Entrainment in calorie-restricted mice: conflicting zeitgebers and free-running conditions

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1Center for Circadian Biology and Medicine, Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60208; and 2Department of Neurobiology of Rhythmic and Seasonal Functions, Centre National de la Recherche Scientifique-UMR 7518, University Louis Pasteur, F-67000 Strasbourg, France

Challet, Etienne, Leah C. Solberg, and Fred W. Turek. Entrainment in calorie-restricted mice: conflicting zeitgebers and free-running conditions. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R1751–R1761, 1998.—Phase-shifting effects of timed calorie restriction were investigated in mice during exposure to a 12:12-h light-dark cycle. Food-anticipatory activity (FAA), the output of a food-entrainable pacemaker, was expressed before the time of feeding whether mice received daily hypocaloric food (3.3 g of chow/day) or normocaloric food (5 g of chow/day) at zeitgeber time (ZT) 2 (ZT12 = lights off). Subsequently, mice were placed in constant darkness and fed ad libitum. The onset of the nocturnal period of locomotor activity was phase advanced by 1 h in calorie-restricted mice compared with normocaloric-fed controls. The phase advance still occurred when FAA was prevented by restraining calorie-restricted mice. Giving hypocaloric food at ZT2, ZT10, ZT14, or ZT22 phase advanced the nocturnal pattern of activity by 1, 3, 1, and 1 h, respectively. After transfer to constant darkness, FAA free ran in parallel with the normal nocturnal period of locomotor activity. A light pulse during the early subjective night phase delayed both components. These results indicate that 1) timed calorie restriction under a light-dark cycle can phase advance the light-entrainable pacemaker with a phase-dependent magnitude, 2) FAA feedback is not crucial for the observed phase advance, and 3) the light-entrainable pacemaker can control the period of the food-entrainable pacemaker in mice fed ad libitum.

Food synchronization; suprachiasmatic nucleus; food-anticipatory activity; circadian rhythm

CIRCADIAN RHYTHMS in mammals are generated by an endogenous time-keeping system. In the absence of external temporal cues, these rhythms free run with a period close to, but generally different from, 24 h. The main circadian pacemaker, located in the suprachiasmatic nuclei (SCN), regulates most circadian rhythms, including those of rest-activity and food intake. The preeminent environmental synchronizer (zeitgeber) is the light-dark (LD) cycle, with photic information entraining the circadian pacemaker in the SCN (i.e., light-entrainable pacemaker, or LEP) via the monosynaptic retinohypothalamic tract (13). Several other (non-photic) temporal cues are also able to phase shift or synchronize the LEP, such as novelty-induced running or injection of benzodiazepines (18, 30), but these are considered to be of secondary relevance when an LD cycle is present. Restricted feeding, however, is one exception. Supplying food for a limited daily period can affect the phase of light-entrained rhythms in the Syrian hamster (15) and the rabbit (12). Also, in rats, timed calorie restriction is capable of competing with photic entrainment (5, 6). Moreover, long-term restricted feeding in constant lighting conditions shortens the circadian period of the LEP (4). Taken together, these data indicate that feeding events may influence the SCN pacemaker.

In most mammals studied, food access for a limited time each day induces a food-anticipatory component of several daily rhythms, including locomotor activity (4, 16, 26), body temperature (19), plasma corticosterone (19), and drinking (17), but not pineal melatonin (6). In SCN-lesioned rats (7, 31), hamsters (2), and C57 mice (14), the food-anticipatory bout of activity (FAA) can still be expressed, suggesting that food anticipation involves a circadian timing system outside the SCN. Entrainment of FAA occurs only when the periods of food access for a daily limited time are in the circadian range, with limits between 22 and 26 h in rats (16). After a phase shift of the synchronizer, an endogenous pacemaker will take several days to reentrain. This gradual phenomenon is also seen in FAA after phase shifts of food access (27). These characteristics have led to the hypothesis of a food-entrainable pacemaker (FEP) separate from the LEP. The location of the FEP is still unknown (see Ref. 16 for review). The LEP and FEP have been assumed to be weakly coupled (4, 16, 24–26). The degree of coupling is dependent on several variables, including the species (16), the strain [i.e., mice (1)], the feeding procedure (16), and the daily amount of ingested food (6).

