Unacylated ghrelin, leptin, and appetite display diurnal rhythmicity in lean adults.

Iain Templeman[1]*, Harry A. Smith[1]*, Jean-Philippe Walhin[1], Benita Middleton[2], Javier T. Gonzalez[1], Leonidas G. Karagounis[3,4], Jonathan D. Johnston[2], James A. Betts[1]

Affiliations:

[1]Centre for Nutrition, Exercise and Metabolism, Department for Health, University of Bath, Bath, United Kingdom, BA2 7AY

[2]Section of Chronobiology, School of Biosciences and Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford GU2 7XH

[3]Nestlé Health Science, Translation Research, Avenue Nestlé 55, CH-1800 Vevey, Switzerland

[4]Institute of Social and Preventive Medicine, University of Bern, 3012 Bern, Switzerland

*These authors contributed equally to this work

Running Head: Diurnal rhythms in appetite

Corresponding Author:

Professor James A. Betts.

Department for Health, University of Bath, Bath, BA2 7AY United Kingdom

Tel: +44 (0) 1225 38 3448

Email: J.Betts@bath.ac.uk

Abstract

Constant routine and forced desynchrony protocols typically remove the effects of behavioural/environmental cues to examine endogenous circadian rhythms, yet this may not reflect rhythms of appetite regulation in the real world. It is therefore important to understand these rhythms within the same subjects under controlled diurnal conditions of light, sleep and feeding.

Ten healthy adults (9M/1F, Mean ±SD: age: 30 ± 10 y; BMI: 24.1 ± 2.7 $kg \cdot m^{-2}$) rested supine in the laboratory for 37 hours. All data were collected during the final 24 hours of this period (i.e. 0800 – 0800 h). Participants were fed hourly isocaloric liquid meal replacements alongside appetite assessments during waking before a sleep opportunity from 2200-0700 h. Hourly blood samples were collected throughout the 24-h period.

A diurnal rhythm in mean plasma unacylated ghrelin concentration was identified (p=0.04), with the acrophase occurring shortly after waking (08:19 h), falling to a nadir in the evening with a relative amplitude of 9%. Plasma leptin concentration also exhibited a diurnal rhythm (p<0.01), with the acrophase occurring shortly after lights-out (00:32 h) and the lowest concentrations at midday. The amplitude for this rhythm was 25%. Diurnal rhythms were established in all dimensions of appetite except for sweet preference (p=0.29), with both hunger (21:03) and prospective food consumption (19:55) reaching their peak in the evening before falling to their nadir shortly after waking.

Under controlled diurnal conditions, simultaneous measurement of leptin, unacylated ghrelin, and subjective appetite over a 24-hour period revealed rhythmicity in appetite regulation in lean, healthy humans.

New and noteworthy

Simultaneous assessment of subjective appetite, unacylated ghrelin, and leptin was carried out over a continuous 37 h protocol for the first time under conditions of controlled light, sleep, and feeding in healthy lean adults. Rhythms were observed in unacylated ghrelin, leptin, and components of subjective appetite, such as hunger, prospective consumption, and fullness. Concurrent measurement of rhythms in these variables is important to fully understand the temporal relationships between components of appetite as well as the influence of diurnal factors such as sleep, light, and feeding.

Key words

Appetite, Circadian rhythms, Ghrelin, Leptin, Diurnal

Introduction

Circadian rhythms describe the periodic oscillations in mammalian physiology and behaviour that occur with approximate 24-hour cycles across most species (53). Such temporal rhythms serve to align physiological processes with anticipated environmental events (22), thereby facilitating survival in an evolutionary context (36).

Recent evidence in humans underlines the importance of the human circadian system in metabolic regulation, including appetite control. Specifically, constant routines and forced desynchrony protocols reveal how ratings of hunger typically peak in the evening, when satiety is generally lowest; whereas hunger is lowest during the early hours of the morning and for the first few hours after waking (42, 44, 55). Daily rhythms have also been identified in the systemic concentrations of hormones implicated in appetite regulation (11, 13, 23), such as ghrelin (31, 44) and leptin (48). However, data on the role of unacylated ghrelin in appetite regulation are uncertain,

and requires investigation especially in the context of subjective appetite (2, 20, 52). Furthermore, due to the necessarily protracted measurement period required to examine daily rhythms, the availability of within-subject human data is limited regarding the temporal relationship between subjective appetite and endocrine appetite regulators.

Constant routine and forced desynchrony protocols are incredibly useful in revealing endogenous circadian rhythms but they also remove the diurnal influence of behavioural and environmental cues, which are critical within a more ecologically valid setting (31). For example, rhythms in plasma ghrelin and leptin concentrations can change in response to sleep (14, 46), feeding (13), and extended fasting (15, 34). The diurnal rhythm of these hormones is therefore subject to modification by behavioural and/or environmental factors, which may independently influence rhythms in subjective appetite. Accordingly, there is an outstanding need to examine 24-hour rhythms in systemic unacylated ghrelin and leptin concentrations, whilst concurrently measuring appetite ratings under controlled diurnal conditions.

To this end, the present study aimed to quantify 24-hour profiles in plasma unacylated ghrelin and leptin concentrations, alongside subjective appetite under a semi-constant routine (i.e. feeding during waking hours), in which light-dark exposure and sleep-wake opportunity were tightly controlled. This was achieved using hourly isocaloric feedings throughout waking hours to suppress the postprandial ghrelin rebound, which may have driven previously reported rhythms (13, 51). It was expected that subjective hunger would be lowest in the biological morning relative to the evening, which would be mirrored by rhythms in unacylated ghrelin. It was also expected that rhythmicity would be present in 24-h leptin.

Materials and Methods

Research Design

Using a time-series design, temporal rhythms in leptin, unacylated ghrelin and appetite were quantified under conditions of semi-constant routine, as previously described (28, 29, 37). Briefly, participants underwent a week of meal and sleep synchronisation prior to a 37-hour laboratory visit. During the final 24-hours of this visit participants had a designated sleeping opportunity and hourly isocaloric feedings during waking periods to preserve diurnal influences. Visual Analogue Scales (VAS) were completed hourly during waking periods to measure appetite ratings, whilst hourly blood samples were collected throughout day and night during sleep to monitor accompanying rhythms in the systemic concentrations of unacylated ghrelin and leptin, along with melatonin to provide a validated internal phase marker. Ethics approval for the experimental protocol was obtained from the NHS research ethics committee (reference: 14/SW/0123). These data were collected as part of a larger study exploring diurnal rhythms in skeletal muscle lipidomics and transcriptomics, which have been reported elsewhere (28, 37).

Participants

Ten healthy participants (9M;1F, **Table 1**) were recruited via local advertisement. Participants were screened via the completion of a general health questionnaire and validated questionnaires to assess habitual sleep patterns and diurnal preferences (8, 19, 40) as described previously (28, 37). All volunteers were fully briefed on the requirements of the study and provided written informed consent for their involvement.

[Table 1]

Experimental Protocol

In the week preceding the laboratory visit participants adhered to a strict routine of feeding and sleeping. Specifically, they woke between 0600 and 0700 h and went to bed between 2200 and 2300 h, which was confirmed using time-stamped voicemail. Furthermore, each day participants ensured at least 15 minutes of natural light exposure within 1.5 hours of waking, compliance with which was affirmed by wrist actigraphy using a light sensor, which further confirmed standardization of sleep-wake patterns (Actiwatch^{TM}, Cambridge Neurotechnology; Cambridge, UK). Self-selected meals were also scheduled at 0800, 1200 and 1800 h, with designated snacking opportunities at 1000, 1500 and 2000 h. For the final two days of this standardisation period, participants completed a weighed record of all food and fluid intake.

