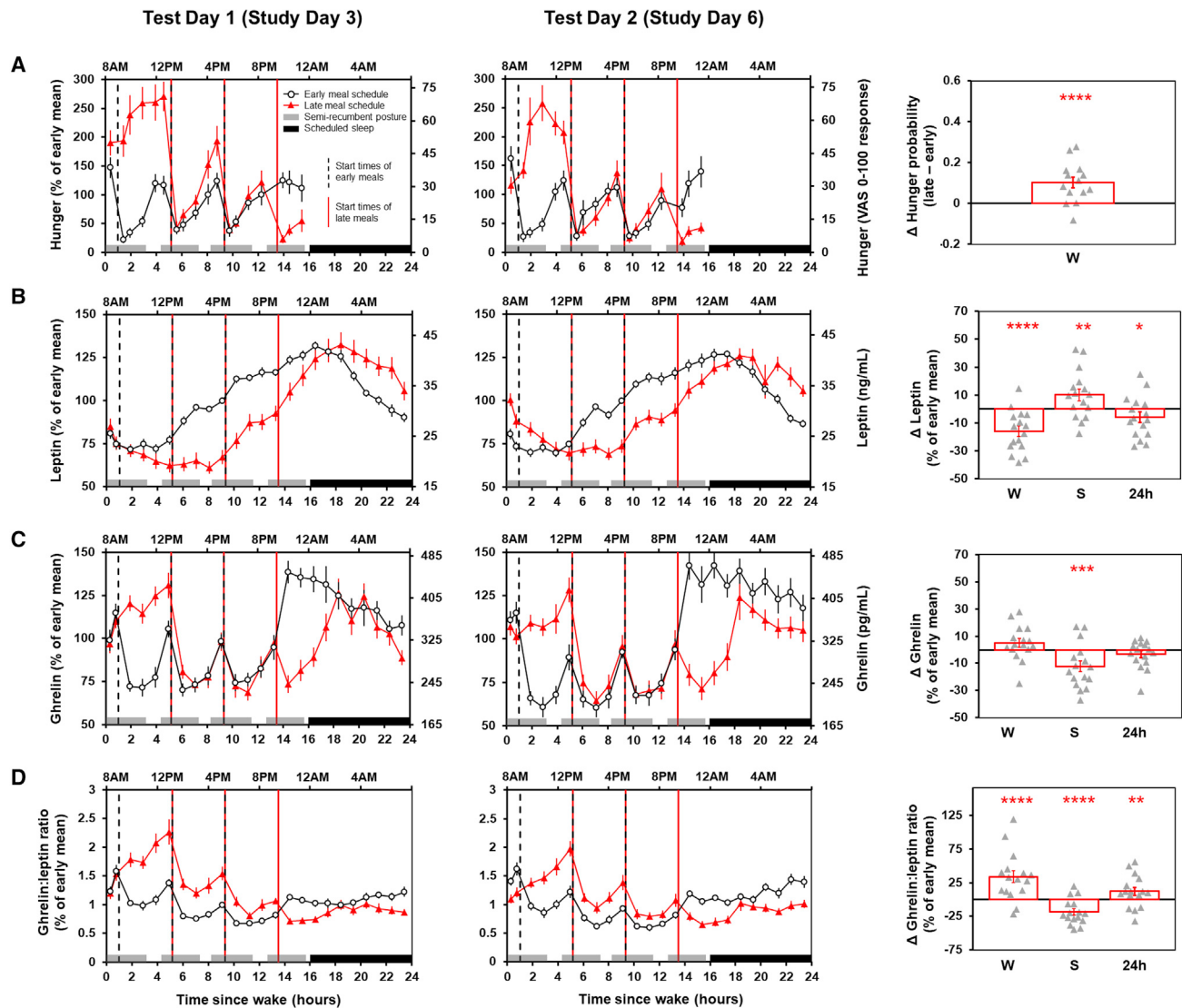


Figure 3. Effect of late eating on energy intake regulation

Effects of late eating schedule on daily profiles in (A) self-reported hunger, (B) serum leptin concentration, (C) plasma acylated (active) ghrelin concentration, and (D) acylated ghrelin:leptin ratio. Data shown as mean $\pm$ SEM; each data point is expressed as the percentage of the mean of all time points collected during the early eating protocol for that same participant. Left panels, test day 1; middle panels, test day 2; right panels, effects of late eating (late eating schedule minus early eating schedule) averaged across test days with asterisks indicating significant differences (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). Vertical black dashed and red solid lines, timing of meals in early and late eating schedule, respectively. Horizontal black bars along x axes, scheduled sleep episodes. Gray bars, semi-recumbent posture.

observation highlights the importance of meal timing for energy regulation.

Late eating altered adipose tissue gene expression consistent with decreased lipolysis/increased adipogenesis

To test the effect of late eating on molecular pathways in adipose tissue, we collected a biopsy of subcutaneous white adipose tissue during both the early eating and late eating protocols. These samples were collected from a subset of participants who specifically consented to the biopsies ($n = 7$; age, 40 ± 4.3 years; 2 women; BMI, 28.95 ± 0.89 kg/m²). After mRNA extraction,

we performed differential expression and pathway enrichment analyses to assess within-participant concordant differences between the early and late eating conditions. These analyses showed several pathways with statistically significant differences in gene expression profiles, including pathways related to lipid metabolism, p38 MAPK signaling, modulation of receptor tyrosine kinases, TGF- β signaling, and autophagy (Figure 5; Table S1).

Lipid metabolism pathways showed the largest absolute number of differentially expressed genes. Late eating downregulated several genes responsible for lipid breakdown, such as *PLD6*, *DECR1*, and *ASAH1*. Consistently, late eating

Table 2. Effect of late eating on measures of energy intake and energy expenditure

Category	Outcome measure	24 h versus wake (W) versus sleep (S)	Quantification of effect of late eating		95% confidence interval	Unadjusted p value	Primary versus secondary outcome	Holm-Bonferroni adjusted p value for primary outcomes
Energy intake: Self-reported appetite via visual analog scale (VAS)	How hungry are you right now?	W	odds ratio of VAS > 50 for late:early eating	2.02	1.46 to 3.35	<0.0001	primary	0.0003
	How much are you craving starchy foods?			2.24	1.5 to 2.99	<0.0001	secondary	
	How much are you craving meat?			2.09	1.46 to 2.4	<0.0001	secondary	
	How much could you eat right now?			1.82	1.38 to 2.38	<0.0001	secondary	
	How strong is your desire to eat right now?			1.73	1.26 to 2.68	0.0007	secondary	
	How much are you craving salty foods?			1.8	1.21 to 2.48	0.0051	secondary	
	How much are you craving vegetables?			1.61	1.05 to 2.39	0.0363	secondary	
	How much are you craving dairy foods?			1.57	1.04 to 1.22	0.0342	secondary	
	Are you experiencing nausea?			0.51	0.21 to 1.97	0.128	secondary	
	How much are you craving sweet foods?			1.32	0.89 to 1.85	0.254	secondary	
	How much are you craving fruits?			1.25	0.85 to 1.78	0.354	secondary	
	How thirsty are you right now?			1.2	0.81 to 1.27	0.621	secondary	
	How full do you feel right now?			0.95	0.71 to 1.27	0.7138	secondary	
Energy intake: Physiological measures	leptin	24 h	% change as effect of late eating	−6.3	−11.98 to −10.37	0.0171	primary	0.0342
		W		−15.95	−21.54 to 16.17	<0.0001	secondary	
		S		9.55	2.93 to 0.65	0.0028	secondary	0.0756
	acylated ghrelin	24 h		−3.35	−7.34 to 10.59	0.0756	primary	
		W		5.14	−0.32 to −5.99	0.0532	secondary	
		S		−12.62	−19.25 to 20.89	0.0002	secondary	
	acylated ghrelin:leptin ratio	24 h		11.72	2.55 to 48.65	0.0063	secondary	
Energy expenditure: Physiological measures		W		34.24	19.82 to −10.73	<0.0001	secondary	
		S		−18.46	−26.18 to −2.46	<0.0001	secondary	
	energy expenditure (via calorimetry)	W	% change as effect of late eating	−4.94	−7.42 to 2.77	0.0002	primary	0.0004
	carbohydrate oxidation (via calorimetry)	W		−4.22	−11.21 to 12.68	0.2087	secondary	
	lipid oxidation (via calorimetry)	W		1.06	−10.57 to −0.02	0.8488	secondary	
	core body temperature	24 h		−0.19	−0.36 to −0.04	0.0185	primary	
		W		−0.21	−0.37 to 0.04	0.0101	secondary	0.0185
		S		−0.19	−0.43 to −0.08	0.0789	secondary	
		last 4 h S		−0.36	−0.64 to 3.35	0.0095	secondary	