The purpose of this study was to investigate the possible synchronizing effects of timed calorie restriction compared with normocaloric feeding in mice exposed to an LD cycle. This study was also designed to give new insights into the internal coupling between the LEP and the hypothetical FEP through their respective outputs, that is, the light-entrained rhythm of locomotor activity and FAA.

The present study was performed in accordance with US Public Health Service regulations under supervision by the Animal Care and Use Committee of Northwestern University.

EXPERIMENT 1

In this preliminary experiment we analyzed possible effects of early morning feeding, with and without added calorie restriction, on the light-entrained activity rhythm in mice. A phase change of the LEP would indicate strong coupling of the LEP to the FEP and/or direct effects of metabolic changes associated with...
calorie restriction on the LEP, as suggested in rats (5, 6).

Methods. Twenty-one adult male C57BL/6j mice (Jackson Laboratories, Bar Harbor, ME) were housed singly in cages equipped with running wheels (11 cm diameter). Mice were maintained in a temperature-controlled room (23 ± 1°C) with a 12:12-h LD cycle. During daytime, light intensity was ~300 lx at the level of the cages. Under LD conditions, times of day were converted to zeitgeber times (ZT), where ZT0 and ZT12 were the onset of light and darkness, respectively. Food (standard laboratory chow, Harlan Teklad) and water were available ad libitum, unless otherwise stated. A fan provided constant fresh airflow and masked noise.

Wheel-running activity was continuously recorded with an on-line data acquisition system (Chronobiology Kit, Stanford Software Systems, Stanford, CA). Body mass was measured weekly. Daily food intake was calculated to assess mass was measured weekly. Daily food intake was calculated to assess

The onset of the nocturnal locomotor activity was determined to assess 1) phase changes of the daily rhythm of activity during calorie restriction under LD conditions (i.e., reflecting changes in the phase angle of photic entrainment) and 2) phase changes of the nocturnal period of activity during ad libitum feeding in DD (i.e., reflecting phase changes of the underlying LEP). Data for five consecutive cycles were divided into 10-min bins of time. The onset of the nocturnal wheel-running activity was defined as the first 10-min bin when 20% of maximal intensity for that cycle was followed by that level of activity in three of the next six bins. Application of this criterion for each 24-h cycle was in good agreement with visual inspections of the actograms. This analysis was performed over three periods: the last 5 days of baseline conditions, the last 5 days of the calorie restriction, and the first 5 days of refeeding in DD. Daily activity was defined as the total wheel revolutions per day averaged for each animal over the three 5-day periods described above. In addition, FAA was analyzed more closely. The cumulative wheel revolutions performed daily between ZT0 and ZT5 were averaged over the last 5 days of baseline conditions and calorie restriction. This temporal window was considered to encompass FAA (when expressed). The same window of time was studied in DD using circadian time (CT), in which CT12 was defined as the onset of locomotor activity. Wheel-running activity was calculated between CT0 and CT5 over the first 5 days in DD. The circadian period (\( \tau \)) was assessed using the \( \chi^2 \) periodogram (Chronobiology Kit software) over the first 10 days in DD.

Values are means ± SE. A one-way ANOVA was used to analyze the effects of a single variable (e.g., \( \tau \)) between groups of mice. A two-way ANOVA with repeated measures was used to compare the effects between the time and the nutritional state (calorie restriction, normocaloric feeding, or ad libitum feeding). If significant main effects or a significant interaction was found, pairwise comparisons were performed with the Student-Newman-Keuls test.

Results and discussion. Body mass was modified significantly by the nutritional state [i.e., calorie restriction, normocaloric feeding, and ad libitum feeding; \( F(2,108) = 21.7, P < 0.01 \) and the weeks \( F(6,108) = 51.5, P < 0.01 \). In addition, the pattern of body mass changes during the experiment differed according to the nutritional state \( F(12,108) = 18.4, P < 0.01 \). Body mass decreased only in calorie-restricted mice (Fig. 1). By the 3rd wk of food restriction, calorie-restricted mice displayed a steady state of lowered body mass. This loss in body mass was recovered during a subsequent period of ad libitum feeding in DD (Fig. 1).