Following this, participants reported to the laboratory at 1900 h the evening prior to the scheduled 24-hour measurement window to acclimatise to the laboratory environment (**Figure 1**). All laboratory conditions were standardised for the duration of their stay, with blackout-blinds to prevent the penetration of natural light and room temperature maintained at 20-25^{o}C. Artificial lighting was set at 800 lux in the direction of gaze during waking hours (0700-2200 h) and turned off (0 lux) during sleeping hours (2200-0700 h), with participants wearing an eye mask for the duration of the sleep opportunity. Participants remained in a semi-recumbent position throughout (i.e. head-end of bed elevated to 30^{o}). Upon arrival, participants were shown to their bed and provided with a prescribed meal composed of a baked potato with butter and cheese, steamed vegetables (broccoli and mini-corn), followed by a bowl of fresh strawberries, raspberries and blueberries (1245 kcal; 31% carbohydrate, 50% fat and 19% protein). An instant hot chocolate made with whole milk was then provided at 21:30 (242 kcal; 56% carbohydrate, 24% fat and 20% protein) before lights out at 2200 h.

141 Participants were woken at 0700 h and resting metabolic rate was immediately
142 measured over 15 minutes using indirect calorimetry via the Douglas bag technique
143 (9). An intravenous cannula was fitted to an antecubital vein to allow for hourly 10 mL
144 blood draws from 0800 h, alongside VAS during waking hours. After each set of
145 measurements, an hourly feed was then ingested in the form of a meal-replacement
146 solution (1.25 kcal·mL^{-1}, 45% carbohydrate, 25% fat, 30% protein; Resource Protein,
147 Nestlé; Vevey, Switzerland). Each hourly dose was prescribed to give 6.66% of
148 measured 24 h resting metabolic rate across the 15 h wake period time, thus meeting
149 ongoing energy requirements and resulting in energy balance for the entire 24 h
150 sampling period. Plain water was consumed *ad libitum* and participants had access
151 to mobile devices, on-demand entertainment, music and reading material throughout
152 waking hours only. Toilet breaks were permitted in the first half of each hour as
153 required.

154 The final set of waking measurements were collected at 2200 h, along with ingestion
155 of the final prescribed feed. Following this, the lights were switched-off and participants
156 were asked to wear an eye mask throughout the lights-out period. Blood samples
157 continued throughout the night at hourly intervals without intentionally waking the
158 participants. At 0700 h, participants were woken and immediately completed a set of
159 VAS before a blood sample was drawn. The final set of measurements were made at
160 0800 h.

161 In accordance with the wider objectives of the study (28, 37), it should be noted that
162 muscle biopsies were collected from the *vastus lateralis* at 4-hourly intervals from 1200
163 until 0800 h (i.e. 6 in total) for transcriptomic and lipidomic analyses (data previously
164 reported). For these night-time tissue biopsies (i.e. 0000 and 0400 h) participants were
165 woken briefly but continued to wear the eye mask while samples were taken by torch-

166 light. Each biopsy took ~5-10 minutes and daytime biopsies were taken following the
167 VAS and blood sample but before the prescribed feed.

168 [Figure 1]

169 *Outcome Measures*

170 **Blood Sampling and Analysis –** At each time-point, 10 mL of whole blood was drawn
171 and immediately distributed into tubes treated with lithium heparin (for melatonin) or
172 ethylenediaminetetraacetic acid (EDTA; for leptin/ghrelin). Both tubes were
173 immediately centrifuged for 10 minutes (3466 x g, 4^{o}C), after which the supernatants
174 were removed and stored at -80^{o}C.

175 Hourly, plasma melatonin concentration was measured in the heparinised samples
176 using a radioimmunoassay (Surrey Assays Ltd; Intra-assay CV: 9.7 $\pm$ 4.9 %, Inter-
177 assay CV: 16.5 $\pm$ 8.7 %). Unacylated ghrelin (SPI-Bio; Intra-assay CV: 5.7 $\pm$ 1.0 %,
178 Inter-assay CV: 15.7 $\pm$ 2.6%) and leptin concentrations (R&D Systems; Intra-assay
179 CV 3.2 $\pm$ 0.2 %, Inter-assay CV: 4.4 $\pm$ 1.0 %) in EDTA-treated plasma were quantified
180 throughout the protocol at 4-hourly intervals starting at 0800 h (i.e. 7 samples total)
181 using commercially available enzyme linked immunosorbent assays.

182 **Appetite Ratings** – Visual analogue scales featured eight scales to assess hunger,
183 desire to eat, fullness, thirst and food preference (sugary, salty, savoury and fatty).
184 Each scale presented a question (e.g. how hungry do you feel?), which participants
185 answered by placing a vertical line on a 100 mm scale to denote their perception
186 relative to the extremes, which were defined as ‘not at all/very low’ to ‘extremely/very
187 high’.

188 *Statistical Analysis*

Due to the high inter-individual variability, values for plasma leptin and unacylated ghrelin were normalised to give a percentage of the 24-hour mean for each participant (raw values in **Figures S1** & **S2**). Values for each participant were then adjusted to dim light melatonin onset (DLMO), as determined by the 25% method with the time of DLMO being assigned at 0º of the circadian phase (5). Values for each outcome were aligned to DLMO by calculating the time in minutes between the DLMO and midnight for each participant and then adjusting 24-h profiles by the calculated difference in minutes. The resulting x-values were binned around half past the hour with average y-values plotted at half past the hour (29, 35, 51). As the study period was one circadian cycle long analysis of rhythmicity in all outcome measures was conducted using the cosine method allowing for calculation of parameters of rhythmicity such as acrophase, amplitude, and MESOR (Prism 8, Graphpad; CA, USA) (6, 39). Analysis of rhythmicity was performed for each individual's profiles, as well as at the group level for both raw and % 24-h mean values. In this approach a cosine wave is fit to the 24-h profile of a given variable and compared against a horizontal line through the mean values (null). If a cosine wave provides a better fit (R^2) for the data than the horizontal line then the dataset characterises diurnal (or 24-h) rhythmicity, with the mesor (rhythm-adjusted mean), amplitude (magnitude of the difference between mesor and peak/trough values) and acrophase (timing of rhythmic peak) all identified and reported (10, 39). For comparison of mean values 24-h apart (i.e. 0800 h day 1 *vs* 0800 h day 2) a paired *t*-test or a Wilcoxon signed rank test was performed depending on the distribution of data (SPSS Statistics 23.0, IBM; NY, USA). To further explore the relationship between measured appetite hormones and subjective appetite simple linear regressions were performed between plasma concentrations of leptin/unacylated ghrelin with subjective ratings of hunger, prospective consumption,

and fullness. Further simple linear regressions were run to explore the relationships between BMI with baseline and peak plasma leptin and unacylated ghrelin respectively. All data are presented as mean ± SD unless otherwise stated (e.g. figures are mean ± SEM).

Results

Melatonin

Individual plasma melatonin responses are reported elsewhere (28) and confirm the presence of neuroendocrine rhythms in all participants.

Leptin Profile

When each individual's data are expressed as a percentage of their 24-h mean, mean plasma leptin of the 10 participants exhibited a significant diurnal rhythm (p<0.001, F = 37.4, R^2 = 0.55, **Figure 2A**). The acrophase occurred at 00:32 h and concentrations were at their lowest following midday. The amplitude for this rhythm was 25%. Leptin concentrations measured 24 hours apart (i.e. same clock time: 08:00 h) were not different (start = 163 ± 242 pg·ml^{-1}, end = 147 ± 216 pg·ml^{-1}; p=0.58, F = 0.77). At the individual level, leptin was rhythmic for six of ten participants (**Table S1** available at https://doi.org/10.6084/m9.figshare.13153190, **Figure S1** available at: https://doi.org/10.6084/m9.figshare.13153187.v3).