The top 13 rows show the effect of late eating on self-reported measures of energy intake regulation, i.e., appetite measured via visual analog scale (VAS), quantified as the odds of score >50 on subjective hunger and appetite measures, with 0 rating meaning “not at all” and 100 rating meaning “extremely.” The next 9 rows show the effect of late eating on physiological (endocrine) measures of energy intake regulation, quantified as % change in waking, 24-h and sleep-episode mean levels of the hormones leptin, acylated (active) ghrelin, and their ratio. The final 7 rows show the effect of late eating on physiological measures of energy expenditure quantified as % change in calorimetrically and thermometrically derived measures. The two rightmost columns indicate primary versus secondary outcome measures and multiple-testing-correction-adjusted p values by false discovery rate (FDR) for primary outcome measures related to energy intake and energy expenditure.

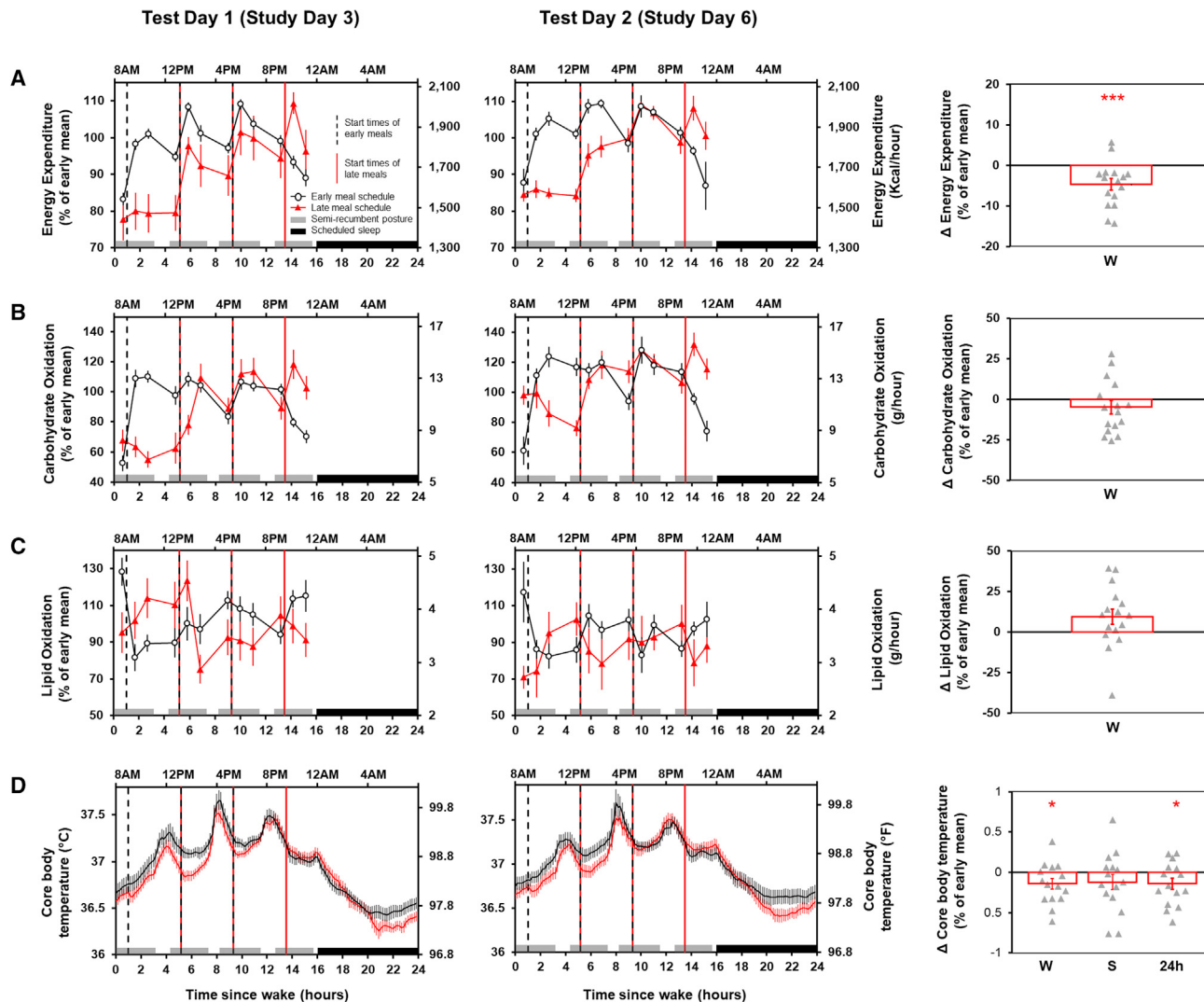


Figure 4. Effect of late eating on energy expenditure regulation

Effects of late eating schedule on daily profiles in (A) energy expenditure, (B) carbohydrate oxidation, (C) lipid oxidation, and (D) CBT. Data shown as mean $\pm$ SEM; (A)–(C) show data normalized to early meal schedule mean (as in Figure 3), and (D) shows absolute levels. Symbols as in Figure 3.

reduced the expression of *ABHD5*, a lipolysis-promoting gene. *ABHD5* is directly and negatively regulated by the protein product of *PERILIPIN1*, whose genetic variants predict the likelihood of adverse metabolic outcomes in response to late meal schedules (Garaulet et al., 2016). Conversely, genes that are responsible for lipid synthesis were consistently upregulated, such as *GPAM*, *ACLY*, *AACS*, and *CERK*. Together, the pathway analysis suggests this set of changes is consistent with a decrease in lipid catabolism and increase in lipid synthesis. However, further studies are needed to empirically confirm whether late eating decreases lipolysis and increases lipogenesis.

Gene expression related to the p38 MAPK pathway was downregulated with late eating. A reduction in this pathway has been shown to increase adipogenesis (Aouadi et al., 2006). *GADD45y*, a positive regulator of the p38 MAPK signaling pathway, particularly showed a large and consistent decrease

in expression in response to late eating. In addition to regulation of adipogenesis, *GADD45y* is also reported to regulate thermogenic energy expenditure of brown adipocytes via the p38 MAPK pathway (Gantner et al., 2014).

Late eating also downregulated expression of genes in the TGF- β signaling pathway, which has been shown to inhibit adipogenesis (Lee, 2018). An activator of the TGF- β pathway, *THBS1*, showed the strongest downregulation of any gene by late eating, which supports a shift toward increased adipogenesis (Kong et al., 2013).

