Alteration of Internal Circadian Phase Relationships after Morning versus Evening Carbohydrate-Rich Meals in Humans

Kurt Kräuchi,[1] Christian Cajochen, Esther Werth, and Anna Wirz-Justice

Centre for Chronobiology, Psychiatric University Clinic, Wilhelm Klein Strasse 27, CH-4025 Basel, Switzerland

Abstract The effects of a single morning and evening carbohydrate-rich meal for 3 consecutive days on circadian phase of core body temperature (CBT), heart rate, and salivary melatonin rhythms were compared under controlled constant routine conditions. In 10 healthy young men entrained to a natural light-dark cycle with regular sleep timing, CBT and heart rate were significantly elevated for approximately 8 h after the last evening carbohydrate-rich meal (EM), and nocturnal melatonin secretion (as measured by salivary melatonin and urinary 6-sulphatoxymelatonin levels) was reduced, compared to the morning carbohydrate-rich meal (MM) condition. Thus, circadian phase could not be measured until the following day due to this acute masking effect. The day after the last meal intervention, MM showed a significant advanced circadian phase position in CBT (+59 ± 12 min) and heart rate (+43 ± 18 min) compared to EM. However, dim-light melatonin onset was not significantly changed (+15 ± 13 min). The results are discussed with respect to central (light-entrainable) and peripheral (food-entrainable) oscillators. Food may be a zeitgeber in humans for the food-entrainable peripheral oscillators, but melatonin data do not support such a conclusion for the light-entrainable oscillator in the suprachiasmatic nucleus.

Key words carbohydrate-rich food, nonphotic zeitgeber, constant routine, circadian phase shifts, core body temperature, heart rate, melatonin, light-entrainable oscillator, food-entrainable oscillator, peripheral oscillators

Circadian rhythmicity in mammals is generated by an endogenous self-sustaining pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (for review, see Kittrell, 1991). However, overt rhythms, such as the circadian profile of core body temperature (CBT), do not simply reflect its output. They result from a complex interaction between the output of the circadian pacemaker, age, gender, light exposure, neuroendocrine feedback mechanisms, and periodic changes in behavior such as the timing of sleep and wakefulness. The most important environmental zeitgeber is the light-dark (LD) cycle, whereby photic information from the retina is transmitted via the retinohypothalamic tract to the SCN. In many species, additional (nonphotic) zeitgebers, such as novelty-induced running activity (Mrosovsky, 1992), social contact (Mrosovsky, 1995), exercise (Mistlberger, 1991; Turek, 1989), and periodic feeding (Stephan et al., 1979), contribute to circadian entrainment. The lateral geniculate nucleus and the intergeniculate leaflet of the subcortical nuclei constitute brain structures that appear to play a major role in mediating nonphotic as well as photic phase shifts of circadian rhythms (Harrington, 1997; Mrosovsky, 1995). The SCN receives a

1. To whom all correspondence should be addressed: Centre for Chronobiology, Psychiatric University Clinic, Wilhelm Klein Strasse 27, CH-4025 Basel, Switzerland; e-mail: kurt.kraeuchi@pukbasel.ch.

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further major input from the midbrain raphe nuclei (Ying and Rusak, 1997). This serotonergic input appears to modulate photic entrainment; for example, tryptophan loading in hamsters 1 h before lights-on delays activity onset relative to controls (Glass et al., 1995). Recent studies in mice emphasize that glucose metabolism is important because it appears to modulate sensitivity to light through activation of the serotonergic system (Challet, Losee-Olsen, et al., 1999; Challet, Van Reeth, et al., 1999), perhaps through activation of the serotonergic system.

In rats, a food-entrainable oscillator has been postulated to communicate between the digestive system and the central nervous system, exclusively entrained by nutritive meals (Mistlberger and Rusak, 1987). Glucose has zeitgeber properties for the food-entrainable oscillator in SCN-lesioned rats, but vegetable oil does not (Stephan and Davidson, 1998). It has long been known that periodic feeding induces anticipatory locomotor activity during constant light (LL) conditions as well as during an LD cycle, and many physiological, hormonal, and behavioral functions develop a similar kind of anticipation or entrainment (Johnson, 1992; Mistlberger, 1994; Stephan, 1986). When feeding is restricted to a limited time each day, anticipatory locomotor activity occurs even under LL or in SCN-lesioned rats (Stephan et al., 1979), suggesting an SCN-independent circadian timing system. Only very few rhythms remain unaffected by periodic feeding, for example those related to pineal melatonin synthesis (Holloway et al., 1979; Nagai et al., 1984), although this is dependent on rat strain (Challet et al., 1997). Furthermore, periodic food restriction in rats under an LD cycle only phase-advances the light-entrainable oscillator; no phase delays have been found (Challet et al., 1998). Large changes in feeding schedules affect the physiological state of the organism and lead to long-term "adaptations" of circadian temporal order.

There are very few, rather uncontrolled studies investigating the effects of meal timing on circadian rhythms in humans (Apfelbaum et al., 1976; Goetz et al., 1976; Sensi and Capani, 1987). In shift workers, meal timing does not appear to be a strong synchronizer of circadian rhythms (Reinberg, 1983). With respect to selective macronutrients as an entraining agent, only protein-rich foods have been tested for their capacity to entrain human circadian rhythms under LD conditions (Apfelbaum et al., 1976; Reinberg, 1983). However, with the exception of urinary nitrogen (phase shifts parallel to the protein-rich meal intake schedule), all variables investigated (e.g., heart rate, plasma cortisol) exhibited no alterations in acrophase or amplitude. To our knowledge, possible zeitgeber properties of carbohydrate-rich food in humans have not yet been investigated. Therefore, using the controlled conditions of a constant routine protocol, we measured circadian rhythms of CBT, heart rate, and melatonin before and after 3 days during which a single carbohydrate-rich meal was given either in the morning or evening of each day. Based on the results of animal studies (e.g., Challet et al., 1998), we hypothesized that circadian phase of CBT, heart rate, and melatonin rhythms would be advanced after three pulses of an MM compared to three pulses of an EM.

METHODS

Subjects

Ten healthy male subjects (age = 25.8 ± 1.3 years, body mass index = 22.97 ± 0.51 kg/m^2) gave signed informed consent to participate in the study and were instructed to refrain from alcohol, drugs, and more than one cup of coffee per day. The experimental protocol was accepted by the Human Research Committee of the Department of Medicine, University of Basel, Switzerland. The subjects were free of self-reported sleep complaints, were neither extreme morning nor evening types (defined by scores < 14 and > 21 on the morning-eveningness questionnaire of Torsvall and Åkerstedt, 1980). Further exclusion criteria included no shift work or transmeridian travel within 1 month prior to the study, no smoking, no medication, and no drug use. Medical disorders were screened by history and physical examination. One week prior to and during the study, subjects were instructed to maintain regular bedtimes between 2400 and 0730 h. Adherence to a regular sleep-wake schedule during the week immediately prior to admission was verified with a wrist actigraph (Gähwiler, Zürich, Switzerland). Only subjects who maintained the regular sleep-wake schedule were admitted to the study. Each subject spent an adaptation night in the sleep laboratory to test his ability to sleep in a new environment and to exclude subjects with sleep apnea.

Design

Subjects underwent two experimental sessions that constituted two 48-h episodes in the laboratory and

were at least 2 weeks apart. The sessions (see Fig. 1) began with a controlled 5-day baseline at home. They were asked to continue their regular sleep-wake cycle, with sleep between 2400 and 0730 h, and maintain a regular eating pattern consisting of three mixed meals per day (about 50% carbohydrates, 25% protein, and 25% fat).