Unacylated Ghrelin

When expressed as a percentage of the 24 h mean, mean plasma unacylated ghrelin was rhythmic (p = 0.04, F = 3.39, R^2 = 0.10, **Figure 2B)**. The acrophase occurred at 08:19 h and fell to the nadir in the evening, with an amplitude of 9%. Unacylated ghrelin concentrations measured 24-h apart (i.e. same clock time: 08:00 h) were lower at the

end of the measurement window when compared to the beginning (start = 41.1 ± 17.8 $pg \cdot ml^{-1}$, end = 35.7 ± 13.2 $pg \cdot ml^{-1}$; p=0.05, F = 0.45). At the individual level, unacylated ghrelin was rhythmic for only one of ten participants (**Table S1** available at https://doi.org/10.6084/m9.figshare.13153190, **Figure S2** available at: https://doi.org/10.6084/m9.figshare.13153193.v3).

[Figure 2]

Ratings of Appetite

As shown in **Table 2**, diurnal rhythms were established in all dimensions of appetite except for sweet preference at the group level. Hunger and prospective consumption both oscillated around the centre of the scale, whilst ratings of fullness tended to oscillate at the lower end of the scale throughout the 24-hour period. Rhythms in desire to eat savoury foods returned the highest mesor and amplitude. Both hunger and prospective consumption were characterised by similar phase relationships, peaking in the evening before falling to their nadirs shortly after waking (**Figures 3A, B**). This pattern was mirrored in the desire to eat salty, savoury, and fatty foods (**Figure 3E, F, G**) all peaking within a 2-hour window shortly before lights out. Fullness was characterised by an approximately antiphasic rhythm to hunger and prospective consumption (**Figure 3C**), peaking shortly after midday and falling to a trough after sleep onset. At the individual level, rhythmicity was present in 3 participants for hunger, 5 for prospective consumption, 4 for fullness, 2 for sweet preference, 4 for savoury preference, 6 for salty preference, and 6 for fatty preference (**Table S1** available at https://doi.org/10.6084/m9.figshare.13153190).

Ratings of hunger (p = 0.04, F = 0.69), prospective consumption (p = 0.03, F = 0.94) and desire to eat savoury foods (p = 0.03, F = 0.92) were higher at the end of the 24-

hour period relative to the beginning but desire to eat fatty (p = 0.06, F = 0.89), sweet (p=0.08), or salty (p=0.08) foods, and or fullness (p = 0.12, F = 0.02) ratings were similar.

[Figure 3]

[Table 2]

Relationships between appetite hormones, subjective appetite, and BMI

Simple linear regression revealed no significant relationships between plasma leptin concentrations and subjective hunger (p =0.60), prospective consumption (p = 0.51), or fullness (p = 0.86) (**Figure 4**). No relationship was observed between plasma unacylated ghrelin with subjective hunger (p = 0.36), or fullness (p = 0.44) but a weak negative relationship between unacylated ghrelin and prospective consumption was evident (R^2 = 0.26, p = 0.04) (**Figure 4**). BMI was not predictive of baseline (P =0.18) or peak unacylated ghrelin (P =0.30) (**Figure 5**). Likewise, BMI and was also not predictive of baseline plasma leptin (P =0.07) however BMI was positively associated with peak plasma leptin however (R^2 = 0.25, P =0.05) (**Figure 5**).

[Figure 4]

[Figure 5]

Discussion

Within a single participant group, this study compares diurnal rhythmicity in systemic unacylated ghrelin and leptin concentrations and the majority of the measured dimensions of appetite. Participants were assessed day and night in highly controlled conditions during a semi-constant routine (i.e. continuous/hourly feeding during waking hours; controlled posture, light-dark and sleep-wake cycles). Dim light

melatonin onset (DLMO) occurred at ~2330 h with individual melatonin profiles confirming the presence of neuroendocrine rhythms in all participants (Figure S1 & S2). Specifically, rhythmic analysis revealed ratings of hunger were highest in the biological evening when unacylated ghrelin was lowest and leptin was highest. Ratings of fullness peaked at midday falling to their lowest levels overnight, with prospective consumption and desire to eat savoury, salty and fatty foods peaking in the evening, before declining overnight to a trough shortly after waking.

Ratings of hunger increased throughout the day to peak at ~2100 h before declining overnight. Despite the diurnal influences of feeding and sleep, the current study agrees with previous constant routine (55) and forced desynchrony (44) protocols, showing lower hunger ratings in the morning with maximum levels in the evening/early night (55). Comparable peaks in the biological evening were also apparent for prospective consumption and the desire to consume salty foods (1910-2030 h). We also observed a diurnal rhythm in feelings of fullness, which were similarly phased to those observed in Sargent *et al* (42) using a 28-hour forced desynchrony protocol. Conversely, the present study did not identify a rhythm in desire to consume sweet foods, which could be due to the sweet taste of the meal-replacement supplement used in this study (18), but also may be driven by habitual diet and behaviour (54). Equally, the sweet taste of the meal replacement could also drive the increase in salty and savoury food preference across the day (17).

Diurnal rhythmicity was identified in unacylated ghrelin, with the acrophase occurring at ~0800 h, before declining throughout waking hours. Previous studies report rhythms in total ghrelin, with the acrophase and nadir reported to be in the region of 2300-0100 h and 0900-1100 h, respectively (13, 14, 31, 57). The rhythm reported in the current study contrasts those reported in studies of continuous fasting, in which total ghrelin

concentrations have been shown to increase prior to habitual meal times before decreasing spontaneously within 1-2 hours (15, 34). Consequently, rhythmicity in unacylated ghrelin in the current study is most likely driven by the diurnal influence of feeding isocaloric meal replacements during waking hours (51). Notably, Solomon *et al* (50) showed that consuming an isocaloric diet through two large meals resulted in more profound peaks and troughs in ghrelin concentration, when compared to consuming the same diet as 12 equally spaced boluses (25). Equally Leidy *et al* (24) observed that when energy-matched diets were consumed as either six or three equally spaced meals, more frequent feeding eliminated the eating-related oscillations in acylated ghrelin over an 11-h period. Whilst acylated ghrelin was not assessed in the current study, Spiegel *et al* (51) observed broad alignment in 24 h profiles of acylated and total ghrelin (reflective of unacylated ghrelin) under controlled diurnal conditions, in which participants were fed 3 identical carbohydrate rich meals across the day, interspersed by 5 h intervals. Furthermore, the gradual increase in unacylated ghrelin reported here during the night is consistent with the reported stimulation of plasma ghrelin during sleep (12, 14, 27, 51). This agrees with previous studies reporting a reduction in the ratio between acylated and total ghrelin overnight thereby supporting a potential decrease in the activity of ghrelin-O-acyl-transferase (GOAT) during sleep (26, 33). It must be noted however that whilst unacylated ghrelin was rhythmic at the group level, at the individual level, only 1/10 participants were rhythmic for unacylated ghrelin and this data must therefore be interpreted with caution.

To the knowledge of the authors, 24-hour unacylated ghrelin concentrations have not been measured under conditions of semi-constant routine (i.e. controlled light-dark, sleep-wake and fed-fasted cycles) with simultaneous assessments of subjective appetite. Whereas unacylated ghrelin was highest in the morning and declined

overnight, ratings of hunger were lowest during the morning and increased throughout the day to peak in the evening. Much debate surrounds the role of unacylated ghrelin in appetite regulation, with studies of analog forms showing no effect (20), increased (52), and decreased food intake (2, 3) in both humans and rodent models. Comparatively less is known about endogenous unacylated ghrelin and its effect upon appetite regulation in humans. Measurement of this hormone over a 24-h period alongside subjective appetite ratings in the current study therefore provides novel human insight *in vivo*. Taken together, the approximate anti-phasic relationship between unacylated ghrelin and subjective hunger ratings supports the idea that unacylated ghrelin plays a role in appetite suppression (2, 3). The negative relationship between unacylated ghrelin and prospective consumption further supports this notion however the current study was not powered for this outcome and future work should continue to investigate the role of endogenous unacylated ghrelin in human appetite regulation in humans.