At the beginning of the study, all mice were fed ad libitum and displayed a rhythm of wheel-running activity synchronized to the LD cycles. The onset of the nocturnal activity occurred around the offset of light (i.e., ZT12; Figs. 2 and 3). Analysis of the nocturnal onset during baseline conditions revealed no significant difference between the nutritional groups (\( P > 0.1 \)) or the days (\( P > 0.1 \), and there was also no significant interaction between the two factors (\( P > 0.1 \); Fig. 3). Calorie-restricted mice showed changes in their day-night pattern of activity, including a strong FAA and a phase advance of the nocturnal pattern of activity (Fig. 2, A and D). All normocalorie-fed mice also expressed FAA, but only minor changes in the nocturnal pattern were visible (Fig. 2, B and E). In particular, the onset of the nocturnal activity remained close to ZT12 in all

![Fig. 1. Changes in body mass (% of initial body mass) in calorie-restricted (CR), normocalorie-fed (NF), and ad libitum-fed (AL) mice. LD, 12:12-h light-dark cycle; DD, constant darkness. *P < 0.05 compared with same week among the 3 groups.](http://ajpregu.physiology.org/Downloadedfrom)
animals. Along with FAA, in five of the seven normocalorie-fed mice, there was also a bout of activity corresponding to the offset of the nocturnal pattern around ZT0, which was clearly separate from FAA (Fig. 2E). During this period the daily organization of wheel-running activity remained essentially unchanged in ad libitum-fed mice (Fig. 2, C and F). During the last 5 days of food restriction the onset of the daily activity rhythm differed significantly according to the nutritional state [F(2,72) = 112.6, P < 0.01]. It occurred 2 h earlier in calorie-restricted than in normocalorie- and ad libitum-fed mice. The main effect of the days was not significant (P > 0.1), nor was the interaction between the nutritional state and the time significant (P > 0.1), indicating that neither the phase of the activity onset nor the differences between the nutritional groups varied over the last 5 days of food restriction (Fig. 3). When mice were transferred to DD and fed ad libitum, the onset of the nocturnal period of locomotor activity occurred significantly earlier (1.3 h) in calorie-restricted than in normocalorie- and ad libitum-fed animals [F(2,72) = 10.6, P < 0.01; Fig. 3]. The treatment × time interaction was not significant (P > 0.1), indicating that the onset of activity in calorie-restricted mice remained advanced compared with that in normocalorie- and ad libitum-fed controls over the first 5 days in DD (Fig. 3).

In calorie-restricted and normocalorie-fed mice that had expressed FAA during food restriction, a free-running bout of FAA was also observed in DD, despite ad libitum feeding. The onset of the bout began from the previous time of feeding in LD (Fig. 2). The free-running FAA persisted throughout the 3 wk in DD in 7 of the 14 animals. In these cases, there was a phase-locked relationship between the free-running FAA and the nocturnal period of locomotor activity (Fig. 2, B and D). This indicates a similar period for these two components of activity in each of these animals. In three other animals, this component merged into the nocturnal period of activity, whereas it damped out after 11–15
days in the last four animals (Fig. 2A). During ad libitum refeeding, τ did not differ significantly (P > 0.1) among calorie-restricted, normocalorie-fed, and ad libitum-fed mice: 23.8 ± 0.1, 23.8 ± 0.1, and 23.9 ± 0.1 h, respectively.

The average total number of wheel revolutions was analyzed across the nutritional state and the stages of the experiment (i.e., initial ad libitum period in LD, food restriction in LD, and final ad libitum period in DD). The main effect of the nutritional state was not significant (P > 0.1), indicating that mean daily activity did not differ among the three groups of mice (Fig. 4A). The main effect of time and the nutritional state × time interaction were significant [F(2,36) = 9.8, P < 0.01 and F(4,36) = 2.7, P = 0.05, respectively], detecting a significant decrease of daily activity in calorie-restricted mice during refeeding. The average activity expressed daily from ZT0 and ZT5 was analyzed similarly. There was a significant effect of the nutritional state [F(2,36) = 12.2, P < 0.01] and the time [F(2,36) = 19.3, P < 0.01] and a significant nutritional state × time interaction [F(4,36) = 8.7, P < 0.01]. The multiple-comparisons test showed that activity corresponding to FAA was higher in calorie-restricted mice during food restriction (calculated from ZT0 to ZT5) and refeeding (calculated from CT0 to CT5) than in all other conditions (Fig. 4A).