The subjects reported to the laboratory on a Wednesday at 0800 h and remained seated (< 100 lux) for lunch at 1200 h and electrode attachment at 1245 h. They remained supine in bed from 1330 h until Friday 0800 h in a noise-attenuated chronobiology laboratory (< 8 lux, room temperature 22 °C, humidity 60%, light bedcover) under the unmasking conditions of a modified constant routine (CR) protocol (baseline CR). In contrast to a classical CR protocol (Czeisler et al., 1986; Mills et al., 1978), sleep was allowed from 2400 to 0730 h. This protocol provided the controlled conditions (no postural changes, regular intake of 100-kcal sandwiches, and 100 mL of water at 1-h intervals) to examine various variables under minimal masking due to behavior or environment (for details, see Kräuchi et al., 1997). After the baseline CR, the subjects went home on Friday morning and consumed the usual three mixed meals per day. On Saturday and Sunday, they were instructed to eat a single, large carbohydrate-rich meal, which had been prepared beforehand (spaghetti, carrots, and tomato sauce) (1600 kcal, 75% carbohydrate, 11% protein, 14% fat) either in the morning (0830 to 0900 h, MM condition) or in the evening (2130 to 2200 h, EM condition). During the 3 days at home the subjects were required to keep their regular sleep timing between 2400 and 0730 h. On Monday morning, the subjects reported to the laboratory at 0800 h and ate the last scheduled carbohydrate-rich meal under controlled conditions either in the morning (sitting position) or in the evening (supine position). This after-intervention CR started on Monday at 1400 h. The protocol was the same as for the baseline CR with the exception that no sandwiches were served on the last intervention day (Monday). Morning and evening meal schedules were applied in a randomized, counterbalanced order.

Measures

CBT was continuously measured and stored in 2-min intervals on a PC. A thermocouple (Ahlborn, Holzkirchen, Germany) was used to measure CBT (probe inserted 10 cm into the rectum). In the electrocardiogram, interbeat intervals were detected off-line and heart rate was calculated (for details, see Kräuchi et al., 1997). Heart rate (beats per minute [bpm]) was smoothed by a 60–min moving average and later collapsed in 30-min bins before entering statistical analysis. During the CRs, subjective sleepiness was assessed at half-hourly intervals on the Karolinska Sleepiness Scale (Gillberg et al., 1994; results reported in Kräuchi et al., 1998), together with collection of saliva for measurement of melatonin using a highly specific direct double-antibody radioimmunoassay (Weber et al., 1998). Urine was collected in 3-h intervals for the assessment of 6-sulphatoxymelatonin (AMT6S, ELISA-Kit, Bühlmann Laboratories, Schönenbuch, Switzerland). Body weight was measured at 1330 h on day 1 and at 0805 h after the last night of each session. Indirect calorimetry was performed for determining energy expenditure and respiratory quotient (RQ) before (1325 to 1400 h) and after (0735 to 0800 h) each session using a ventilated hood system (Deltatrac, MBM-100, Datex Instrumentarium, Helsinki, Finland).

Data Analysis

Data were analyzed using the statistical packages StatView 4.5 and SuperANOVA (Abacus Concepts, Berkeley, CA).

Because there was no third experimental session without any feeding interventions between the two CRs, possible order effects between the baseline and after-intervention CRs could not be assessed. Comparison of the baseline CRs of MM and EM conditions was a prerequisite for the present design in order to test for similarity of circadian phase and amplitude. Two parts of the CR were distinguished: day 1 of the CR from 1400 to 2400 h, followed by a nocturnal sleep episode (2400 to 0730 h), and day 2 of the CR from 0730 to 2400 h, again followed by a nocturnal sleep episode (2400 to 0730 h). The acute effects of the last EM were compared to those of the last MM during the period between 1800 h of day 1 and 1330 h of day 2. Differences in circadian phase and waveform between MM and EM conditions were compared in the after-intervention CR on day 2.

Statistically significant changes in the time courses were tested by two-way analyses of variance for repeated measures (rANOVAs) with time of day (time) and meals (morning vs. evening) as factors. Huynh-Feldt statistics were used to adjust the covariance matrix for violations of sphericity. Huynh-

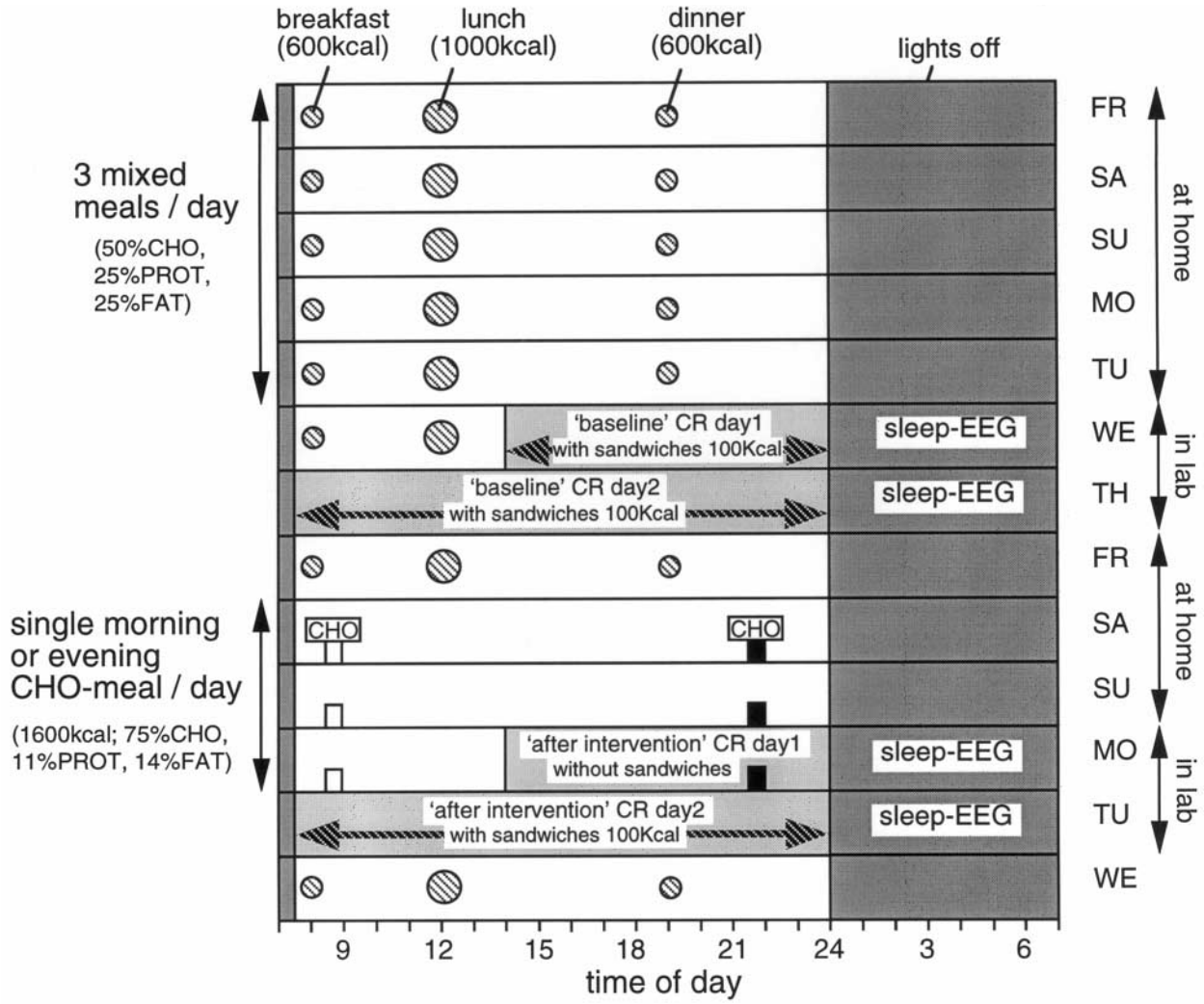

Figure 1. Design of the study for the morning and evening carbohydrate-rich meal condition. The second session started after a 2-week interval. The order of the morning and evening meal schedules was counterbalanced and randomized. CHO = carbohydrate, PROT = protein, CR = constant routine, EEG = electroencephalogram.