Leptin also exhibited diurnal oscillations in the present study, peaking within the hour after midnight and declining to its lowest concentrations at midday. This is consistent with previous studies of 24-h profiles in systemic leptin (43, 45, 48). Across a 24-h period in which participants consumed 3-meals and a snack Sinha *et al* (48) reported a similar profile of leptin across the day, declining across the day before peaking overnight peak (~0200 h). Likewise, Schoeller *et al* (45) also demonstrated that lower values of leptin occur during the day before rising to peak overnight (~0000 h). Under conditions of forced desynchrony, Scheer *et al* (43) established that leptin rhythms track the behavioural rather than the circadian phase, rising throughout waking hours from a trough prior to breakfast to a peak at the onset of sleep, several hours after the final meal. Data from Schoeller *et al* (45) suggests that the rhythm in systemic leptin

359 is particularly influenced by meal timing, with a 6-hour phase shift in the leptin rhythm
360 occurring in response to a 6.5-h delay in meal times. Shea *et al* (46) demonstrated a
361 clear distinction between the circadian and diurnal profiles of plasma leptin, indicating
362 a strong effect of behaviour in the diurnal profiles. Furthermore, the slight delay in the
363 timing of the nadir in the leptin rhythm in the present study (occurring at midday rather
364 than breakfast) is remarkably similar to Mäntele *et al* (29), who employed essentially
365 an identical schedule of sleeping and feeding, as emphasised by the similar DLMO.
366 Sleep also plays an important role in the nocturnal peak in leptin, which is thought to
367 facilitate prolonged fasting overnight (33, 47). Whilst chronic insufficient sleep does
368 not appear to meaningfully alter rhythmic leptin, a recent study that removed the
369 diurnal influence of sleep through continual wakefulness across 26-h did not report
370 significant rhythmicity in leptin (41). The agreement of rhythmic parameters of leptin
371 between the current study and previous literature therefore further supports the notion
372 that 24-h profiles of leptin are driven by behavioural, rather than circadian factors (38).
373 Interestingly, BMI appeared to have a weak predictive ability for peak leptin
374 concentrations across the 24-h period, however the study was not directly powered
375 for this outcome and therefore warrants further exploration.

376 Considering the proposed role of leptin in inducing satiety (4, 23) the evening rise in
377 leptin reported here is seemingly misaligned with subjective hunger and fullness,
378 which also increased during the evening. The evening rise in both leptin and subjective
379 hunger are well-supported by prior literature when measured independently (7, 29, 47,
380 56) and simultaneously (32). Speculatively this misalignment may hint at the longer-
381 term effects of leptin in signalling energy balance rather than acute hunger/fullness
382 (21) but may also be due to the primarily circadian drivers of rhythms subjective hunger
383 relative to the predominant behavioural drivers of plasma leptin rhythms (38).

Whilst the pattern of feeding in the current study was more reflective of real-life patterns of eating relative to constant routine studies the even distribution of energy intake to be consumed hourly across waking hours is still somewhat artificial and future studies should build upon these findings. Furthermore, the use of a liquid meal replacement rather than solid would necessarily alter gastric emptying and even appetite (1, 30), however it is not yet known whether or not this would influence rhythmicity over a 24-h period. Whereas hourly sleep fragmentation *per se* has been shown to not influence ghrelin levels (16, 49) we cannot rule out an effect of night time sampling procedures (i.e. biopsies) on sleep quality and therefore unacylated ghrelin/leptin. The recruitment of predominantly male, lean subjects limits the generalisability of the current data to women and populations with overweight/obesity. It should also be noted that the current data are published secondary to previous work (28, 37), and therefore no formal power calculation was performed. However, the complexity of our primary transcriptomic/lipidomic for often subtle rhythms in multiple genes/metabolites means that the same sample size was more than adequate to detect meaningful changes in systemic endocrine responses (28, 37).

In summary, this study demonstrated 24-hour rhythmicity in systemic concentrations of unacylated ghrelin and leptin, as well as appetite under conditions of semi-constant routine. Lower appetite in the morning compared to the evening was observed, whereas unacylated ghrelin peaked in the morning, declining through waking hours. Furthermore, the 24 h profile of leptin was such that plasma leptin was highest during the night relative to the day. This manuscript provides novel context for rhythmicity in appetite in measuring appetite regulatory hormones alongside subjective ratings of appetite.

Acknowledgements

J.A.B., J.D.J., and J-P.W. designed the research; I.T., J.A.B., J-P.W., conducted the research.; I.T., H.A.S., J.T.G., J.A.B., B.M., and J.D.J. analysed data., H.A.S., I.T., J-P.W., J.T.G., J.D.J., J.A.B., wrote the paper; J.A.B. had primary responsibility for final content. All authors read and approved the final manuscript.

Grants

The work was part funded by the Biotechnology and Biological Sciences Research Council Grant BB/I008470/1 (to J.D.J.). Nestlé Institute of Health Sciences provided funding for consumables.

Disclosures

I.T., H.A.S., J.P.W. declare no conflicts of interest. B.M. is a co-director of Stockgrand Ltd J.T.G. has received research funding and/or has acted as a consultant for Arla Foods Ingredients, Lucozade Ribena Suntory, Kenniscentrum Suiker and Voeding, and PepsiCo. L.G.K. is an employee of Nestle Health Sciences. J. D. J. has performed consultancy work for Kellogg Marketing and Sales Company (UK) Limited, and has collaborated with the Nestlé Institute of Health Sciences. J.A.B. has received research funding from BBSRC, GlaxoSmithKline, Nestlé Institute of Health Sciences, Lucozade Ribena Suntory, Kellogg's, PepsiCo and the Rare Disease Foundation.

References

1. **Achour L, Méance S, and Briend A**. Comparison of gastric emptying of a solid and a liquid nutritional rehabilitation food. *Eur J Clin Nutr* 55: 769-772, 2001.
2. **Allas S, Caixas A, Poitou C, Coupaye M, Thuilleaux D, Lorenzini F, Diene G, Crino A, Illouz F, Grugni G, Potvin D, Bocchini S, Delale T, Abribat T, and Tauber M**. AZP-531, an unacylated ghrelin analog, improves food-related behavior in patients with Prader-Willi syndrome: A randomized placebo-controlled trial. *PLoS One* 13: 2018.
3. **Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, and Kasuga M**. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. *Gut* 54: 18-24, 2005.
4. **Baicy K, London ED, Monterosso J, Wong ML, Delibasi T, Sharma A, and Licinio J**. Leptin replacement alters brain response to food cues in genetically leptin-deficient adults. *Proc Natl Acad Sci U S A* 104: 18276-18279, 2007.

5. **Benloucif S, Burgess HJ, Klerman EB, Lewy AJ, Middleton B, Murphy PJ, Parry BL, and Revell VL**. Measuring melatonin in humans. *Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine* 4: 66-69, 2008.
6. **Bingham C, Arbogast B, Guillaume GC, Lee JK, and Halberg F**. Inferential statistical methods for estimating and comparing cosinor parameters. *Chronobiologia* 9: 397-439, 1982.
7. **Boden G, Ruiz J, Urbain JL, and Chen X**. Evidence for a circadian rhythm of insulin secretion. *The American journal of physiology* 271: 1996.
8. **Buysse DJ, Reynolds CF, 3rd, Monk TH, Berman SR, and Kupfer DJ**. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry research* 28: 193-213, 1989.
9. **Compher C, Frankenfield D, Keim N, and Roth-Yousey L**. Best practice methods to apply to measurement of resting metabolic rate in adults: a systematic review. *J Am Diet Assoc* 106: 881-903, 2006.
10. **Cornelissen G**. Cosinor-based rhythmometry. *Theor Biol Med Model* 11: 16, 2014.
11. **Cummings DE**. Ghrelin and the short- and long-term regulation of appetite and body weight. *Physiol Behav* 89: 71-84, 2006.
12. **Cummings DE, Frayo RS, Marmonier C, Aubert R, and Chapelot D**. Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. *American journal of physiology Endocrinology and metabolism* 287: E297-304, 2004.
13. **Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, and Weigle DS**. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 50: 1714-1719, 2001.
14. **Dzaja A, Dalal MA, Himmerich H, Uhr M, Pollmacher T, and Schuld A**. Sleep enhances nocturnal plasma ghrelin levels in healthy subjects. *American journal of physiology Endocrinology and metabolism* 286: E963-967, 2004.
15. **Espelund U, Hansen TK, Hojlund K, Beck-Nielsen H, Clausen JT, Hansen BS, Orskov H, Jorgensen JO, and Frystyk J**. Fasting unmasks a strong inverse association between ghrelin and cortisol in serum: studies in obese and normal-weight subjects. *The Journal of clinical endocrinology and metabolism* 90: 741-746, 2005.
16. **Gonnissen HK, Hursel R, Rutters F, Martens EA, and Westerterp-Plantenga MS**. Effects of sleep fragmentation on appetite and related hormone concentrations over 24 h in healthy men. *Br J Nutr* 109: 748-756, 2013.
17. **Griffioen-Roose S, Hogenkamp PS, Mars M, Finlayson G, and de Graaf C**. Taste of a 24-h diet and its effect on subsequent food preferences and satiety. *Appetite* 59: 1-8, 2012.
18. **Hetherington M, Rolls BJ, and Burley VJ**. The time course of sensory-specific satiety. *Appetite* 12: 57-68, 1989.
19. **Horne JA, and Ostberg O**. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* 4: 97-110, 1976.
20. **Inhoff T, Mönnikes H, Noetzel S, Stengel A, Goebel M, Dinh QT, Riedl A, Bannert N, Wisser AS, Wiedenmann B, Klapp BF, Taché Y, and Kobelt P**. Desacyl ghrelin inhibits the orexigenic effect of peripherally injected ghrelin in rats. *Peptides* 29: 2159-2168, 2008.