These results indicate that timed calorie restriction is a potent phase-shifting factor capable of competing with the photic cues for entrainment in mice. Accordingly, a 1-h phase advance of the onset of the nocturnal period of locomotor activity was found in calorie-restricted mice transferred to DD and fed ad libitum. A 2-h phase advance of daily rhythm of activity, however, was seen in these mice during calorie restriction. This difference might be due to a rapid phase delay associated with refeeding, therefore reducing the overall phase advance of the underlying LEP. On the other hand, this discrepancy may be ascribed to some positive masking effects of activity downstream of the SCN pacemaker (i.e., at the output level) during calorie restriction. In either case, with the onset of the circadian rhythm of activity under DD conditions as a reliable phase marker of the underlying LEP, there was a ≥1-h phase advance of the LEP in calorie-restricted mice fed in the morning. Timed normocaloric feeding had no effect on the photic entrainment of the LEP, and no phase advance was seen when normocalorie-fed mice were transferred to DD.

The FAA expressed by calorie-restricted and normocalorie-fed mice during food restriction was found to free run in parallel with the nocturnal period of locomotor activity in most animals. This observation rules out any hourglass-like timer for the expression of daily FAA and confirms the circadian nature of FAA. Moreover, the phase-locked relationship between the two free-running components suggests that the FEP may be coupled to the LEP in mice.

**EXPERIMENT 2**

Bouts of hyperactivity during the inactive period can induce phase shifts of the LEP in hamsters (18, 30). Daily access to a running wheel can also entrain circadian rhythms in C57 mice (10). Therefore, FAA, independently of feeding events, may be involved in the entraining properties of timed calorie restriction (6). To test this hypothesis, daily expression of FAA was prevented by restraining mice between the onset of light (i.e., ZT0) and the time of feeding (i.e., ZT2).

**Methods.** Twenty-one adult male C57BL/6J mice were used under lighting and temperature conditions similar to those used for experiment 1. The same data acquisition methods were also used. After 3 wk of baseline conditions, mice were divided into three groups of seven animals each. The calorie-restricted group was...
given 66% of daily food intake during baseline conditions (i.e., 3.3 g of chow/day) at ZT2.

The normocalorie-fed group received 100% of daily food intake (i.e., 5 g of chow/day) at ZT2. Food remained available ad libitum in the ad libitum-fed group. Between ZT0 and ZT2, all mice were immobilized in closed transparent polypropylene tubes (11 cm long \( \times \) 3 cm diameter) with air holes for breathing. The ends of the tubes were filled with cotton so that the animals were completely restrained. The duration of timed food restriction and immobilization was 4 wk. Thereafter, all animals were transferred to DD and fed ad libitum for 3 wk. The parameters of wheel-running activity were quantified as in experiment 1. Despite the daily period of immobilization, the level of activity was also determined between ZT0 and ZT5 to account for the bouts of activity after immobilization.

Figure 5. Wheel-running activity in 2 calorie-restricted mice (A and D), 2 normocalorie-fed mice (B and E), and 2 ad libitum-fed mice (C and F). Successive 24-h periods are double plotted (48-h horizontal time scale). Days 1–14, initial period of ad libitum feeding under LD conditions; days 15–42, daily immobilization between ZT0 and ZT2 and calorie restriction under LD conditions; days 43–63, final period of ad libitum feeding in DD. Nighttime when mice were kept under LD conditions is indicated by black bar on abscissa. Schedule of immobilization is indicated by stippled area on abscissa. Time of feeding during calorie restriction is indicated by vertical arrow at ZT2 on abscissa.

Figure 6. Onset of nocturnal pattern of wheel-running activity in calorie-restricted, normocalorie-fed, and ad libitum-fed mice during last 5 days of initial period of ad libitum feeding under LD conditions, last 5 days of daily immobilization between ZT0 and ZT2 and calorie restriction under LD conditions, and first 5 days of final period of ad libitum feeding in DD. Nighttime when mice were kept under LD conditions is indicated by black bar on top abscissa. Schedule of immobilization is indicated by stippled area on top abscissa. Time of feeding during calorie restriction is indicated by vertical arrow at ZT2 on top abscissa. For statistical comparisons see text.
these mice. However, most mice, whether food restricted or not, displayed a small bout of activity after daily immobilization that was concomitant with the time of feeding (ZT2) in calorie-restricted and normocalorie-fed mice (Fig. 5 and see below).