Expression of several genes that modulate tyrosine kinase signaling also significantly changed with late eating. The effects of tyrosine kinases are diverse, but in adipose tissue the insulin receptor tyrosine kinase promotes energy uptake and storage (Cignarelli et al., 2019). The expression of negative regulators of tyrosine kinases was downregulated, implying increased sensitivity of tyrosine kinase

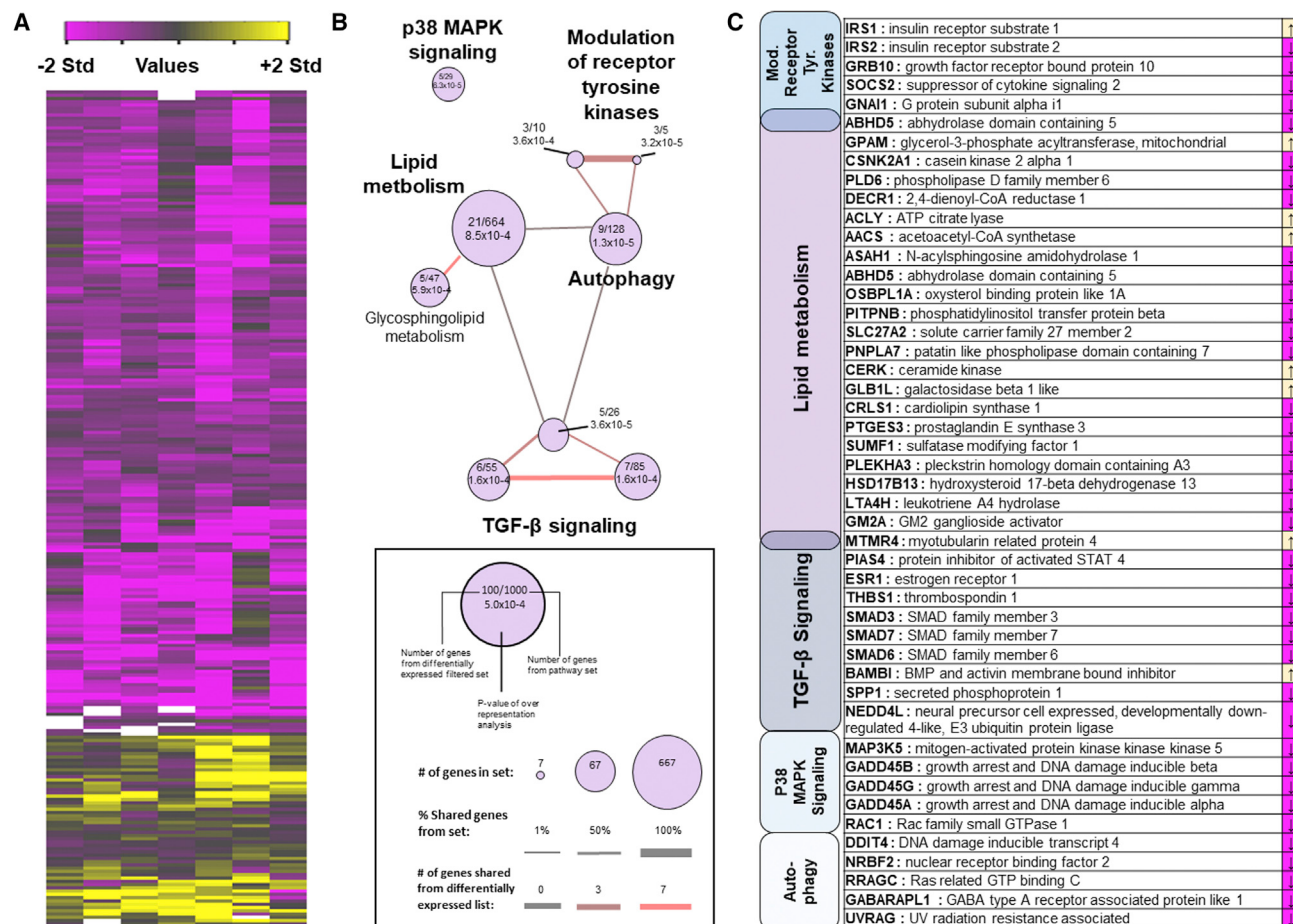


Figure 5. Effect of late eating on directional gene expression change in adipose tissue

(A) Heatmap indicating direction and magnitude of change of gene expression (late divided by early eating) capped at ± 2 SDs of the mean of all data. Fuchsia, decreased gene expression with late eating; yellow, increased gene expression; gray, no change; white, missing data.

(B) Network diagram summarizing pathways altered by late eating. Network diagram was generated by running an over-representation analysis on the filtered list of genes that were significantly differentially expressed. Node pathway clusters were named based upon the narrowest ontological category that could define all the pathway nodes included within it. This diagram is based on databases accessed on November 1, 2020.

(C) Genes from pathway analysis and their direction of change with late eating. Each fuchsia down arrow indicates a decrease in expression; yellow up arrow indicates an increase in expression.

pathways with late eating, thus favoring energy uptake and storage.

Expression of several genes that promote autophagy also decreased with late eating. In general, decreases in autophagy pathway signaling coincide with increases in adipogenesis (Rabanal-Ruiz et al., 2017; Tao et al., 2019).

Moreover, the changes in circulating leptin or ghrelin concentration due to late eating, measured at a similar clock time as when the associated biopsies were taken (STAR Methods), were directly correlated with the magnitude of change in expression of several adipocyte genes (Figures S3A–S3H). Of those genes, two were significantly changed with late eating: *RPL7L1* and *C4orf19* (Figures S3A and S3B) ($p = 0.0001$). Furthermore, the correlation of leptin changes with those of *NDUFAF1* is of particular interest, as the latter is an assembly factor of complex 1 in mitochondria and has been associated with altered feeding behavior and metabolism in transgenic mouse models (Lindfors et al., 2011).

Sleep duration/stages did not differ between early and late eating conditions

Dietary modifications may impact sleep (St-Onge et al., 2016; Afaghi et al., 2007), and sleep restriction can alter appetite (Nedeltcheva et al., 2009; Nedeltcheva and Scheer, 2014; Spiegel et al., 2004; St-Onge et al., 2011), energy expenditure (Markwald et al., 2013), and molecular processes in adipose tissue (Brousard et al., 2012). To control for impacts of diet on sleep, we kept the amount and composition of nutrient intake identical between early and late eating protocols. Even in the late eating protocol, the last meal was started 2.5 h prior to the scheduled sleep episode. Nonetheless, we still tested whether the meal schedule intervention caused any changes in total sleep time or sleep stages. This was measured during the sleep episodes prior to the fourth day on each meal schedule intervention, i.e., the night prior to the second test day (Figure 2). Total sleep time did not significantly differ between the early (6.87 ± 0.23 h) and late (6.85 ± 0.17 h) meal schedules, nor did sleep efficiency or

percentage/duration of wake, N1, N2, N3, REM, sleep-onset latency, or wake after sleep onset (Figure S4). Thus, changes in sleep time or sleep stages were unlikely to be an underlying mechanism for the observed effects of meal schedule on our outcome measures in this protocol (where, it is worth reiterating, sleep opportunity duration was clamped between the early and late eating conditions). Furthermore, there was no significant correlation between sleep duration and our key outcome variables: hunger, acylated ghrelin, leptin, energy expenditure, and CBT (all $p > 0.05$).

Integration of physiological and molecular results

Our results show that late eating consistently altered physiological functions and biological processes involved in regulation of energy intake, expenditure, and storage—each of these three in a direction favoring weight gain. Furthermore, even within each of these three categories, we found that the direction of change was consistent across their individual outcome measures. Late eating caused increased hunger and an increase in the ratio of (acylated) ghrelin:leptin concentrations, even though the 24-h nutritional intake was kept identical, indicating an increased drive for energy intake. At the same time, late eating also caused a decrease in energy expenditure across the wake episode, combined with a decrease in CBT across the full 24-h sleep-wake cycle. We also found, in a subset of biopsied participants, that late eating impacted molecular pathways to promote adipose tissue expansion via both adipogenesis and inhibition of lipolysis. Together, these results present a comprehensive picture of energy balance control, indicating that late eating—while carefully controlling caloric intake and other behavioral and environmental factors—leads to physiological and molecular changes favoring a positive energy balance.