21. **Joannic JL, Oppert JM, Lahlou N, Basdevant A, Auboiron S, Raison J, Bornet F, and Guy-Grand B**. Plasma leptin and hunger ratings in healthy humans. *Appetite* 30: 129-138, 1998.
22. **Johnston JD**. Physiological links between circadian rhythms, metabolism and nutrition. *Exp Physiol* 99: 1133-1137, 2014.
23. **Klok MD, Jakobsdottir S, and Drent ML**. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obesity reviews : an official journal of the International Association for the Study of Obesity* 8: 21-34, 2007.
24. **Leidy HJ, Armstrong CL, Tang M, Mattes RD, and Campbell WW**. The influence of higher protein intake and greater eating frequency on appetite control in overweight and obese men. *Obesity (Silver Spring, Md)* 18: 1725-1732, 2010.
25. **Leidy HJ, and Campbell WW**. The effect of eating frequency on appetite control and food intake: brief synopsis of controlled feeding studies. *The Journal of nutrition* 141: 154-157, 2011.
26. **Lim CT, Kola B, Korbonits M, and Grossman AB**. Ghrelin's role as a major regulator of appetite and its other functions in neuroendocrinology. *Prog Brain Res* 182: 189-205, 2010.
27. **Liu J, Prudom CE, Nass R, Pezzoli SS, Oliveri MC, Johnson ML, Veldhuis P, Gordon DA, Howard AD, Witcher DR, Geysen HM, Gaylinn BD, and Thorner MO**. Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. *The Journal of clinical endocrinology and metabolism* 93: 1980-1987, 2008.
28. **Loizides-Mangold U, Perrin L, Vandereycken B, Betts JA, Walhin JP, Templeman I, Chanon S, Weger BD, Durand C, Robert M, Paz Montoya J, Moniatte M, Karagounis LG, Johnston JD, Gachon F, Lefai E, Riezman H, and Dibner C**. Lipidomics reveals diurnal lipid oscillations in human skeletal muscle persisting in cellular myotubes cultured in vitro. *Proc Natl Acad Sci U S A* 114: E8565-E8574, 2017.
29. **Mantele S, Otway DT, Middleton B, Bretschneider S, Wright J, Robertson MD, Skene DJ, and Johnston JD**. Daily rhythms of plasma melatonin, but not plasma leptin or leptin mRNA, vary between lean, obese and type 2 diabetic men. *PLoS One* 7: e37123, 2012.
30. **Martens MJ, Lemmens SG, Born JM, and Westerterp-Plantenga MS**. A solid high-protein meal evokes stronger hunger suppression than a liquefied high-protein meal. *Obesity (Silver Spring)* 19: 522-527, 2011.
31. **McHill AW, Hull JT, McMullan CJ, and Klerman EB**. Chronic Insufficient Sleep Has a Limited Impact on Circadian Rhythmicity of Subjective Hunger and Awakening Fasted Metabolic Hormones. *Frontiers in endocrinology* 9: 319, 2018.
32. **McHill AW, Hull JT, McMullan CJ, and Klerman EB**. Chronic Insufficient Sleep Has a Limited Impact on Circadian Rhythmicity of Subjective Hunger and Awakening Fasted Metabolic Hormones. *Front Endocrinol* 9: 2018.
33. **Morselli LL, Guyon A, and Spiegel K**. Sleep and metabolic function. *Pflugers Arch* 463: 139-160, 2012.
34. **Natalucci G, Riedl S, Gleiss A, Zidek T, and Frisch H**. Spontaneous 24-h ghrelin secretion pattern in fasting subjects: maintenance of a meal-related pattern. *Eur J Endocrinol* 152: 845-850, 2005.
35. **Otway DT, Mantele S, Bretschneider S, Wright J, Trayhurn P, Skene DJ, Robertson MD, and Johnston JD**. Rhythmic diurnal gene expression in human

adipose tissue from individuals who are lean, overweight, and type 2 diabetic. *Diabetes* 60: 1577-1581, 2011.
36. **Panda S, Hogenesch JB, and Kay SA**. Circadian rhythms from flies to human. *Nature* 417: 329-335, 2002.
37. **Perrin L, Loizides-Mangold U, Chanon S, Gobet C, Hulo N, Isenegger L, Weger BD, Migliavacca E, Charpagne A, Betts JA, Walhin JP, Templeman I, Stokes K, Thompson D, Tsintzas K, Robert M, Howald C, Riezman H, Feige JN, Karagounis LG, Johnston JD, Dermitzakis ET, Gachon F, Lefai E, and Dibner C**. Transcriptomic analyses reveal rhythmic and CLOCK-driven pathways in human skeletal muscle. *eLife* 16: 34114, 2018.
38. **Poggiogalle E, Jamshed H, and Peterson CM**. Circadian regulation of glucose, lipid, and energy metabolism in humans. *Metabolism* 84: 11-27, 2018.
39. **Refinetti R, Lissen GC, and Halberg F**. Procedures for numerical analysis of circadian rhythms. *Biol Rhythm Res* 38: 275-325, 2007.
40. **Roenneberg T, Wirz-Justice A, and Merrow M**. Life between clocks: daily temporal patterns of human chronotypes. *Journal of biological rhythms* 18: 80-90, 2003.
41. **Rynders CA, Morton SJ, Bessesen DH, Wright KP, Jr., and Broussard JL**. Circadian Rhythm of Substrate Oxidation and Hormonal Regulators of Energy Balance. *Obesity (Silver Spring)* 28 Suppl 1: S104-s113, 2020.
42. **Sargent C, Zhou X, Matthews RW, Darwent D, and Roach GD**. Daily Rhythms of Hunger and Satiety in Healthy Men during One Week of Sleep Restriction and Circadian Misalignment. *International journal of environmental research and public health* 13: 170, 2016.
43. **Scheer FA, Hilton MF, Mantzoros CS, and Shea SA**. Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc Natl Acad Sci U S A* 106: 4453-4458, 2009.
44. **Scheer FA, Morris CJ, and Shea SA**. The internal circadian clock increases hunger and appetite in the evening independent of food intake and other behaviors. *Obesity* 21: 421-423, 2013.
45. **Schoeller DA, Cella LK, Sinha MK, and Caro JF**. Entrainment of the diurnal rhythm of plasma leptin to meal timing. *J Clin Invest* 100: 1882-1887, 1997.
46. **Shea SA, Hilton MF, Orlova C, Ayers RT, and Mantzoros CS**. Independent circadian and sleep/wake regulation of adipokines and glucose in humans. *The Journal of clinical endocrinology and metabolism* 90: 2537-2544, 2005.
47. **Simon C, Gronfier C, Schlienger JL, and Brandenberger G**. Circadian and ultradian variations of leptin in normal man under continuous enteral nutrition: relationship to sleep and body temperature. *J Clin Endocrinol Metab* 83: 1893-1899, 1998.
48. **Sinha MK, Ohannesian JP, Heiman ML, Kriauciunas A, Stephens TW, Magosin S, Marco C, and Caro JF**. Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. *J Clin Invest* 97: 1344-1347, 1996.
49. **Smith HA, Hengist A, Thomas J, Walhin JP, Heath P, Perkin O, Chen YC, Gonzalez JT, and Betts JA**. Glucose control upon waking is unaffected by hourly sleep fragmentation during the night, but is impaired by morning caffeinated coffee. *Br J Nutr* 1-20, 2020.
50. **Solomon TP, Chambers ES, Jeukendrup AE, Toogood AA, and Blannin AK**. The effect of feeding frequency on insulin and ghrelin responses in human subjects. *The British journal of nutrition* 100: 810-819, 2008.