Feldt's p values were based on corrected degrees of freedom, but the original degrees of freedom are reported. Linear contrasts with the additional false discovery rate procedure for multiple comparisons (Curran-Everett, 2000) were performed to localize differences between MM and EM conditions. To calculate differences in phase position, circadian phase markers were extracted and statistically analyzed by one- or two-way rANOVAs.

The following circadian phase markers were determined on day 2. The midrange crossing times (MRCTs) of the morning rise and the nocturnal decline in CBT and heart rate were obtained after smoothing by 1 h moving averages. MRCT was determined as follows: for each individual curve, the maximum values and the minimum value (in °C and beats/min) were averaged and taken to determine the midrange crossing time on the abscissa (for details, see Kräuchi et al., 1997). The minimum value of CBT and heart rate during the first night was masked by long-lasting, diet-induced thermogenesis (see Fig. 2); by substitution, the first (unmasked) value of the CR on day 2 at 0730 h was taken. Dim-light melatonin onset (DLMOn) and offset (DLMOff) times were calculated using a threshold value of 3 pg/mL (Weber et al., 1998).

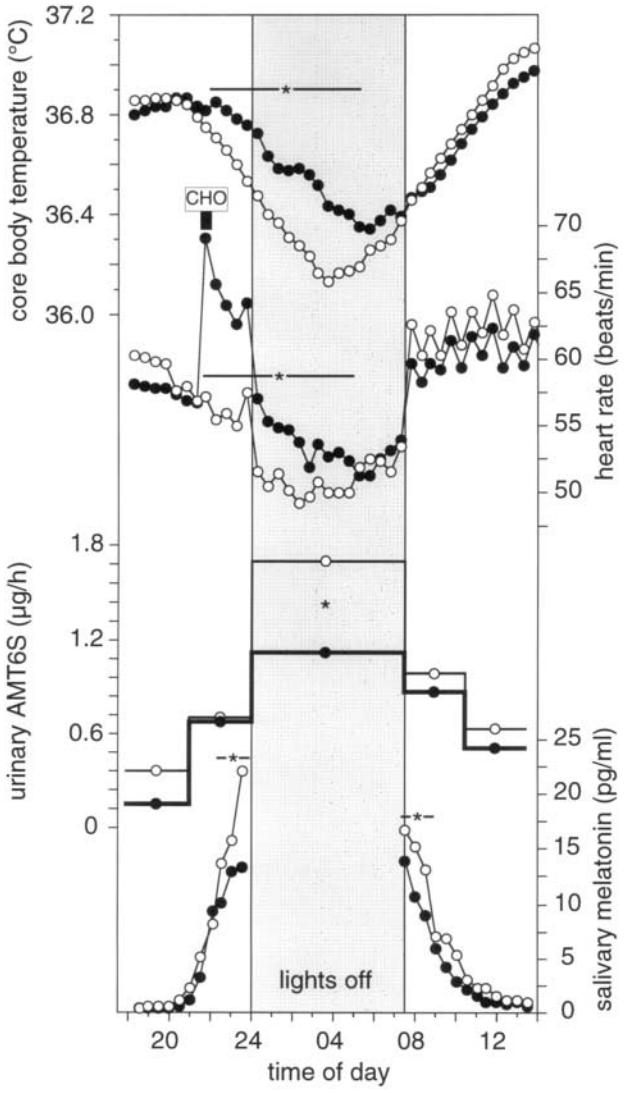

Figure 2. Acute effects of the last evening carbohydrate-rich meal (filled circles) were compared to those of the last morning carbohydrate-rich meal (open circles) from 1800 h on day 1 until 1330 h on day 2: the mean time course of core body temperature, heart rate, salivary melatonin, and urinary 6-sulphatoxymelatonin. Significant differences ($p < 0.05$) are indicated with a horizontal line and an asterisk.

RESULTS

Acute Effects of the Last EM on Day 1

Figure 2 shows the mean time course of the acute effects. The horizontal lines with asterisks indicate the times of significant differences. In comparison to the MM condition, CBT and heart rate values were significantly increased after EM. This increase lasted until the last third of the night. In contrast, salivary melatonin and urinary AMT6S secretion were decreased. All interaction terms of the rANOVAs reached significance (EM/MM × Time, $p < 0.05$), making possible detailed post hoc analyses between EM and MM at different time points (see Fig. 2).

Periodic feeding can also be interpreted as periodic fasting. These effects were analyzed by comparing the average value between 1800 and 2130 h on day 1 (directly before intake of the last EM) to the same time segment on day 2 of the CR (where regular 100-kcal sandwiches were given) (see Fig. 2). Before intake of the last EM on day 1, both CBT and heart rate exhibited lower values compared to day 2 of the CR (CBT day 1, 36.83 ± 0.05 °C vs. day 2, 37.09 ± 0.06 °C, *t* test, $p < 0.01$; heart rate day 1, 57.81 ± 1.64 bpm vs. day 2, 62.54 ± 2.10 bpm, *t* test, $p < 0.01$). Similar lower values were found for the MM condition (CBT day 1, 36.86 ± 0.04 °C vs. day 2, 37.08 ± 0.04 °C, *t* test, $p < 0.01$; heart rate day 1, 59.56 ± 2.48 bpm vs. day 2, 61.25 ± 1.81 bpm, *t* test, $p > 0.1$ [n.s.]). Salivary melatonin and urinary AMT6S levels in this time segment were uniformly low and did not differ between day 1 and day 2.

Baseline CR on Day 2

Time Course Analysis (Figs. 3A, 4A, 5A)

During the CR between 0730 and 2400 h, the dynamics of CBT, heart rate, and melatonin revealed a significant time-of-day effect. None of these variables showed significant differences during the baseline CRs of EM and MM conditions. In addition, no significant differences between EM and MM at baseline were found for baseline body weight (EM, 75 ± 3 kg; MM, 75 ± 3 kg), energy expenditure (EM, 1766 ± 80 kcal/day; MM, 1735 ± 74 kcal/day), RQ values (EM, 0.91 ± 0.02; MM, 0.90 ± 0.02), or urinary AMT6S secretion (during the night periods before [EM, 0.97 ± 0.26 µg/h; MM, 1.11 ± 0.17 µg/h], after [EM, 1.30 ± 0.21 µg/h; MM, 1.28 ± 0.27 µg/h], and during the daytime CR [EM, 0.45 ± 0.05 µg/h; MM, 0.44 ± 0.05 µg/h]).