Interestingly, the results suggest that—despite the identical 24-h caloric intake—late eating resulted in a decrease in 24-h circulating leptin levels, a simultaneous increase in the drive for energy intake, and the concurrent decrease in energy expenditure. Late eating also altered human adipose tissue gene expression toward increased adipogenesis and decreased lipolysis, which may promote fat mass accumulation. To our knowledge, this is the first study demonstrating that effect of late eating on adipose tissue. Therefore, future studies would be needed to understand how multiple signals are integrated at the level of adipose tissue to result in the changes in gene expression observed. The potential mechanisms underlying the effect of late eating on measures of energy balance control are currently not understood. Typically, during a state of true negative energy balance, coordinated physiological responses act to correct this disbalance by increasing energy intake driven by increased hunger/appetite and by decreasing energy expenditure (Spiegelman and Flier, 2001). While these changes are adaptive in the face of a negative energy balance, they appear maladaptive when meal timing is delayed while the 24-h energy intake is clamped, as is the case in the current study.

Leptin and acylated ghrelin—in addition to their role in energy intake—act as important feedback signals to the brain to influence energy expenditure, sympathetic tone, and propensity for anabolic versus catabolic processes throughout the body (Abizaid and Horvath, 2012; Spiegelman and Flier, 2001; St-Pierre et al., 2004). The observed increases in the acylated

ghrelin:leptin ratio with late eating were—as expected—concurrent with increased hunger and decreased energy expenditure and 24-h CBT. The increase in the ghrelin:leptin ratio during the waking hours and across 24 h was explained primarily by a decrease in leptin, without a significant change in ghrelin. This decrease in leptin could, at least partially, be responsible for the observed increase in hunger and appetite as well as for the decrease in energy expenditure and CBT (Friedman, 2016, 2019).

This result is of special interest considering that identical meals were consumed in both the early and late eating condition. Given our concurrent observation of increased drive for food intake, in a real-world setting—where people have *ad libitum* access to food—the effects of late eating may be even more pronounced.

Limitations of study

A key strength of our protocol is the concomitant measurement of many relevant markers of the body's systems for regulation of energy intake, energy expenditure, and energy storage, at both the molecular/adipocyte and systems level. Our findings are consistent across outcomes and shed new light on mechanisms that may help explain increased obesity risk with late eating. This study was conducted in adults with overweight or obesity, a highly relevant population. Our cohort also had good representation of racial/ethnic minorities. Finally, the randomized, crossover, and highly controlled laboratory protocol enabled us to carefully test within-subject changes, without confounding by interindividual differences and while minimizing environmental and behavioral interference.

Although the study duration and sample size are limited, and adipose tissue biopsies were collected in a subset of participants, these parameters are in line with similar studies using highly controlled, multi-day, in-laboratory, crossover trials with intensive monitoring. We were unable to test the effect of meal timing on self-selected food intake because *ad libitum* food access would have confounded all subsequent physiological assessments; however, our assessment methods for hunger and appetite have been shown to accurately predict subsequent food intake (Barkeling et al., 1995).

Fasting duration alone or in combination with changes in meal timing within the 24-h day can affect metabolic measures related to energy balance in humans (Gill and Panda, 2015; Moro et al., 2016; Ravussin et al., 2019; Sutton et al., 2018). For this reason, fasting duration in the current protocol was matched for all meals and assessments on test day 2 (study day 6). Although fasting duration was not exactly matched for the first meal on test day 1 (study day 3), when transitioning into the late eating schedule, this could not be the driver of any key study outcomes, as our statistical analysis showed that the effect of late eating was not significantly different between test days for any key outcome measure (Figure S2; supplemental information). Relatedly, the duration of fast experienced during waking versus sleeping hours may have relevance for our findings on hunger/appetite and adipose tissue gene expression. Future studies, e.g., using an experimental nap design, would be needed to quantify and understand such potential influences.

Adipose biopsies were timed to occur right before the first meal of the wake episode with identical fasting duration in both protocols, i.e., collected about 4 h later in the late eating

condition (Figures 2B and 2C). Therefore, differences in adipose tissue gene expression seen with late eating could be due to differences in timing of the biopsy relative to the timing of (1) the behavioral/environmental cycle, (2) the central circadian clock, and (3) the adipocyte peripheral clock or any interaction of the above (including internal desynchrony). Prior work suggests that while markers of the central circadian clock are not shifted by a late eating schedule, molecular circadian clocks in adipocytes can be delayed, although only by ~ 1 h, when meals were delayed by ~ 4 h (Wehrens et al., 2017). Therefore, in a late eating condition, there could be misalignment between central and peripheral clocks. Such misalignment may contribute to the adipocyte gene expression changes reported here. That said, if differences in circadian phase of peripheral clocks in adipose tissue were driving the effects of late eating on gene expression, we would expect to see commensurate changes in the expression of at least some circadian clock genes, and this was not the observed. No clock gene mRNA expression significantly changed between the late versus early condition (Figure 5; Table S1). Sequential biopsies across 24 h and simultaneous assessments of the phase of the central and peripheral clock as well as metabolically relevant genetic pathways would be required to fully test the relative role of these three mechanisms.

The use of indirect calorimetry to assess energy expenditure precluded us from collecting a full, 24-h profile for this measure. That said, our observed decrease in CBT during the wake episode, the end of the sleep episode, and across the full 24-h sleep/wake cycle suggests there was no increase in energy expenditure during the night to offset the decrease in energy expenditure measured during the day. Nonetheless, future studies using methods such as whole-room calorimetry or doubly labelled water would be of value for both 24-h assessment and detection of potential changes in carbohydrate versus lipid versus protein oxidation.

Note that the present study aimed to test the acute effects of late eating on energy balance regulation, and not whether long-term adherence to such a schedule would result in weight gain over time, or whether the body would adapt allostatically. Long-term and highly controlled studies with comprehensive assessments are needed to test if the observed physiological and molecular changes persist long term and test their relative role in body weight regulation. To minimize potential confounds, our study excluded potential participants with comorbidities or medication use (other than oral contraceptives). We had an underrepresentation of women, and some women were postmenopausal. Thus, future studies are needed to test generalizability of these findings to a broader population, and to study interactions with sex, age, BMI, and physical activity level.

Furthermore, it would be of value to test if genetic variance in those genes whose expression was shown here to change with late eating modifies the effect of late eating on obesity risk (Garaulet et al., 2016) and, more broadly, to identify individuals and groups most likely to benefit from meal-timing interventions. Additional important future directions include testing whether those genes identified to show changes in expression with late eating may serve as drug targets for obesity prevention and treatment. Finally, our data provide physiological and molecular mechanistic insights that may help in the development of timing-

based dietary interventions in the prevention, management, and treatment of obesity.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cmet.2022.09.007>.

ACKNOWLEDGMENTS

We thank the research volunteers and Center for Clinical Investigation nursing and technical staff. We thank Karen Yee, RD and Leigh K. Keating, RD for the diet preparation. This study was funded by R01DK099512, UL1TR001102, and UL1TR002541. F.A.J.L.S. was supported by NIH grants R01DK099512, R01HL118601, R01DK102696, R01DK105072, and R01HL140574. M.J.P. and M.J.B. were supported by DK020595. M.G. was supported by the Spanish Government of Investigation, Development and Innovation (SAF2017-84135-R) including FEDER co-funding; the Autonomous Community of the Region of Murcia through the Seneca Foundation (20795/PI/18); and NIDDK R01DK099512. S.L.C. was supported by the Alexander von Humboldt Foundation. J.Q. was supported by the American Diabetes Association (award 1-17-PDF-103) and by the NIH (grants K99HL148500 and R01DK102696). We also thank Prof. Susan Fried, PhD and members of her laboratory for generously providing guidance related to *ex vivo* adipose tissue collection, handling, and analysis.