51. **Spiegel K, Tasali E, Leproult R, Scherberg N, and Van Cauter E**. Twenty-four-hour profiles of acylated and total ghrelin: relationship with glucose levels and impact of time of day and sleep. *The Journal of clinical endocrinology and metabolism* 96: 486-493, 2011.
52. **Toshinai K, Yamaguchi H, Sun Y, Smith RG, Yamanaka A, Sakurai T, Date Y, Mondal MS, Shimbara T, Kawagoe T, Murakami N, Miyazato M, Kangawa K, and Nakazato M**. Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. *Endocrinology* 147: 2306-2314, 2006.
53. **Van Gelder RN, and Buhr ED**. Ocular Photoreception for Circadian Rhythm Entrainment in Mammals. *Annual review of vision science* 2: 153-169, 2016.
54. **Venditti C, Musa-Veloso K, Lee HY, Poon T, Mak A, Darch M, Juana J, Fronda D, Noori D, Pateman E, and Jack M**. Determinants of Sweetness Preference: A Scoping Review of Human Studies. *Nutrients* 12: 2020.
55. **Wehrens SMT, Christou S, Isherwood C, Middleton B, Gibbs MA, Archer SN, Skene DJ, and Johnston JD**. Meal Timing Regulates the Human Circadian System. *Curr Biol* 27: 1768-1775, 2017.
56. **Westerterp-Plantenga MS**. Sleep, circadian rhythm and body weight: parallel developments. *The Proceedings of the Nutrition Society* 75: 431-439, 2016.
57. **Yildiz BO, Suchard MA, Wong ML, McCann SM, and Licinio J**. Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. *Proc Natl Acad Sci U S A* 101: 10434-10439, 2004.

Figure 1. Schematic representation of the study protocol. d1/d2/d3 = day 1/2/3 respectively.

Figure 2. Dim light melatonin onset (DLMO) adjusted 24-hour profiles of (A) Plasma concentration of leptin % of 24-h mean (B) Plasma concentration of unacylated ghrelin % of 24-h mean. Values are presented as mean ± SEM. The solid line denotes the regression that best fits the data. The dotted vertical line denotes DLMO whereas the dotted horizontal line denotes the mesor. The grey shaded areas represent 24-h melatonin profile.

Figure 3: Dim light melatonin onset (DLMO) adjusted 24-hour profile for ratings of: (A) hunger (B) prospective consumption (C) fullness (D) sweet preference (E) savoury preference (F) fatty preference (G) salty preference Values are presented as mean ± SEM. The solid line denotes the regression that best fits the data and the dotted horizontal line shows the 24-hour mean concentration used for the null comparison. The dotted vertical line denotes DLMO whereas the dotted horizontal line denotes the mesor. The shaded areas represent 24-h melatonin profile.

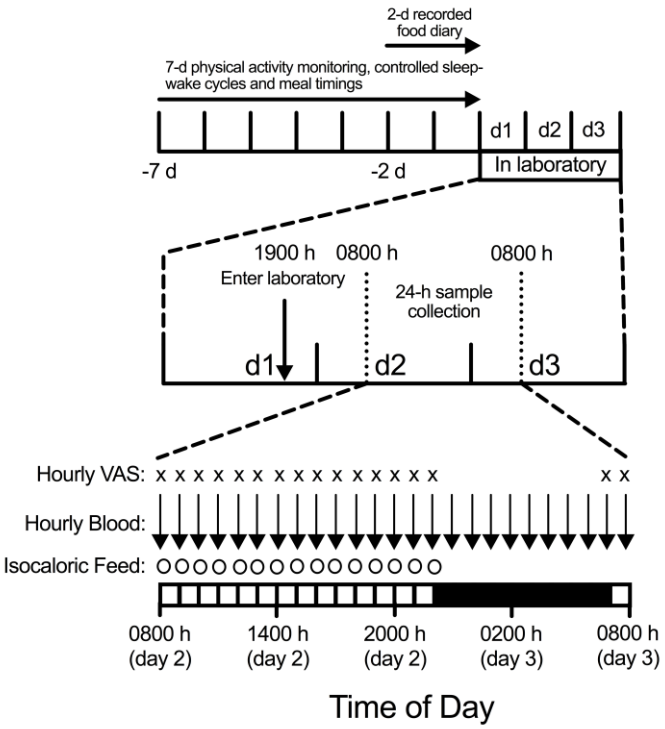
Figure 4. Simple linear regression between plasma unacylated ghrelin/leptin and (A/B) hunger, (C/D) prospective consumption, (E/F) fullness.

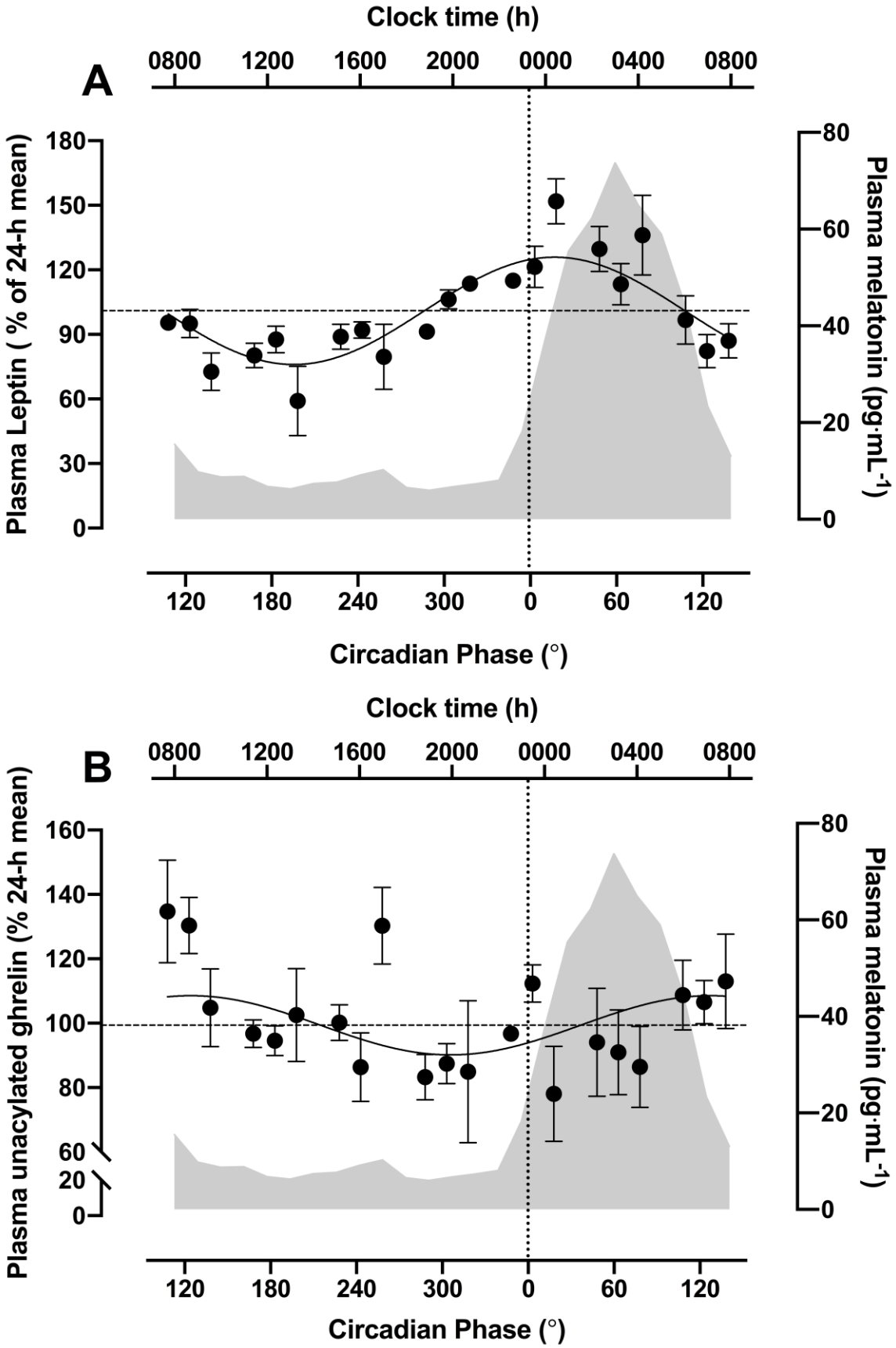
Figure 5. Simple linear regression between body mass index ($kg.m^2$) and peak plasma unacylated ghrelin/leptin and (A/B), body mass index and baseline plasma unacylated ghrelin/leptin (C/D).