Circadian Phase Marker Analysis (Fig. 6A)

Statistical analysis (rANOVA) of all circadian phase markers did not reveal any significant differences ($p > 0.5$) between baseline EM and MM conditions (neither main effect, EM vs. MM, nor interaction term, EM/MM vs. dusk/dawn). Additionally, the time of maxi-

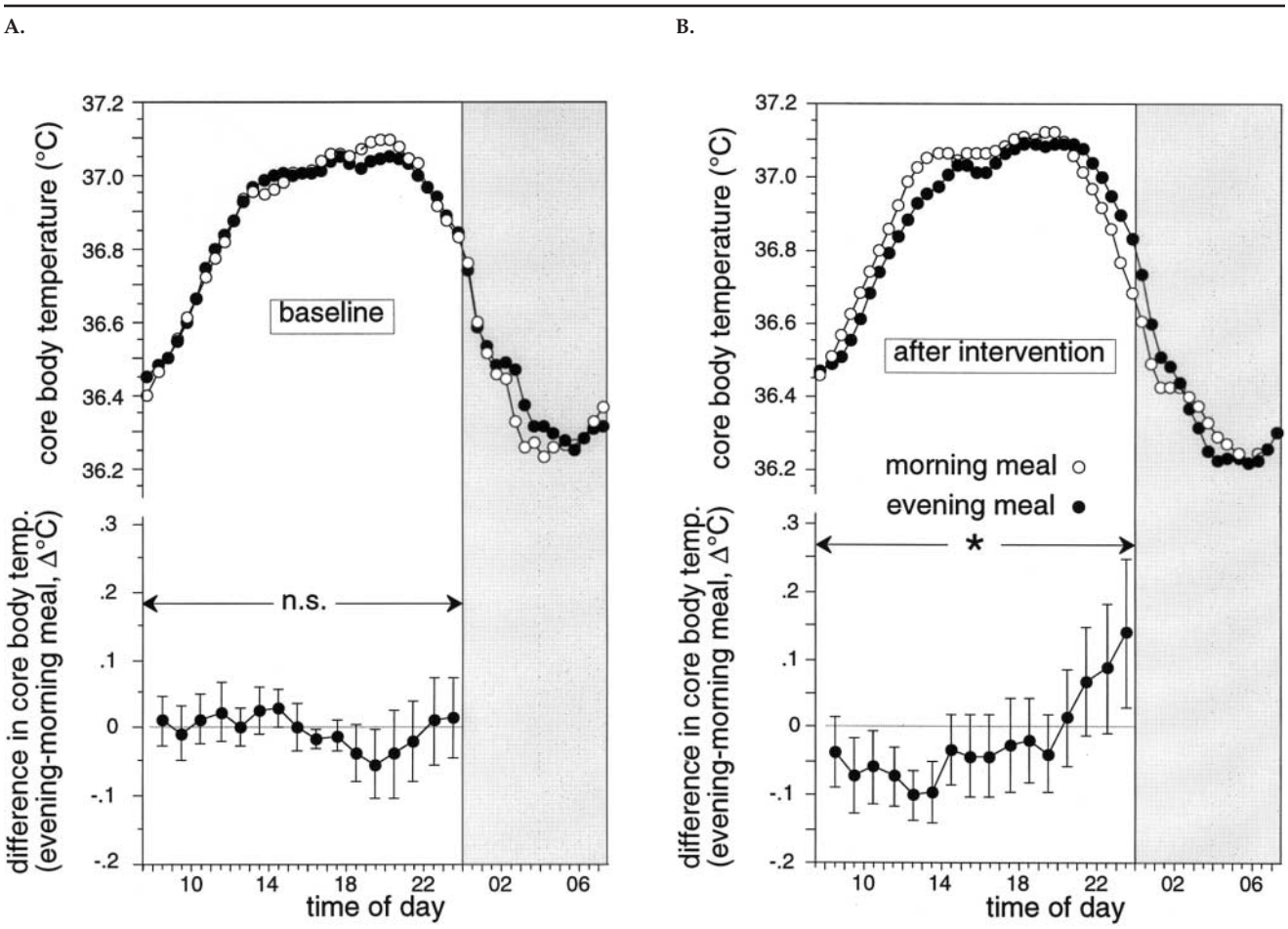

Figure 3. (A) The upper panel shows the baseline mean time course (30-min bins) of core body temperature during the constant routine (< 8 lux) and the following night episode (0 lux) of morning (MM) (open circles) and evening (EM) (filled circles) carbohydrate-rich meal conditions. Additionally, for visualization of the interaction term (EM/MM × Time), the difference between MM and EM is presented (lower panel). (B) After-intervention mean time course (details as in Fig. 3A). Significant differences ($p < 0.05$) are indicated with a horizontal line with an asterisk. Note that there are no differences during the *baseline* constant routine and that a significant interaction term *after intervention* indicates a significant phase shift.

NOTE: Two-way analyses of variance for repeated measures for the time courses (time segment = 0800 to 2400 h). (A) Baseline constant routine: evening carbohydrate-rich meal (EM)/morning carbohydrate-rich meal (MM), $F(1, 9) = 0.02$, n.s.; time of day, $F(31, 279) = 24.35$, $p < 0.0001$; EM/MM × Time, $F(31, 279) = 0.56$, n.s. (B) After-intervention constant routine: EM/MM, $F(1, 9) = 0.02$, n.s.; time of day, $F(31, 279) = 40.19$, $p < 0.0001$; EM/MM × Time, $F(31, 279) = 2.55$, $p < 0.05$.

mum CBT and heart rate did not differ, indicating similar shapes of the profiles (mean maximum time: CBT, 1848 h ± 27 min; heart rate, 1510 h ± 65 min; main effect: EM/MM, n.s.).

After-Intervention CR on Day 2

Time Course Analysis (Figs. 3B, 4B, 5B)

A separate two-way rANOVA for the time course of CBT and heart rate revealed significant interaction terms (EM/MM vs. time of day), making possible a more detailed analysis (linear contrasts) of the differences between morning and evening meals with respect to time of day. Only salivary melatonin secretion showed a significant main effect between EM and MM.

CBT values between 0800 and 2030 h were significantly ($p < 0.05$) higher in the MM condition and lower between 2030 and 2400 h compared to EM (Fig. 3B). This increase lasted until 0230 h—the masked data during sleep, however, were not statistically analyzed. The shapes of the profiles were very similar and appear to differ only in phase.

MM heart rate was increased from 0800 to 1600 h and was reduced between 1600 and 2400 h compared