AUTHOR CONTRIBUTIONS

F.A.J.L.S., M.J.B., and M.G. conceptualized the study (i.e., were involved in formulation/evolution of overarching research goals and aims). F.A.J.L.S. acquired funding and supervised research activity. F.A.J.L.S., D.B., A.N., N.V., and B.S. developed the study design and methodology for human

inpatient experiments. M.J.P. and M.J.B. developed the study design and methodology for molecular analyses. N.V., J.Q., S.L.C., A.N., D.B., and F.A.J.L.S. coordinated research activity planning and execution. K.K., S.W.H., and S.S. recruited study participants and collected data. N.V., M.J.P., S.L.C., and J.Q. were responsible for visualization and presentation of data. N.V., M.J.P., and W.W. performed statistical analyses. N.V., M.J.P., M.J.B., and F.A.J.L.S. wrote the manuscript, with editorial input from M.G., J.Q., S.L.C., A.N., D.B., K.K., S.W.H., S.S., W.W., and B.S. Original draft was prepared by N.V.

DECLARATION OF INTERESTS

During the execution of this project, F.A.J.L.S. received lecture fees from Bayer HealthCare, Sentara HealthCare, Philips, Vanda Pharmaceuticals, and Pfizer Pharmaceuticals; received consulting fees from the University of Alabama at Birmingham; and served on the Board of Directors for the Sleep Research Society. F.A.J.L.S.'s interests were reviewed and managed by Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies. None of these are related to the current work. N.V. has been compensated for consulting services provided to the Novartis Institutes of Biomedical Research, also unrelated to the current work.

INCLUSION AND DIVERSITY

We worked to ensure gender balance in the recruitment of human subjects. We worked to ensure ethnic or other types of diversity in the recruitment of human subjects. We worked to ensure that the study questionnaires were prepared in an inclusive way. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community.

Received: February 14, 2022

Revised: July 28, 2022

Accepted: September 12, 2022

Published: October 4, 2022

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human Blood Samples	This study	N/A
Human Adipose Samples	This study	N/A
Critical commercial assays		
E.Z.N.A. Total RNA Kit II	Omega bio-tek	R6934-01
E.Z.N.A. Micro Elute RNA Clean Up Kit	Omega bio-tek	R6247-01
HumanHT-12 v4 Bead Chip	Illumina	BD-901-1001
TrueSeq stranded mRNA kit	Illumina	20020594
Leptin RIA (RA010/HL-81K)	Millipore	https://www.emdmillipore.com/
EZGRA-88BK Human Ghrelin ELISA	Millipore	https://www.emdmillipore.com/
GHRA-88HK Human Ghrelin RIA	Millipore	https://www.emdmillipore.com/
Deposited data		
Gene Expression Data	Gene Expression Omnibus (GEO)	GEO: GSE190168
Software and algorithms		
Heatmapper	Babicki et al. (2016)	Heatmapper.ca
ConsensusPathDB-human	Max Plank Institute	http://cpdb.molgen.mpg.de/CPDB
DAVID Bioinformatics Resources	Laboratory of Human Retrovirology and Immunoinformatics	https://david.ncifcrf.gov/
GenomeStudio	Illumina	https://support.illumina.com/array/array_software/genomestudio/downloads.html
BaseSpace-Sequence Hub	Illumina	https://basespace.illumina.com/
Microsoft Excel 2013-17, 365	Microsoft	https://www.microsoft.com/en-us/microsoft-365/excel
SAS (Statistical Analysis Software) version 14.3 or above	SAS Institute	RRID: SCR_008567
Vitascore (Sleep scoring and analysis software)	Temec Technologies	https://vitascore.nl/
Respironics Actiware (Software package to analyze, manage, and export recorded activity data from all Actiwatch models)	Phillips Respironics	RRID: SCR_016440
Other		
Vmax Encore 29N indirect calorimeter	VIASYS Healthcare	http://www.viasyshealthcare.com/
Actiwatch	Phillips Respironics	https://www.usa.philips.com/healthcare/product/HC1046964/actiwatch-spectrum-activity-monitor

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Frank A.J.L. Scheer (fscheer@bwh.harvard.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- FAIR Standards Data Availability: In accordance with MIAME and MINSEQE standards, raw data files (FASTQ, IDAT) used for the analysis of gene expression data in this paper were uploaded to the public data repository Gene Expression Omnibus (GEO). Data can be reached at GEO: GSE190168.

- All other data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials, see [Data S1](#).
- As per the NIH Policy on Data Sharing, we will make the datasets available to other investigators following publication of the final study results. These datasets will not contain identifying information per the regulations outlined in HIPPA. Per standard Partners HealthCare System policies, we will require from any investigator or entity requesting the data a data-sharing agreement that provides for: (i) a commitment to using the data only for research purposes and not to identify any individual participant; (ii) a commitment to securing the data using appropriate computer technology; and (iii) a commitment to destroying or returning the data after analyses are completed.
- This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Participant details

The protocol was approved by the institutional review board at the Partners Human Research Committee, performed in accordance with the principles of the Declaration of Helsinki, and registered as [ClinicalTrials.gov](#) number: NCT02298790. Participants provided written informed consent. Sixteen participants (5 female) completed the study, all of them met the following inclusion criteria: 20–60 years of age (mean $\pm$ SEM 37.3 ± 2.8 years, range 25–59 years), BMI in the overweight or obese range between 25 and 34.9 kg/m^2 (mean $28.7 \pm 0.6 \text{ kg/m}^2$, $25.5\text{--}32.1 \text{ kg/m}^2$) and no diabetes, i.e., hemoglobin A1c $<6.5\%$ (mean $5.4 \pm 0.1\%$, $4.7\text{--}5.9\%$). Participants were in good health, were habitual breakfast eaters (based on 7-day food diaries), reported stable levels of physical activity, and no history of shift work in the prior 12 months. They also abstained from the use of tobacco, caffeine, alcohol, and (medical or recreational) drug use for at least 2 weeks prior to each laboratory visit, with an exception made for one participant who was taking a stable dose of antihypertensive medication throughout the whole study. For all outcome measures, sensitivity analyses were performed, and it was determined that exclusion of this individual did not change statistical outcome. To avoid any confounding effects of menopause on metabolic markers ([Ko and Jung, 2021](#)), no peri-menopausal women were enrolled in this within-subject crossover study. Furthermore, because menstrual cycle phase may affect resting metabolism ([Benton et al., 2020](#)), premenopausal women ($n = 3$) were scheduled to be studied at a similar menstrual phase for both their visits (i.e., phase-matched to either early follicular for both visits or late luteal for both visits to avoid hormone surges around ovulation). However, for logistical reasons one of these women could only be studied during follicular phase for one visit (randomized to early eating) and during luteal phase for the other (late eating) visit. Based on CBT data, we infer that ovulation did not occur during either visit. This participant also did not complete all hunger/appetite questions on time and was fully excluded from all appetite analyses. For all outcome measures besides hunger/appetite questionnaires, sensitivity analyses were performed and confirm that our statistical outcomes were unchanged regardless of this participant's inclusion as part of the cohort.

METHOD DETAILS

Overall experimental protocol

Participants completed two in-laboratory stays to examine the effects of late eating and early eating (in randomized order) on metabolic outcome measures. For the purposes of this study, we present data from the first 6 days of a longer (9-day) inpatient protocol. A schematic of the in-laboratory protocol is given in [Figure 2](#). Between the first and second laboratory visit, there was a washout period during which participants were instructed to resume their normal meal schedules and dietary habits. The two in-laboratory protocols were scheduled 3–12 weeks apart and each was preceded by a 2–3-week pre-inpatient lead-in designed to minimize potential confounding effects of prior sleep/wake schedule and a 3-day lead-in designed to minimize potential confounding effects of dietary habits ([Figure 2A](#)). Participants' sleep in the lab was scheduled according to their individual habitual sleep/wake cycle maintained in the weeks prior.