641

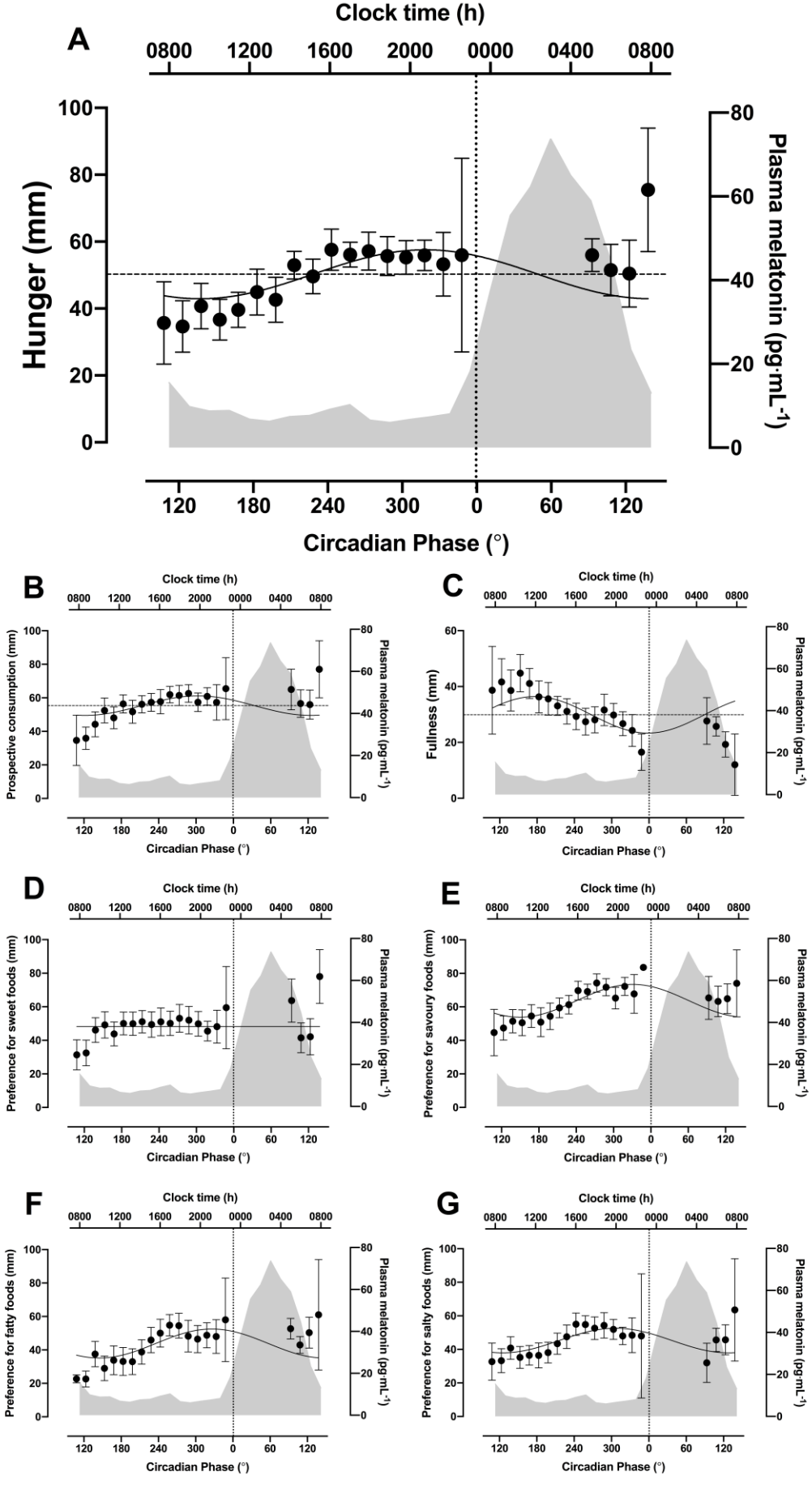
642

643

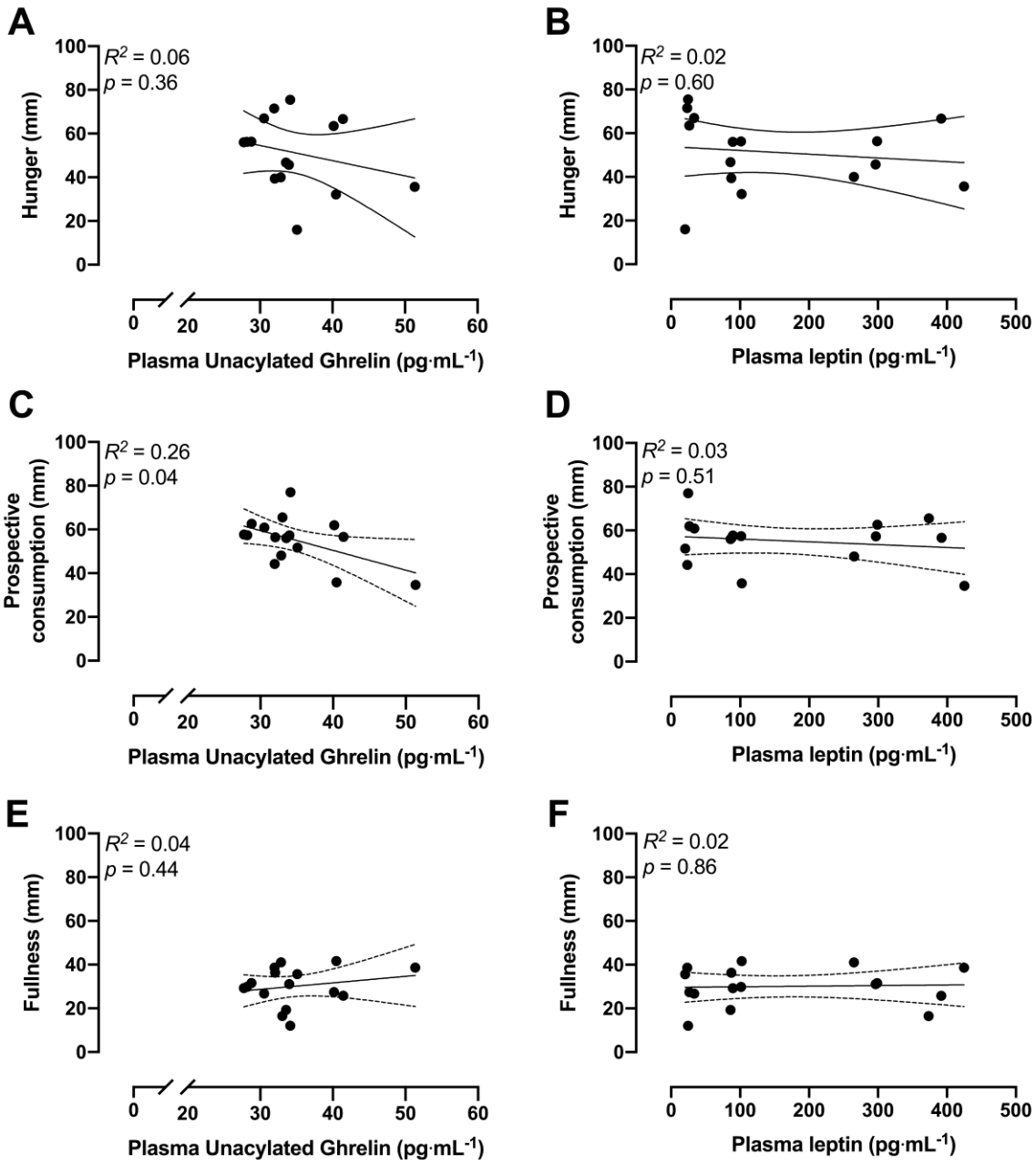

2-d recorded food diary
7-d physical activity monitoring, controlled sleep-wake cycles and meal timings
d1
d2
d3
-7 d
-2 d
In laboratory
1900 h
0800 h
0800 h
Enter laboratory
24-h sample collection
d1
d2
d3
Hourly VAS:
Hourly Blood:
Isocaloric Feed:
0800 h (day 2)
1400 h (day 2)
2000 h (day 2)
0200 h (day 3)
0800 h (day 3)
Time of Day


A
Clock time (h)
0800
1200
1600
2000
0000
0400
0800
Plasma Leptin (% of 24-h mean)
180
150
120
90
60
30
0
Plasma melatonin (pg·mL-1)
80
60
40
20
0
120
180
240
300
0
60
120
Circadian Phase (°)
B
Clock time (h)
0800
1200
1600
2000
0000
0400
0800
Plasma unacylated ghrelin (% 24-h mean)
160
140
120
100
80
60
20
0
Plasma melatonin (pg·mL-1)
80
60
40
20
0
120
180
240
300
0
60
120
Circadian Phase (°)

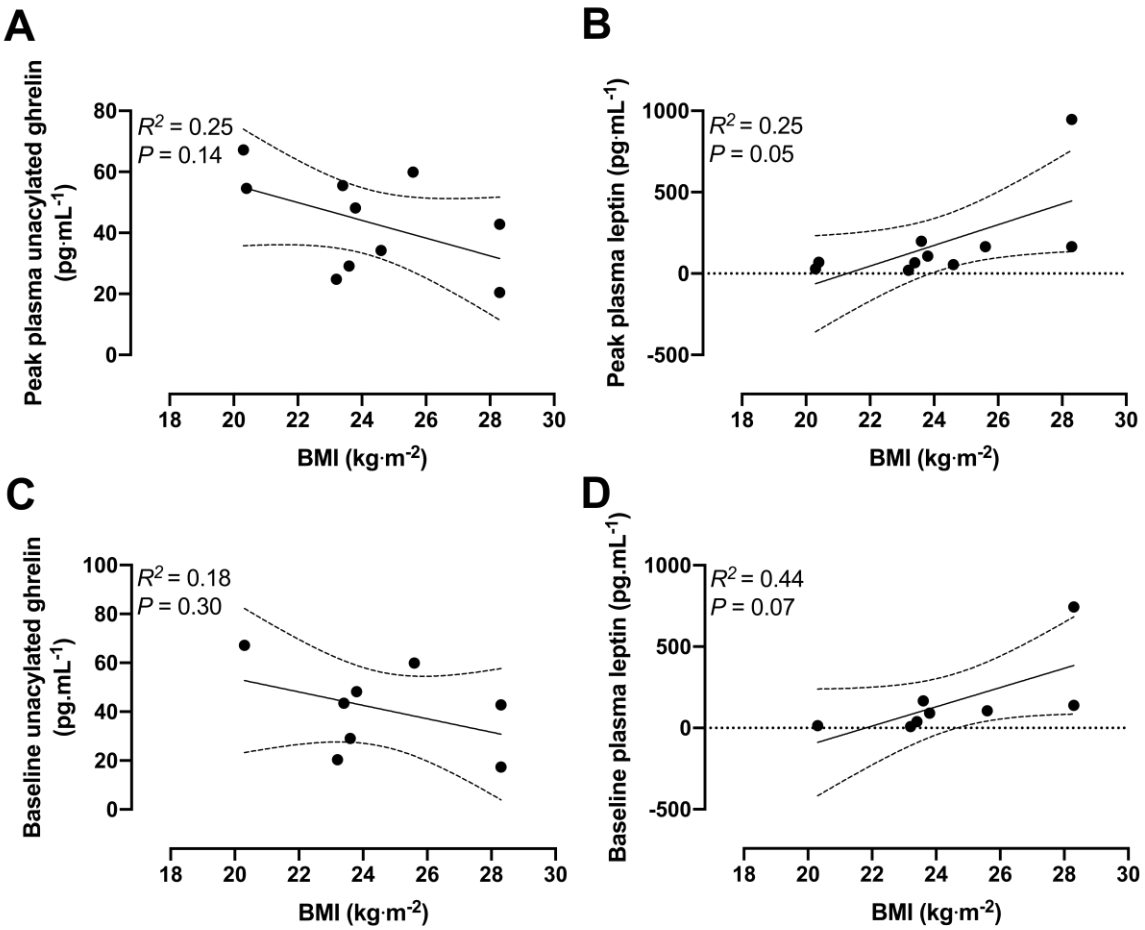
644


A
Clock time (h)
0800 1200 1600 2000 0000 0400 0800
Hunger (mm)
Plasma melatonin (pg·mL-1)