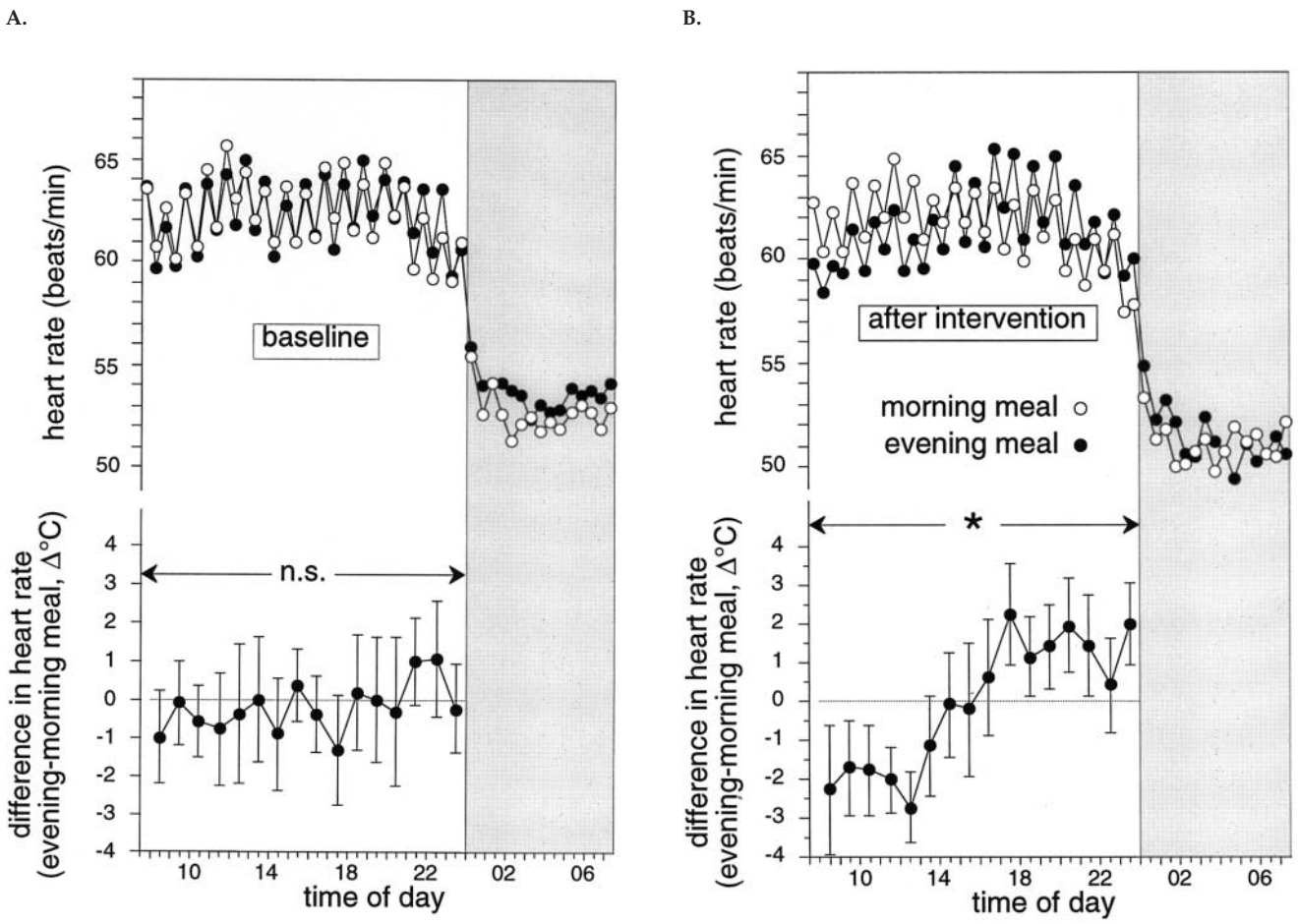

Figure 4. Time course of heart rate during the constant routine and the following night (details as in Fig. 3). The data (30-min bins) during the constant routine show the small but clear effects of the protocol as an increase after each isocaloric meal.
NOTE: Two-way analyses of variance for repeated measures for the time courses (time segment = 0800 to 2400 h). (A) Baseline constant routine: evening carbohydrate-rich meal (EM)/morning carbohydrate-rich meal (MM), $F(1, 9) = 0.04$, n.s.; time of day, $F(31, 279) = 4.57$, $p < 0.002$; EM/MM × Time, $F(31, 279) = 0.62$, n.s. (B) After-intervention constant routine: EM/MM, $F(1, 9) = 0.002$, n.s.; time of day, $F(31, 279) = 4.57$, $p < 0.0001$; EM/MM × Time, $F(31, 279) = 2.99$, $p < 0.005$.

to EM ($p < 0.05$) (Fig. 4B). This reduction lasted throughout the following night until 0400 h (again, the masked data during sleep were not statistically analyzed). Visual inspection suggests a dissimilar time course of heart rate in MM compared to EM.

Salivary melatonin secretion was significantly increased after MM compared to EM (main effect: EM vs. MM, $p < 0.05$; MM-EM: 1.55 ± 0.68 pg/mL) (Fig. 5B). This result was confirmed by analysis of urinary AMT6S secretion, which also tended to be higher in MM during the CR on day 2 (EM, 0.45 ± 0.04 µg/h; MM, 0.54 ± 0.04 µg/h; MM-EM, 0.09 ± 0.09 µg/h) ($p = 0.13$). During the preceding night, this increase was significant (EM, 1.11 ± 0.25 µg/h vs. MM, 1.70 ± 0.32 µg/h; MM-EM, 0.58 ± 0.18 µg/h) ($p = 0.01$) (Fig. 2). However, AMT6S secretion during the night after the CR on day 2 was no longer different (EM, 1.39 ± 0.22 µg/h; MM, 1.02 ± 0.15 µg/h) (n.s.).

Both groups showed similar energy expenditure (EM, 1661 ± 58 kcal/day; MM, 1676 ± 50 kcal/day), RQ values (EM, 0.89 ± 0.02; MM, 0.88 ± 0.03), and body weight (EM, 74 ± 3 kg; MM, 74 ± 3 kg).

Circadian Phase Marker Analysis (Fig. 6B)

CBT and heart rate of MM showed an earlier phase position of the MRCT in both the dawn and dusk portion of the circadian rhythm (Table 1) in comparison to EM (by 59 ± 12 min and 43 ± 18 min, respectively). Two-way rANOVAs indicate similar phase shifts at dusk and dawn (no significant interaction terms: dawn/dusk vs. EM/MM) (Table 1). However, the