For each inpatient stay ([Figures 2B and 2C](#)), the first two days allowed participants to adapt to laboratory environment and procedures. Beginning on day 3, late and early eating protocols were established with an interval of 250 min between meals. In the early-eating protocol, participants ate meals at 1h0 m, 5h10 m and 9h20 m following wake time. In the late-eating protocol, subjects consumed meals at 5h10 m, 9h20 m and 13h30 m after wake. Study day 3 and 6 served as test days for key outcome measures including calorimetry-derived assessment of energy expenditure and subjective hunger/appetite during wake episode, as well as 24-h profiles of CBT, leptin and ghrelin. Subjects were allotted 20 min to complete each meal on study day 3 and 6, and 30 min on the other days. On study day 5 (at least three days into the late or early eating schedule) a fat biopsy was performed after an equal overnight fasting duration of 15 h in both protocols for analysis of gene expression.

Recruitment and screening

Subjects were recruited from the general population via internet, newspapers and flyers. Medical suitability was determined on the basis of phone screens, medical history, biochemical and toxicology screens of blood and urine, and a physical exam. Furthermore, psychiatric suitability was determined using several tests of mental state and a clinical interview with a clinical psychologist. During the first inpatient night, it was verified that none of the participants had sleep apnea (all AHI $\leq 3.1/\text{h}$).

Randomization

“Minimization” was used to minimize imbalance in the sequence of the early and late eating protocols based on sex, BMI, and age (in order of importance, from high to low) to optimize balanced numbers of male versus female, those with BMI defined as obese ($>30 \text{ kg/m}^2$) versus BMI defined as overweight ($25\text{--}30 \text{ kg/m}^2$), and those of younger age (20–40y) versus older age (41–60y) (Altman and Bland, 2005). This randomization-minimization program was run separately for participants with prediabetes (hemoglobin A1c 5.7–6.5%) versus those with normoglycemic A1c levels ($<5.7\%$), effectively making hemoglobin A1c level the highest priority stratification variable. Following completion of enrollment for the whole study, a Pearson’s Chi squared test showed no significant difference in the distribution of participants into early-late versus late-early visit order according to any of the above dichotomized variables.

Pre-inpatient study conditions

To ensure the initial stability of circadian rhythms and sleep patterns, subjects were required to maintain a regular sleep/wake schedule for at least 2–3 weeks prior to each laboratory admission. For both 2–3-week pre-inpatient segments, participants selected a fixed 8-h time in bed according to their habitual bedtime. Compliance was verified by wrist actigraphy (Actiwatch, Respironics), review of a sleep diary, and daily call-ins to a time-stamped voicemailbox when going to bed and getting out of bed. If more than one deviation ($>1 \text{ h}$) per week from the target schedule was detected, the participant was either disempanelled or asked to repeat all or part of the pre-inpatient study segment until schedule adherence was achieved. If washout time between study visits was longer than 3 weeks, participants were encouraged (but not strictly required) to maintain their regular sleep-wake schedule during this window.

Participants were provided with all meals for the last 3 days before both laboratory admissions in order to standardize diet quantity, composition, and timing. During these 3 days, participants were instructed to only eat the provided food and to eat the food at pre-arranged times as close as possible to the anticipated in-lab baseline mealtimes (see Figure 2A). This was verified by having participants call in to a time-stamped voice-mailbox when starting each meal, and by review of a meal diary. In this diary, we requested that participants document the timing, size and approximate content of all meals for at least 2 weeks prior to each laboratory visit. Of note, during the 3–12-week washout period between the two laboratory protocols, participants were instructed to resume their habitual meal schedules at home (with no caloric or dietary restrictions) up until the 3-day lead-in to the next study visit.

Inpatient study conditions

Participants spent their inpatient stay in individual laboratory suites at the Brigham and Women’s Hospital Center for Clinical Investigation with closely controlled environmental conditions, set up to minimize potential changes in environmental and behavioral factors. Room temperature was kept at $23 \pm 1^\circ\text{C}$, with relative humidity at 50–55%. Light levels were tightly controlled in their study suite where no windows were present. Participants did not have access to phones, internet, radio or visitors. Experimenters were present 24 h per day to monitor data acquisition, collect biological specimens and initiate tests. Participants abstained from unscheduled exercise and naps/sleep and adhered to instructions regarding timing and completion of all test meals and study procedures. A closed-circuit video camera and microphone were available in each suite for real-time participant safety monitoring and to ensure study protocol compliance. During times when participants were not involved in a study-related procedure, they were able to enjoy leisure activities, such as reading, writing, watching movies, crafts, playing musical instruments, etc. During these segments, participants were free to move about the suite as desired. Each participant’s activity level was monitored via continuous wrist actigraphy.

Both admissions to the CCI lab began on day 1 and throughout the whole in-laboratory stay, habitual, self-selected, fixed 8-h sleep episodes ($<0.02 \text{ lux}$, darkness) and 16-h wake episodes (90 lux, normal room light) were maintained, and the timing matched that maintained during the 2–3-week ambulatory segments. The room light exposure during scheduled wakefulness was designed to help keep the central circadian pacemaker entrained to the sleep/wake cycle, despite the difference in meal timing between the two protocols (Zeitzer et al., 2000). Throughout study day 1–6, each meal was of equal size, similar macronutrient composition and part of a calculated eucaloric diet (see Diet section below for details). On non-test days, participants were able to move freely about their suites, however shower schedules were strictly maintained, with two exceptions: on study day 2 showers could be delayed by up to 1 h to allow for IV insert and on study day 5, a washcloth was used at the scheduled shower time, to allow for healing of the adipose biopsy incision site.

The experimental eating protocol began on study day 3 and ended on study day 6, with an inter-meal interval of 250 min. In the early eating protocol, subjects consumed identical meals at 1h00 m, 5h10 m, and 9h20 m following scheduled time of awakening (times similar to breakfast, lunch, and dinner, with the last meal occurring 6h40 m before bedtime) (Figure 2). In the late eating protocol, subjects skipped breakfast and consumed identical meals at 5h10 m, 9h20 m, and 13h30 m after waking (times similar to lunch, dinner, and supper, with the last meal occurring 2h30 m before bedtime). To minimize confounding acute effects of physical activity or postural change on metabolic outcome variables, physical activity was controlled and kept to a minimum during in-lab visits; most of the wake episodes were spent sedentary and exercise was prohibited during the in-laboratory stays. On test days (study day 3 and 6) subjects were seated in a semi-recumbent position for most of the wake period, with only brief breaks to allow use of the bathroom/shower (see Figure 2, also gray bars on X axes in Figures 3 and 4). During all semi-recumbent protocol segments, participants were provided with a urinal or bedpan if needed to avoid upright posture during these times. Posture schedules were identical for the early and late protocol.

Diet

The Brigham and Women's Hospital Center for Clinical Investigations Dietary office calculated and provided a eucaloric standardized diet to participants both leading up to and during each in-laboratory study visit. The Mifflin St. Jeor formula was used to calculate daily target energy intake with an activity factor of 1.5 for the pre-inpatient portion and 1.4 for inpatient portion of the study, appropriate for the lower activity in the laboratory. A controlled nutrient diet using weighed foods was provided throughout the study. Participants' diet was comprised of 2.5 L water, ~100 mEq potassium, 150 mEq sodium, 45-50% carbohydrates, 15-20% protein, and 30-35% fat, glycemic index 60-65, per 24 h. Participants consented to eat all food provided and were given an explanation of how critical this was to the experiment. During screening, participants completed a study specific food preference form which allowed dietitians to calculate and prepare a menu cycle that met the diet orders and maintained the participants' weight. Because dietary composition and caloric intake were identical on each day and between both protocols, these factors could be ruled out as modulators of the outcome variables. Furthermore, daily in-laboratory body weight measurements from both the early and late eating protocols indicate that body weight was successfully kept consistent throughout the study, with no significant differences between early and late visits or between visit 1 and 2 regardless of randomization order (Figure S5).