120 180 240 300 0 60 120
Circadian Phase (°)
B
Prospective consumption (mm)
C
Fullness (mm)
D
Preference for sweet foods (mm)
E
Preference for savoury foods (mm)
F
Preference for fatty foods (mm)
G
Preference for salty foods (mm)

645


A
R^2 = 0.06
p = 0.36
Hunger (mm)
Plasma Unacylated Ghrelin (pg·mL^{-1})
B
R^2 = 0.02
p = 0.60
Hunger (mm)
Plasma leptin (pg·mL^{-1})
C
R^2 = 0.26
p = 0.04
Prospective consumption (mm)
Plasma Unacylated Ghrelin (pg·mL^{-1})
D
R^2 = 0.03
p = 0.51
Prospective consumption (mm)
Plasma leptin (pg·mL^{-1})
E
R^2 = 0.04
p = 0.44
Fullness (mm)
Plasma Unacylated Ghrelin (pg·mL^{-1})
F
R^2 = 0.02
p = 0.86
Fullness (mm)
Plasma leptin (pg·mL^{-1})

646


A
Peak plasma unacylated ghrelin (pg·mL-1)
R2 = 0.25
P = 0.14
BMI (kg·m-2)
B
Peak plasma leptin (pg·mL-1)
R2 = 0.25
P = 0.05
BMI (kg·m-2)
C
Baseline unacylated ghrelin (pg.mL-1)
R2 = 0.18
P = 0.30
BMI (kg·m-2)
D
Baseline plasma leptin (pg.mL-1)
R2 = 0.44
P = 0.07
BMI (kg·m-2)