At the end of timed food restriction and immobilization under LD conditions, mice were transferred to DD and fed ad libitum. At that time, there was a main effect of the nutritional state and the days for the onset of the nocturnal period of activity [F(2,72) = 4.4, P < 0.05 and F(4,72) = 20.0, P < 0.01, respectively]. The activity onset was phase advanced by 1 h in calorie-restricted mice compared with normocalorie-fed and ad libitum-fed animals (Fig. 6). The nutritional state × time interaction was not significant [F(8,72) = 0.8, P > 0.1], indicating that the pattern of changes was similar among the three nutritional groups during this period. In addition to the free-running nocturnal period of activity, a separate free-running bout of activity was also seen in most (15 of 21) of the mice. The onset of this distinct bout began from the previous time of feeding (and the end of the immobilization period) in calorie-restricted and normocalorie-fed mice and at the end of the immobilization period in ad libitum-fed mice (Fig. 5). As in experiment 1, this free-running bout of activity was phase locked with the free-running nocturnal period of locomotor activity. It persisted over the 3 wk of DD in 9 of the 15 animals (Fig. 5, D–F), whereas it damped out after several days in the 6 other animals (Fig. 5, A and B). During ad libitum refeeding, z did not differ significantly (P > 0.1) among calorie-restricted, normocalorie-fed, and ad libitum-fed mice: 23.8 ± 0.1, 23.8 ± 0.1, and 23.9 ± 0.1 h, respectively.

The average total number of wheel revolutions did not differ among the three nutritional groups of mice (P > 0.1). The main effect of time and the nutritional state × time interaction were significant [F(2,36) = 38.6, P < 0.01 and F(4,36) = 8.4, P < 0.01, respectively]. There was a significant decrease of daily activity in normocalorie-fed and ad libitum-fed mice during the period of daily immobilization compared with baseline conditions, but not in calorie-restricted mice (data not shown). In the latter group, however, daily activity was significantly decreased during ad libitum refeeding in DD compared with calorie restriction under LD conditions. The average activity expressed daily from ZT0 to ZT5 was also analyzed. There was no significant effect of the nutritional state (P > 0.1), indicating that the amount of activity expressed during this temporal window was similar in the three groups of mice during all periods analyzed (Fig. 4B). Moreover, the main effect of time was significant [F(2,36) = 14.2, P < 0.01], in contrast to the nutritional state × time interaction (P > 0.1). The multiple-comparisons test showed that activity after time of feeding and/or immobilization was higher in DD, when it free ran in most mice, than during the food restriction and/or immobilization under LD conditions (Fig. 4B).

These results demonstrate that the combination of timed calorie restriction at ZT2 and daily immobilization between ZT0 and ZT2 (to prevent the expression of FAA) still induces a 1-h phase advance of the LEP, as found in calorie-restricted mice fed at ZT2 without restraint (experiment 1). This demonstrates that FAA is not a critical variable in the phase-shifting effects of timed calorie restriction when hypocaloric food is given at ZT2. Furthermore, the finding that mice fed ad libitum and immobilized daily showed no phase advance of the LEP indicates that a daily stress and associated arousal cannot account for the phase-shifting effects of timed calorie restriction.

No anticipatory bout of activity was expressed before the time of daily immobilization in calorie-restricted mice, indicating that FAA is specifically related to feeding events. In most calorie-restricted and normocalorie-fed mice, a free-running component of FAA was expressed in DD, despite the previous immobilization. This again supports the circadian nature of FAA. In ad libitum-fed mice, activity after daily immobilization may also free run in parallel with the nocturnal period of locomotor activity during several days in DD, as does FAA in normocalorie-fed or calorie-restricted mice (experiments 1 and 2). This suggests that several repetitive nonphotic events can entrain a subcomponent of the LEP and/or a subordinate pacemaker to the LEP in mice. The nature of these components free running apart from the nocturnal pattern of activity will be investigated in SCN-lesioned mice under similar conditions.