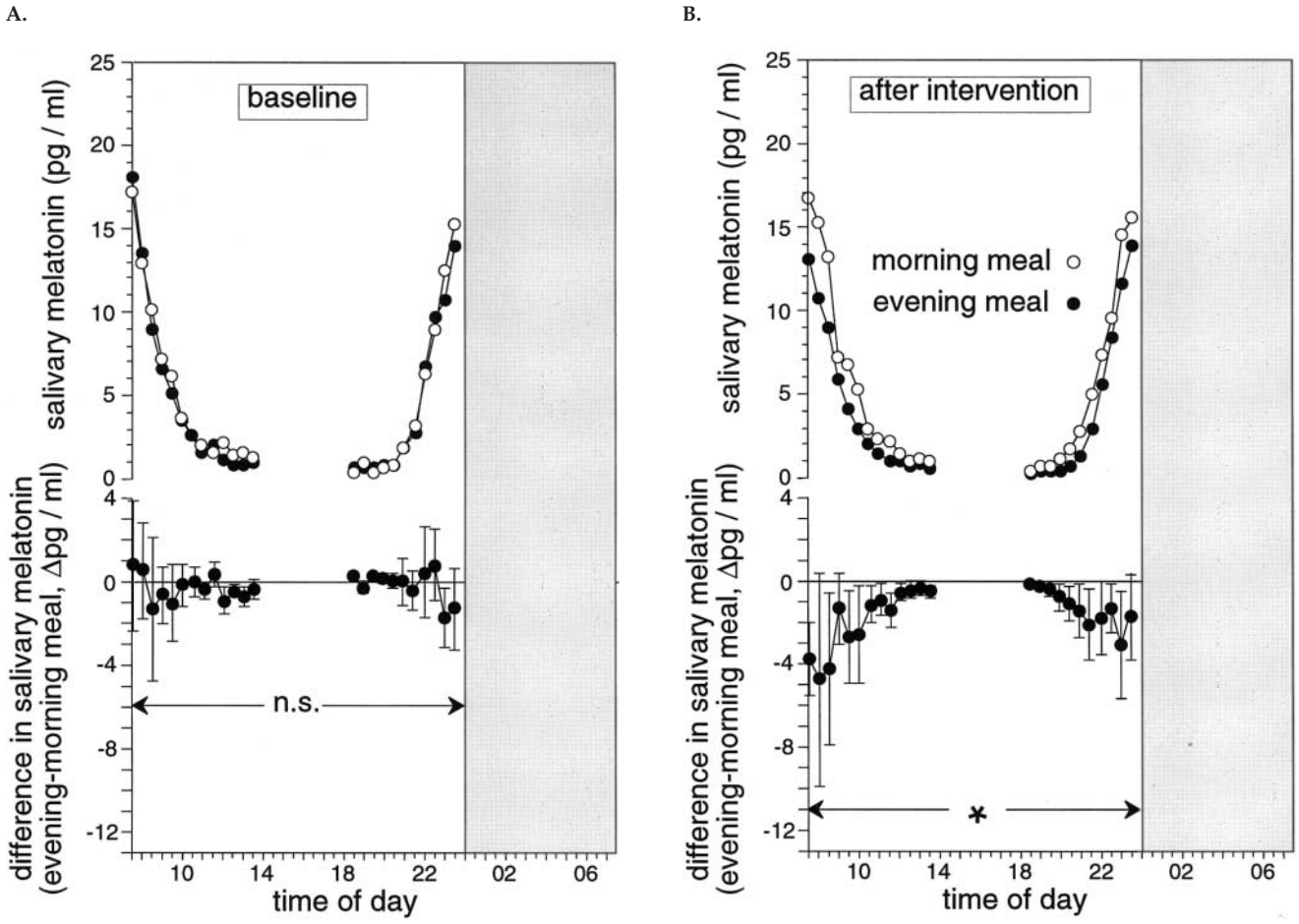

Figure 5. Time course of salivary melatonin during the constant routine showing dim-light melatonin offset in the morning and dim-light melatonin onset in the evening (details as in Fig. 3).
NOTE: Two-way analyses of variance for repeated measures for the time courses (time segments = 0730 to 1330 h and 1800 to 2330 h). (A) Baseline constant routine: evening carbohydrate-rich meal (EM)/morning carbohydrate-rich meal (MM), $F(1, 9) = 0.49$, n.s.; time of day, $F(23, 207) = 12.24$, $p < 0.0001$; EM/MM × Time, $F(10, 90) = 0.22$, n.s. (B) After-intervention constant routine: EM/MM, $F(1, 9) = 5.18$, $p < 0.05$; time of day, $F(23, 207) = 8.60$, $p < 0.0007$; EM/MM × Time, $F(10, 90) = 0.54$, n.s.

time of maximum heart rate values after EM (1730 h ± 65 min) occurred significantly later than after MM (1257 h ± 61 min; EM-MM, –273 ± 71 min), $F(1, 9) = 14.6$, $p < 0.005$. This phase difference is six times as great as the difference in MRCT of heart rate. Thus, the circadian heart rate rhythm was not only phase-shifted but also changed in waveform. CBT did not show significant different maximum values (mean maximum time 1840 h ± 32 min).

In a two-way rANOVA of DLMOff and DLMOn time, there were no significant differences between EM and MM. However, a nearly significant interaction term (dusk/dawn vs. EM/MM, $p < 0.06$) (see Fig. 2) was found, indicating different influences of the meal interventions on DLMOff and DLMOn. MM showed a nonsignificant later DLMOff (EM-MM, –27 ± 20 min) and an earlier DLMOn (EM-MM, 15 ± 13 min) in comparison to EM. There was a tendency for MM to have a shorter duration of the nonsecretory period between dawn and dusk (11 h 48 min ± 20 min) than EM (12 h 30 min ± 23 min) (EM/MM × Dusk/Dawn, $p < 0.06$).

DISCUSSION

This study demonstrates that under normal LD conditions, daily timed single carbohydrate-rich meals in humans can markedly influence the circadian phase position of core body temperature and heart rate without significantly shifting melatonin. Our data suggest that timed carbohydrate-rich meals may

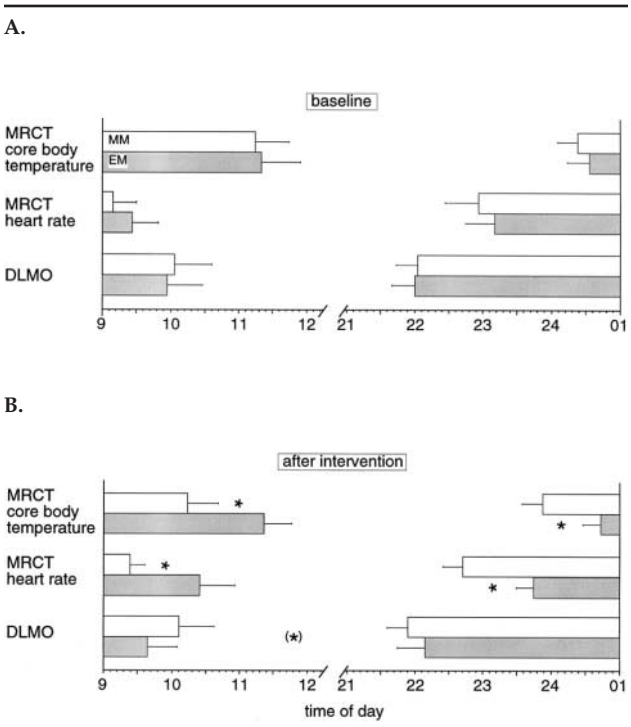

Figure 6. Mean (± SEM error bars) midrange crossing times (MRCTs) of core body temperature and heart rate extracted from the declining (evening) and rising (morning) portion of their circadian profile, respectively. Additionally, dim-light melatonin offset and onset (DLMO) time (threshold = 3 pg/mL) for the morning and evening portion of the circadian profile are shown. (A) Baseline values. (B) After-intervention values. MM indicates the morning and EM the evening carbohydrate-rich meal condition.

Table 1. Two-Way Analyses of Variance for Repeated Measures for Dusk and Dawn Phase Markers (After-Intervention Constant Routine)

Core body temperature (midrange crossing time)		
EM/MM	$F(1, 9) = 20.1$, $p < 0.002$	EM-MM: 59 ± 12 min
Dusk/dawn	$F(1, 9) = 1472$, $p < 0.0001$	
EM/MM × Dusk/dawn	$F(1, 9) = 0.24$, n.s.	
Heart rate (midrange crossing time)		
EM/MM	$F(1, 9) = 5.37$, $p < 0.05$	EM-MM: 43 ± 18 min
Dusk/dawn	$F(1, 9) = 760$, $p < 0.0001$	
EM/MM × Dusk/dawn	$F(1, 9) = 0.12$, n.s.	
Salivary melatonin		
EM/MM	$F(1, 9) = 0.11$, n.s.	
Dusk/dawn	$F(1, 9) = 1395$, $p < 0.0001$	
EM/MM × Dusk/dawn	$F(1, 9) = 4.85$, $p < 0.06$	EM-MM: dawn: 15 ± 13 min; dusk: –27 ± 20 min; dawn-dusk: EM: 11 h 48 min ± 20 min; MM: 12 h 30 min ± 23 min

NOTE: EM = evening carbohydrate-rich meal, MM = morning carbohydrate-rich meal.

differentially affect internal circadian phase relationships. The effects may be on a food-entrainable oscillator rather than on the light-entrainable oscillator.

Circadian phase of CBT, heart rate, and salivary melatonin secretion did not differ during the baseline CRs, confirming similar starting conditions before meal interventions. The design of the study limits interpretation in two ways. Because there was not a third "placebo" group without any interventions between the two CRs separated by 2 weeks to control for an order effect, we cannot distinguish whether EM phase-delayed and/or MM phase-advanced the circadian system with respect to baseline conditions. A comparison of the after-intervention conditions to the baseline CRs would not serve as a comparison to a "normal" three meal per day condition, since in the latter condition the subjects received the characteristic one-hourly isocaloric snacks throughout rather than a normal big dinner. In addition, we cannot state conclusively whether the observed effects are specific for carbohydrate-rich meals (as opposed to protein- or fat-rich meals) or related to limiting food intake to a single large, timed meal. Nevertheless, the shifted rhythms of CBT and heart rate indicate that food may have zeitgeber properties in humans.