All in-laboratory meals were served according to protocol schedule and consumed within 30 min. On Test Days, each test meal was actively supervised and timed via stopwatch by a study staff member present in the room, who ensured all items were started within 1 min of scheduled time (as indicated in Figure 2) and consumed within 20 min and in the exact same order every time. Completed written records verify this. All other meals during laboratory visits were started within 5 min of the scheduled time and fully consumed within 30 min. Furthermore, for all meals (i.e., including test meals and non-test meals), the consumption was also recorded by measuring and analyzing the exact food content of the meal tray before and after the meal was completed. On the rare occasions that a meal was not fully completed, our Dietary Research Office calculated the actual food consumption by subtracting any remaining food items from what was provided as the targeted meal. Analysis of the food consumption compared to the targeted food intake shows that average difference in consumption compared to provided food across individuals was $0.16\% \pm 0.36\%$ (3 ± 6 kcal/day), with a maximum of 1.3% difference.

Assessing perceived hunger and appetite

Intermittently over the course of study day 3 and 6, participants completed visual analog scales to assess subjective hunger/appetite including the questions: "How HUNGRY are you?", "How STRONG is your desire eat?", "How MUCH could you eat?", "How FULL do you feel?", "How much are you craving STARCHY foods?", "How much are you craving MEAT?", "How much are you craving DAIRY foods?", "How much are you craving VEGETABLES?", "How much are you craving FRUITS?", "How much are you craving SWEETS?", "How much are you craving SALTY foods?", "How THIRSTY are you?", and "To what extent are you experiencing NAUSEA?" They responded by clicking a point on a sliding scale between the 0 and 100 mm extremes (i.e., not at all hungry to very hungry) to indicate their feelings at that time-point. This tool has been previously used and validated (Flint et al., 2000). Because responses on this scale did not meet criteria for normality, statistical analyses were performed on both hunger probability data, and logit transformed dichotomized data, where answers from 0-50 were treated as "not hungry", "not a strong desire to eat" or "not craving starchy food", etc. while answers above 50 were treated as "hungry", "stronger desire to eat than usual" or "craving starchy food" etc. This dichotomization approach has been previously described and validated (Molfino et al., 2016; Zabel et al., 2009) with being hungry defined as VAS score of ≥ 50 .

Blood sampling and hormone assays

The experimental suites were equipped with a porthole to enable 24-h blood sample collection without disturbing the participant's sleep, and participants were outfitted with forearm IV catheters to collect blood samples. Designated CCI staff drew and processed blood samples at scheduled times, separating serum from plasma and ensuring that appropriate cooling and protease inhibitors were used to preserve specimens. Leptin assays on serum samples were performed using the Millipore Leptin RIA (RA010/HL-81K) at the BWH research assay core. Absolute leptin and ghrelin levels vary with demographic differences such as BMI, sex, ancestry, age, etc. Such demographic differences may have contributed to any differences between absolute hormone levels reported in the current and other studies. Subject P07 had a considerable number of leptin readings which were at the upper range of this assay (>100 ng/mL), which represent more than 1/3 of values for WP6 for late eating schedule visit. As a sensitivity analysis we proceeded with analysis either treating all these values as 100 ng/mL or excluding WP6 for this subject altogether and saw no difference in the outcome of our statistical model.

Active (acylated) ghrelin was initially (for the first $n = 7$ participants) measured from plasma using EZGRA-88BK Human Ghrelin ELISA at the BWH research assay core. After this kit was discontinued, plasma specimens for the remaining ($n = 9$) participants were assayed with GHRA-88HK Human Ghrelin RIA at the UPENN research assay core. Because this experiment had a within-subject-control design and all analyses were based on within-subject comparisons, this does not pose a concern for data integrity. One participant (P06) had a considerable number of ghrelin readings which appeared to be invalid due to technical issues. These represent more than 1/3 of values for study day 6 for late eating schedule visit and thus study day 6 (both early and late) for this participant was dropped altogether from the analysis.

Calorimetry

Using an indirect calorimeter (Vmax Encore 29N, VIASYS Healthcare, Yorba Linda, CA) energy expenditure was measured 12 times on each test day (see Figure 2). This included recordings immediately preceding each meal, and then 30 and 90 min after start of meal, as well as recordings at corresponding times for the “skipped meal” (i.e., recordings around a “phantom breakfast” in the late eating protocol or around a “phantom supper” in the early eating protocol). All participants were in bed, resting in a standardized semi-recumbent posture ≥ 30 min before the start of each recording. Recordings lasted 15–25 min with average duration of approximately 20 min. Energy expenditure was calculated on a minute-by-minute basis in kcals/day from gas expiration. Carbohydrate and lipid oxidation were calculated based on the Frayn equation, and because urine nitrogen was not measured, we estimated protein oxidation (Frayn, 1983). Calories expended, grams of carbohydrates oxidized, or grams of lipids oxidized during the waking segment of each test day were quantified as area under the curve for that set of 12 recordings (using trapezoid rule with imputed data points).

Only data points with an associated fraction of exhaled carbon dioxide (FeCO_2) between 0.5 and 1% were included in the analysis in accordance with recommendations of prior literature (Schadewaldt et al., 2013) and Vmax technical guidelines indicating that instrument/flow sensor detection is not reliable outside that FeCO_2 range. Data points during which participants fell asleep, made large movements, started coughing or which met none of the above criteria but constituted outliers outside the 3 standard deviation range within a single recording were excluded. After these preprocessing steps, recordings with less than 5 min of usable data were also excluded.

Missing/excluded recording values were imputed from the participant's average energy expenditure (i.e. mean of all recordings from both late and early conditions) and considering fasting duration. This imputation approach is based on classical statistical approaches (Riffenburgh, 2006) and was done blind to whether the missing data point was from early or late eating schedule, making it a conservative approach, biasing results toward the null hypothesis. The same procedure was repeated to impute missing values for carbohydrate and lipid oxidation. Of note, one participant was missing more than 1/3 of recordings on study day 6 of their early eating visit, thus data from study day 6 of both this subjects early and late visit were dropped altogether from the analysis and not imputed.

Core body temperature measurement

Core body temperature (CBT) was measured continuously via rectal thermometer throughout each test day. This system was configured to take readings every second, which were reduced to 10-min averages before subsequent analysis. Study staff performed routine sensor checks and monitored CBT data output and check sensor if the temperature was low. Trained research personnel filtered temperature data for non-physiological readings (temperature below 95 F or above 102 F) and excised segments where the temperature sensor was removed intentionally (e.g., use of bathroom) or appeared to have been inadvertently dislodged (i.e., sharp drops of body temperature observed at a rate that could not be physiologically possible). These filtered data were then subject to another level of pre-processing where investigators identified segments that appeared physiologically implausible/artefactual for reasons other than those listed above (e.g., those caused by environmental or experimental errors). One participant (P03) was not included in final analysis because large segments of data were composed of physiologically implausible values, indicating the sensor was not worn properly.

Adipose tissue biopsy

Adipose tissue biopsies were included in our study protocol as an opt-in procedure performed on a subset of participants. On both visits (i.e., early eating and late eating schedule), an abdominal subcutaneous adipose tissue sample was collected through surgical biopsy on day 5 of the protocol after an identical overnight fast duration. This 2–4 g sample was obtained from the lateral lower abdominal wall (below the level of the umbilicus) via dissection using sterile surgical procedures. Local anesthesia without epinephrine was used and the second biopsy was always taken from the contralateral side of the abdomen (relative to the first). Adipose specimens were dissected into several pieces that were flash frozen in liquid nitrogen immediately following excision and stored at -80°C until analysis. A pair of adipose tissue samples were acquired (one from each protocol) in 7 subjects. The other participants either didn't consent to the optional biopsy or the availability of the surgeons performing the biopsy didn't allow collection of the pair of biopsies in the other participants. The demographics of the subset of participants who provided biopsies (age: 40 ± 4.3 y; gender: 2F; 5M 40%F; BMI: 28.95 ± 0.89 kg/m²; A1c: $5.4 \pm 0.2\%$) did not significantly differ from the total group of 16 individuals who completed the study.