EXPERIMENT 3

Experiment 1 showed the phase-shifting effects of a hypocaloric diet given 2 h after lights on. Whether the time of hypocaloric feeding relative to the LD cycle may have an impact on food entrainment is unknown. Therefore, limited food was supplied daily at four different times of the day surrounding the dark-light and light-dark transitions (i.e., 2 h before or after lights on and 2 h before or after lights off). Because FAA was found to free run in mice transferred to DD (experiment 1), we can use this as an experimental tool to further study the coupling of the FEP to the LEP. Thus, under subsequent free-running conditions (i.e., DD and food provided ad libitum), the coupling of the FEP to the LEP was assessed by means of a light pulse known to phase delay the circadian (i.e., light-entrainable) rhythm of locomotor activity. If the FAA is expressed independently of the LEP (i.e., the coupling between the LEP and FEP is only weak), FAA may not be expected to be shifted by the light pulse. Conversely, if FAA is shifted after a light pulse, this would indicate that FAA is strongly coupled to the LEP. In the case where FAA is superimposed on the onset of the nocturnal period of activity (i.e., in those mice fed toward the end of afternoon or during early night), we hypothesize, similarly, that if FAA is not shifted after a light pulse, this would dissociate or uncouple the FEP and the LEP and, therefore, lead to two distinct free-running components. On the contrary, a complete phase shift would suggest a strong coupling of the FEP to the LEP.

Methods. Twenty-four adult male C57BL/6j mice were used under lighting and temperature conditions...
similar to those used for experiment 1, and the same data acquisition methods were utilized. At the end of baseline conditions, mice were divided into four groups of six animals each. All animals were calorie restricted so that they were given 66% of daily food intake during baseline conditions (i.e., 3.3 g of chow/day). The first group received a daily hypocaloric diet at ZT2, similar to calorie-restricted mice in experiment 1. The three other groups received a daily hypocaloric diet at ZT10, ZT14, and ZT22. For the twolatter groups (i.e., mice fed during the dark phase), food was given with the aid of an infrared viewer. After 4 wk of calorie restriction, all animals were transferred to DD on the same day at ZT0 and fed ad libitum for 3 wk. When switched to ad libitum feeding, mice were provided food beginning close to the time when hypocaloric food was given previously. Therefore, animals that received food around lights on (i.e., at ZT2 and ZT22) were fed ad libitum at ZT0. Animals that received food at ZT10 and ZT14 spent 10 and 14 h, respectively, in DD before ad libitum feeding. On the 11th day in DD, mice received a 30-min light pulse (300 lx of fluorescent white light) at CT15. Animals were returned to DD after the light pulse. To restore the previously calorie-restricted mice to their normal body condition, the light pulse was not given until after 10 days of ad libitum refeeding in DD. This is important, because photic responses of the SCN, assessed by Fos expression, are altered in calorie-restricted rats (5), and that may impair the light-induced behavioral phase shift in calorie-restricted mice.

Quantitative analysis of the rhythm of wheel-running activity was performed as in experiment 1. To quantify the light pulse-induced phase delays, lines were fitted by eye to the onsets of locomotor activity and FAA (if any) for the first 7 days before the light pulse. These lines were projected to the day of the pulse. Similarly, lines were fitted to the onsets of activity and FAA during the 7 days after the photic pulse. These lines were retroprojected to the day of the pulse. The magnitude of the phase shifts was calculated as the difference between the respective two lines for locomotor activity rhythm and FAA. \( \tau \) was calculated as in experiment 1.

Results and discussion. The onset of the nocturnal activity under LD baseline conditions did not differ among the four groups of calorie-restricted mice \( (P > 0.1) \) or among the days \( (P > 0.05; \text{Figs. } 7 \text{ and } 8) \). During calorie restriction the time of food supply led to changes in the phase of wheel-running activity. Similarly, this phase delay was observed as a result of the light pulse applied at CT15 on day 53. The light pulse applied at CT15 on day 53 is indicated by circles. Nighttime when mice were kept under LD conditions is indicated by black bar on abscissa. Time of feeding during calorie restriction is indicated by vertical arrow on abscissa. Light pulse applied at CT15 on day 53 is indicated by circles.
in the day-night pattern of activity in all groups. These include a strong FAA and a phase advance of the nocturnal pattern of activity. In calorie-restricted mice fed at ZT10 or ZT14 the onset of the nocturnal locomotor activity cannot be discriminated from the superimposed expression of FAA (Fig. 7, B and C, respectively). Another interpretation is that the nocturnal onset has been delayed to the middle of the night. However, this seems unlikely in view of the phase advance of the onset of the nocturnal period of activity after transfer to DD. Therefore, in further analysis of these groups, the onset of the nocturnal activity was defined as the first 10-min bout of activity in the late afternoon that fulfilled our criterion for the onset of nocturnal wheel-running activity (see methods in experiment 1). Inter-group comparison of the onset of the daily rhythm of activity showed a marked effect of the time of food supply [F(3,80) = 30.6, P < 0.01]. No significant effects were detected for the days (P > 0.1) and the time of food supply × days interaction (P > 0.05). A 5-h phase advance of the daily onset of nocturnal activity was found in calorie-restricted mice fed in late afternoon (i.e., at ZT10), whereas a 1-h mean phase advance was observed in the three other groups of mice, irrespective of the time of feeding (Figs. 7 and 8).