Core Body Temperature

CBT was approximately phase-advanced 1 h after MM compared to EM, with no change in waveform. One counterargument for a circadian phase shift could be that in terms of thermoregulation, the 3 intervention days induced periodic masking by increasing heart rate and CBT acutely after each carbohydrate-rich meal. However, this kind of hourglass mechanism would be balanced by the one-hourly isocaloric sandwiches of the CR protocol on day 2 (Mistlberger, 1994). In addition, the diet-induced thermogenesis after the last evening meal had disappeared by the beginning of the CR protocol on day 2, providing identical initial values. A second counterargument could be that greater sensitivity to the CR sandwiches after the long fasting episode following an MM resulted in a higher

metabolic rate and earlier CBT rise in the morning. However, this alone cannot explain the relative phase advance in the evening CBT decline after MM versus EM.

Heart Rate

Heart rate showed a similar approximately 45 min phase advance of the MRCT in both the rise and decline portion of the circadian rhythm after MM compared to EM. In contrast to the waveform of the CBT rhythm, the waveform of the heart rate rhythm was changed. During baseline CRs, the heart rate rhythm was bimodal with peaks at approximately 1200 h and 2000 h. Interestingly, after MM no evening peak was present, and after EM there was no morning peak. These waveform changes could reflect direct long-lasting effects of periodic food intake on metabolism (e.g., increased oxygen consumption) and hormonal and neuronal function. These processes are oxygen dependent and need increased blood supply that is regulated by an increase in heart rate. Thus, the waveform changes in heart rate may be a result of metabolic masking of the underlying phase shift, which was parallel to that seen for CBT.

Melatonin

The third phase marker, melatonin, did not simply follow the above pattern of a phase advance after MM compared to EM. Nocturnal melatonin secretion (measured by salivary melatonin and urinary 6-sulphatoxymelatonin levels) after the last morning carbohydrate-rich meal was elevated in comparison to EM. The parallels between salivary melatonin and AMT6S secretion indicate that there were no differences in liver metabolism of melatonin between the two conditions; rather, melatonin synthesis and secretion was modified. It is known that such amplitude differences can mask the timing of DLMOff (Lewy, 1999), so that higher melatonin levels observed after MM may be the cause of a slightly but not significantly later DLMOff.

Similar to our EM condition, a recent "naturalistic" study of the metabolic effects of Ramadan—1 month of daytime fasting with a single large meal in the evening—has shown that at the end of 3 weeks under such a regimen, plasma melatonin had lower total secretion than under usual meal timing (Bogdan et al., 2001).

Melatonin is considered to be a sensitive and reliable circadian phase marker, masked only by light. Body posture has also been found to modify secretion (or blood volume) (Deacon and Arendt, 1994), and our findings suggest that periodic carbohydrate-rich meals with approximately 24 h fasting periods may also be a further masking factor that must be controlled for.

Given this interpretation, only the final DLMOn, 24 h and 36 h, respectively, after the last meal could be considered as an unmasked circadian phase marker. At this time, no significant differences in DLMOn were found, which is not in line with the altered phase position of the CBT and heart rate rhythms. However, there is still the possibility that a small relative phase advance in DLMOn induced by MM may not have been maintained at this late measuring time.

Energy Expenditure and Body Weight

Under baseline conditions, the subjects ate three regular mixed meals per day (each separated by 4 to 6 h) and fasted overnight. In the entrainment protocol, the food constituents were changed and the timing of food intake differed by 13 h, with a fasting interval of approximately 23 h. Additionally, daily caloric intake was slightly reduced by approximately 200 to 400 kcal/day. The timing of meals did not change energy expenditure differentially. Heat production was significantly reduced to a similar extent (approximately 80 kcal below the baseline levels) after both EM and MM. In addition, the reduction in body weight of approximately 1 kg after both EM and MM indicates that, in both conditions, the hypocaloric diet during the 3-day protocol had a similar effect on energy expenditure. Whether a longer schedule of periodic feeding or a greater degree of caloric restriction may induce differences between EM and MM in energy expenditure and body weight loss, as shown in a previous study (Graeber et al., 1978), remains to be studied. No conclusive evidence for the interpretation of the observed circadian phase shifts of CBT and heart rate can be drawn from the energy expenditure and body weight data.

Putative Mechanisms of a Carbohydrate-Rich Meal as Zeitgeber

There are no systematically controlled studies of food as a zeitgeber in humans. Thus, the majority of

data relating to putative mechanisms are drawn from animal studies.

It has long been recognized that during ad lib conditions, the food-entrainable oscillator is controlled and coupled to the central light-entrainable oscillator localized in the SCN (Stephan, 1986; Stephan et al., 1979). Food-entrainable oscillators have recently been found in many peripheral organs (peripheral circadian oscillators, Damiola et al., 2000; Stokkan, et al., 2001). The memory for feeding time requires that the coupling between food-entrainable oscillators and the SCN depend on environmental conditions, in particular the presence or absence of a restricted feeding schedule (e.g., the 24 h food anticipatory activity in rats remains concurrently under free-running conditions; Stephan, 1986). Such a flexible coupling relationship between circadian oscillators may allow animals to entrain their behavioral (and physiological) activity ("foraging") with both geophysically stable and predictable environmental periodicities (e.g., LD cycle) as well as unstable environmental periodicities (e.g., food availability) (Rosenwasser et al., 1984). This coupled oscillator system may function as a "continuously consulted clock" in the adaptive temporal coordination of behavior (Pittendrigh, 1958).

Feeding cycles can entrain the circadian rhythm of gene expression in peripheral organs independent of the central oscillator in the SCN and the LD cycle (Damiola et al., 2000; Stokkan et al., 2001). It has been suggested that peripheral circadian oscillators such as those in the liver may be coupled to the SCN primarily through rhythmic behavior, such as feeding (Damiola et al., 2000; Stokkan et al., 2001). However, the signaling pathways for the coupling of peripheral circadian clocks to the central circadian clock have not yet been identified. Several neuronal and humoral signals have been suggested to be important, as well as physiological inputs such as food ingestion (Balsalobre et al., 2000; Damiola et al., 2000; Buijs and Kalsbeek, 2001). It is known that timed temperature pulses in rats can change phase relationships of hormonal, behavioral, and physiological circadian rhythms in vivo (Francis and Cleman, 1997; De Souza and Meier, 1993). The uncoupling of the central and peripheral circadian oscillators may be facilitated by changes in body temperature rhythms (via diet-induced thermogenesis) that accompany the altered feeding behavior (Damiola et al., 2000). Thus, the temperature fluctuations elicited by restricted feeding are either less dramatic in the SCN region, or the central oscillator is more resistant to temperature variations than peripheral oscillators (Damiola et al., 2000). The observed phase shifts of CBT and heart rate induced by timed carbohydrate-rich meals could have resulted from phase shifts of peripheral circadian oscillators indirectly via diet-induced thermogenesis.

In summary, periodic MMs result in an approximately 1 h circadian phase advance of CBT and heart rate relative to EMs without significantly advancing the dim-light melatonin onset. Thus, food intake can be considered as an internal zeitgeber to which different circadian systems are differentially sensitive. Periodic food restriction may be a zeitgeber for the peripheral circadian oscillators (see CBT and heart rate data) but not the central oscillator (see melatonin data), leading to a state of altered phase relationships. Under normal entrainment or free-running conditions, the main circadian pacemaker in the SCN synchronizes all body functions. Confirmation of a true zeitgeber property of carbohydrates must await comparison with other macronutrients and mixed single meals to separate out nutrient categories from periodic feeding and fasting. Furthermore, studies with longer schedules of periodic feeding or a greater degree of caloric restriction may be required to induce larger phase shifts of the peripheral circadian oscillators, or even phase shifts in the central circadian oscillator.

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REFERENCES

Apfelbaum M, Reinberg A, and Lacatis D (1976) Effects of meal timing on circadian rhythms in nine physiological variables of young healthy but obese women on a calorie restricted diet. Int J Chronobiol 4:29-37.