Sequencing and differential gene analysis

Fourteen adipose tissue samples (7 from the early eating protocol and 7 from the late eating protocol) were shipped on dry ice from Boston to Chicago for analysis. Tissue was homogenized in RNA Solv via a rotor homogenizer. RNA isolation from samples was performed using an E.Z.N.A. Total RNA Kit II (Omega bio-tek). This is a phenol chloroform chemistry-based isolation that utilizes nucleic acid binding spin columns. RNA was eluted from columns using 35 μL of 1.0 mM sodium citrate (6.4 pH). Eluted RNA concentrations were measured via nano spectrometer. If RNA concentrations were under 100 ng/ μL , samples were concentrated using an E.Z.N.A. Micro Elute RNA Clean Up Kit (Omega bio-tek). The integrity of the RNA was assessed via an Agilent Bioanalyzer. Samples were submitted to the genomics core at the University of Chicago for Micro-array/RNA-Seq analysis. The samples from the first four subjects had their expression profiles assessed via micro-array. The HumanHT-12 v4 Bead Chip (Illumina) was used. All samples were run in duplicate. Initial analysis and differential expression data were generated using GenomeStudio software. Quantile normalization was used across the samples. Missing bead data was imputed. Probes that had a low signal:noise ratio (detection p value greater

than 0.01) were excluded from analysis. Samples from the last three subjects were submitted for RNA-seq analysis. Library preparation was conducted using the Illumina TrueSeq stranded mRNA kit. Samples were run on a flow cell in the HiSeq 4000 system. Samples were run twice, with samples being rebalanced on the second run to even out total read counts across the samples. Project management and downstream analysis was done on the web based BaseSpace-Sequence Hub (Illumina) platform. Read alignment/mapping was done with the RNA-seq Alignment app (Illumina).

Gene expression data from the two platforms (micro-array/RNA-Seq) were combined and expressed in fold change between groups, with the early eating group being set as the reference group. Expression data that was not present in both the platforms was excluded from further analysis. Differential expression analysis was done by running a two-tailed t test assuming unequal variances between the two groups for each expressed gene and having the resulting p values rank ordered. All genes that had a lower p value than 0.05 constituted a narrowed gene list used for further analysis. Heat maps were generated using Heatmapper.ca web-based platform (Babicki et al., 2016). For the heat maps, data was clustered by average linkage and distance measurement method was Euclidean.

Pathway analysis

Narrowed gene lists from our differential gene analysis were used for overrepresentation analysis. ConsensusPathDB-human (Max Plank Institute) and DAVID Bioinformatics Resources web-based platform was used to determine signaling pathway interactions, protein-protein interactions, and ontological group relationships between the genes submitted for analysis (Huang da et al., 2009a, 2009b; Kamburov et al., 2013). Final pathway networks depicted were selected by passing a false discovery rate threshold of ($q < 0.05$).

Correlation of gene expression changes with hormone level changes

Pearson correlation coefficients were generated from comparison of gene expression changes of biopsied tissue to the changes in serum leptin or plasma acylated ghrelin levels from blood samples collected one day following the adipose tissue biopsy but at the same clock time and after a similar approximately 15 h of fasting (± 30 min, see Figure 2). This correlation was only performed for genes which had adequate readout from all participants ($n = 7$ for leptin and $n = 6$ for ghrelin). T-statistics and p values were generated from the Pearson coefficients. The p values were adjusted to generate q values to satisfy a false discovery rate (Storey, 2003). Q values of < 0.05 were considered significant.

Polysomnography

Sleep was measured on night 1 and night 5 of each laboratory visit by EEG, EOG, and submental EMG using American Academy of Sleep Medicine recommended EEG derivations (C3/4, F3/4, O1/2, referenced to M2/1) (Vitaport recorder, Temec, Netherlands). Two channels of this montage were devoted to EKG recording. On night 1, additional equipment was used to allow for detection of obstructive sleep apnea, including thoracic and abdominal resistance bands, a pulse oximeter, nasal flow cannula and snore microphone. Designated staff checked PSG recording signals visible on a monitor outside each participant's suite once per hour on each scheduled night of recording, to troubleshoot any problematic signals to the extent possible from outside the suite, and if subject request a urinal/bedpan during the sleep opportunity, to take advantage of any such instance to briefly attempt any necessary PSG troubleshooting within the suite. EEG files were exported and viewed using the Vitascore program and visually scored as wake, non-rapid eye movement (NREM, stages N1, N2, or N3) sleep, or REM sleep by a single registered PSG technician according to American Academy of Sleep Medicine guidelines (Berry et al., 2012). Wake after sleep onset (WASO) duration was calculated as minutes of total wake minus sleep-onset latency (SOL). SOL was calculated as minutes from scheduled lights off until first full minute spent in either N1 or N2 sleep stage.

QUANTIFICATION AND STATISTICAL ANALYSIS

Figures 3 and 4 and Table 2 summarize all planned analyses of primary and secondary physiological outcome measures. Figure 5 and Table S1 summarize all planned analyses for molecular outcome measures. Additional analyses included assessing sleep (Figure S4) and body weight (Figure S5) as potential confounding/mediating variables and a correlational analysis between hormone and gene expression data (Figure S3) conducted to gain further mechanistic insight into the observed effects of late eating.

Systemic physiological data were analyzed using a linear mixed model or generalized linear model, treating participant as a random effect and early (versus late) eating protocol, study day (3 versus 6) and the interaction of protocol and study day as fixed effects. This was done using SAS statistical analysis software (STAT version 14.3 or above). *A priori*, we estimated that 16 participants in a randomized crossover design would be sufficient to test our hypotheses with $> 90\%$ power based on paired, two tailed tests, with $\alpha = 0.05$.

Because visual analog scale data did not meet all criteria for parametric analysis, they were dichotomized and logit transformed, and a generalized linear mixed model was used to calculate the odds ratio of hunger (or other VAS outcome) being > 50 . Participants were treated as a random effect and protocol (early versus late eating schedule), study day (3 versus 6) and the interaction of protocol and study day as fixed effects. Although results text and Table 2 report odds ratios, for the sake of clarity, right-hand panels of Figures 3 and S1 show probability of VAS score being > 50 .

A linear mixed model was used to model levels of leptin (or ghrelin) normalized to the baseline (early eating) average of a given subject, treating participant as a random effect and protocol (early versus late eating schedule), study day (3 versus 6) and the interaction of protocol and study day as fixed effects. This was done for the wake-time average, 24 h average and sleep episode average of each hormone as well as the ratio of acylated ghrelin:leptin. The same type of model was applied to wake-time average, 24-h average and sleep episode average of CBT as well as wake-time normalized data for calories expended, grams of carbohydrates oxidized, and grams of lipids oxidized. Family-wise error rate correction (Holm-Bonferroni) was applied to adjust for multiple testing of primary outcome measures within the category of energy intake (self-reported hunger during waking hours, 24-h leptin and 24-h acylated ghrelin) and energy expenditure (calorimetrically assessed energy expenditure during waking hours and 24-h CBT). A paired t-test was used to determine whether there were differences in sleep on study night 5 between the early and late protocol. Adipose tissue gene expression data were analyzed as described in the “[pathway analysis](#)” and “[correlation of gene expression changes with hormone level changes](#)” sections.