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schütz G, and Schibler U (2000) Resetting of circadian time in peripheral tissue by glucocorticoid signaling. Science 289:2344-2347.

Bogdan A, Bouchareb B, and Touitou Y (2001) Ramadan fasting alters endocrine and neuroendocrine circadian patterns: Meal-time as a synchronizer in humans? Life Sci 68:1607-1615.

Buijs RM and Kalsbeek A (2001) Hypothalamic integration of central and peripheral clocks. Nat Rev Neurosci 2:521-526.

Challet E, Losee-Olsen S, and Turek FW (1999) Reduced glucose availability attenuates circadian responses to light in mice. Am J Physiol 276:R1063-R1070.

Challet E, Pévet P, Vivien-Roels B, and Malan A (1997) Phase-advanced daily rhythms of melatonin, body temperature, and locomotor activity in food-restricted rats fed during daytime. J Biol Rhythms 12:65-79.

Challet E, Solberg LC, and Turek FW (1998) Entrainment in calorie-restricted mice: Conflicting zeitgebers and free-running conditions. Am J Physiol 274:R1751-R1761.

Challet E, Van Reeth O, and Turek FW (1999) Altered circadian responses to light in streptozotocin-induced diabetic mice. Am J Physiol 277:E232-E237.

Curran-Everett D (2000) Multiple comparisons: Philosophies and illustrations. Am J Physiol 279:R1-R8.

Czeisler CA, Allen JS, Strogatz SH, Ronda JM, Sanchez R, Rios CD, Freitag WO, Richardson GS, and Kronauer RE (1986) Bright light resets the human circadian pacemaker independent of the timing of the sleep-wake cycle. Science 233:667-671.

Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, and Schibler U (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 14:2950-2961.

Deacon S and Arendt J (1994) Posture influences melatonin concentrations in plasma and saliva in humans. Neurosci Lett 167:191-194.

De Souza CJ and Meier AH (1993) Daily intervals of heat exposure alter the phase of the daily variation of plasma hormones in Holtzman rats. Am J Physiol 265:R1115-R1120.

Francis AJP and Cleman GJ (1997) Phase response curves to ambient temperature pulses in rats. Physiol Behav 62:1211-1217.

Gillberg M, Kecklund G, and Åkerstedt T (1994) Relations between performance and subjective ratings of sleepiness during a night awake. Sleep 17:236-241.

Glass JD, Selim M, Srkalovic G, and Rea MA (1995) Tryptophan loading modulates light-induced responses in the mammalian circadian system. J Biol Rhythms 10:80-90.

Goetz F, Bishop J, Halberg F, et al (1976) Timing of single daily meal influences relations among human circadian rhythms in urinary cyclic AMP and hemic glucagon, insulin and iron. Experientia 32:1081-1084.

Graeber RC, Gatty R, Halberg F, and Levine H (1978) Human eating behavior: Preferences, consumption patterns, and biorhythms. Food Science Laboratories Technical Report No. DAAK03-74-C-0233, US Army Research and Development Command, Natick, MA.

Harrington ME (1997) The ventral lateral geniculate nucleus and the intergeniculate leaflet: interrelated structures in the visual and circadian systems. *Neurosci Biobehav Rev* 21: 705-727.

Holloway WR, Tsui HW, Grota LJ, and Brown GM (1979) Melatonin and corticosterone regulation: Feeding time or the light:dark cycle? Life Sci 25:1837-1842.

Johnson BC (1992) Nutrient intake as a time signal for circadian rhythm. J Nutr 122:1753-1759.

Kittrell EMW (1991) The suprachiasmatic nucleus and temperature rhythms. In *Suprachiasmatic Nucleus: The Mind's Clock*, DC Klein and RY Moore, eds, pp 233-245, Oxford University Press, New York.

Kräuchi K, Cajochen C, Möri D, Hetsch C, and Wirz-Justice A (1997) Early evening melatonin and S-20098 advance circadian phase and nocturnal regulation of core body temperature. Am J Physiol 272:1178-1188.

Kräuchi K, Werth E, Cajochen C, et al (1998) Effects of timed carbohydrate rich-meals on thermoregulation and sleepiness. J Sleep Res 7(suppl 2):140.

Lewy AJ (1999) The endogenous melatonin profile as a marker for circadian phase position. J Biol Rhythms 14:227-236.

Mills JN, Minors DS, and Waterhouse JM (1978) Adaptation to abrupt time shifts of the oscillator(s) controlling human circadian rhythms. J Physiol (London) 285:455-470.

Mistlberger R (1991) Scheduled daily exercise or feeding alters the phase of photic entrainment in Syrian hamsters. Physiol Behav 50:1257-1260.

Mistlberger R (1994) Circadian food-anticipatory activity: Formal models and physiological mechanisms. Neurosci Biobehav Rev 18:171-195.

Mistelberger R and Rusak B (1987) Palatable daily meals entrain anticipatory activity rhythms in free-feeding rats: Dependence on meal size and nutrient content. Physiol Behav 41:219-226.

Mrosovsky N, Salmon PA, Menaker M, and Ralph MR (1992) Nonphotic phase shifting in hamster clock mutants. J Biol Rhythms 7:41-49.

Mrosovsky N (1995) A non-photic gateway to the circadian clock of hamsters. Ciba Found Symp 183:154-174.

Nagai K, Yamazaki K, Tsujimoto H, Inoue S, and Nakagawa H (1984) Meal feeding schedule and ventromedial hypothalamic lesions do not affect rhythm of pineal serotonin N-acetyltransferase activity in rats. Life Sci 35:769-774.

Pittendrigh CP (1958) Perspectives in the study of biological rhythms. In *Perspectives in Marine Biology*, AA Buzzati-Travero, ed, pp 239-268, University of California Press, Berkeley.

Reinberg A (1983) Chronobiology and nutrition. In *Biological Rhythms and Medicine: Cellular, Metabolic, Physiologic, and Pharmacologic Aspects*, A Reinberg and MH Smolensky, eds, pp 265-300, Springer-Verlag, New York.

Rosenwasser AM, Pelchat RJ, and Adler NT (1984) Memory for feeding time: Possible dependence on coupled circadian oscillators. Physiol Behav 32:25-30.

Sensi S and Capani F (1987) Chronobiological aspects of weight loss in obesity: Effects of different meal timing regimens. Chronobiol Int 4:251-261.

Stephan FK (1986) The role of period and phase in interactions between feeding- and light-entrainable circadian rhythms. Physiol Behav 36:151-158.

Stephan FK and Davidson AJ (1998) Glucose, but not fat, phase shifts the feeding-entrained circadian clock. Physiol Behav 65:277-288.

Stephan FK, Swann JM, and Sisk CL (1979) Anticipation of 24-hr feeding schedules in rats with lesions of the suprachiasmatic nucleus. Behav Neural Biol 25:346-363.

Stokkan KA, Yamazaki S, Tei H, Sakaki Y, and Menaker M (2001) Entrainment of the circadian clock in the liver by feeding. Science 291:490-493.

Torsvall L and Åkerstedt T (1980) A diurnal type scale: Construction, consistency, and validation in shift work. Scand J Work Environ Health 6:283-290.

Turek FW (1989) Effects of stimulated physical activity on the circadian pacemaker of vertebrates. J Biol Rhythms 4:135-147.

Weber J, Unger I, Wirz-Justice A, and Schwander JC (1998) Sensitive monitoring of alterations in circadian rhythms by supersensitive melatonin immunoassays. J Sleep Res 7(suppl 1):302.

Ying SW and Rusak B (1997) 5-HT7 receptors mediate serotonergic effects on light-sensitive suprachiasmatic nucleus neurons. Brain Res 755:246-254.