When mice were transferred to DD and fed ad libitum, the onset of the nocturnal pattern of locomotor activity occurred significantly earlier (2 h) in calorie-restricted mice previously fed at ZT10 than in the three other groups of calorie-restricted mice previously fed at ZT2, ZT14, or ZT22 [F(3,80) = 27.2, P < 0.01; Fig. 8]. In the three latter groups, there was a 1-h phase advance of the onset of the nocturnal period of locomotor activity compared with the ad libitum-fed state during baseline conditions. In calorie-restricted mice previously fed at ZT10, there was a 3-h phase advance of the onset of nocturnal wheel-running activity compared with baseline conditions. The time of food supply × days interaction was also significant [F(12,80) = 2.2, P < 0.05]. All mice were transferred to DD at ZT0. Only animals that received food around lights on (i.e., at ZT2 and ZT22) were fed ad libitum at the same time (i.e., ZT0). Animals that received food at ZT10 and ZT14 were placed into DD at 10 and 14 h, respectively, before ad libitum feeding. FAA during the 1st day of refeeding may have masked the onset of the nocturnal period of activity in these mice (Fig. 8), therefore explaining the significant interaction between the time of food supply and the days. A free-running bout of FAA was observed in mice previously fed at ZT2 (Fig. 7A), as in experiment 1, as well as in those mice previously fed at ZT22 (Fig. 7D). In 8 of these 12 mice, FAA was expressed until the end of the experiment. It damped out before the 10th day of DD in the four other mice. No separate free-running bout of FAA was detectable in the 12 calorie-restricted mice previously fed at ZT10 (Fig. 7B) and ZT14 (Fig. 7C). The differences between t values were not significant among the four groups of previously calorie-restricted mice: 23.6 ± 0.1, 23.5 ± 0.2, 23.6 ± 0.1, and 23.8 ± 0.1 h in mice fed at ZT2, ZT10, ZT14, and ZT22, respectively (P > 0.1).

A light pulse applied at CT15 induced a phase delay of the nocturnal period of locomotor activity in the four groups of mice (Fig. 7). There was no difference in the magnitude of light-induced phase delays among the groups: 2.3 ± 0.2, 1.8 ± 0.2, 2.2 ± 0.2, and 2.0 ± 0.1 h in mice fed at ZT2, ZT10, ZT14, and ZT22, respectively (P > 0.1). In the two groups of mice in which no free-running bout of FAA was detectable before the light pulse (i.e., in calorie-restricted mice previously fed at ZT10 and ZT14), the light pulse did not unmask any free-running FAA. In other words, no masking effects due to FAA prevent the phase delay of the onset of the nocturnal period of activity rhythm in these mice (Fig. 7, B and C). By contrast, in mice in which FAA was observed to free run before the light pulse (i.e., in calorie-restricted mice previously fed at ZT2 and ZT22), the two free-running components were phase delayed by a light pulse starting at CT15 (Fig. 7, A and D). In the eight animals that showed a free-running FAA after the light pulse, a paired comparison indicated that the phase delay of FAA was less than that of the nocturnal period of locomotor activity: 1.6 ± 0.2 and 2.2 ± 0.1 h, respectively (n = 8, t = 2.5, P < 0.05).

The average total wheel revolutions did not differ among the four groups of calorie-restricted mice over the experiment (P > 0.1; data not shown). The main effect of time and the time of food supply × time interaction were significant [F(2,40) = 50.7, P < 0.01 and F(6,40) = 3.0, P < 0.05, respectively]. There was a significant decrease of daily activity in the four calorie-restricted groups during ad libitum refeeding in DD compared with calorie restriction and baseline under LD conditions (data not shown).

The present results confirm that timed calorie restriction is capable of phase shifting the LEP in mice (see experiment 1). Furthermore, the magnitude of the phase change induced by the timed calorie restriction is phase dependent. The largest (3 h) phase advance of the onset of the nocturnal period of locomotor activity was found in calorie-restricted mice fed in late afternoon. In calorie-restricted mice fed during early night, late night, or early morning, there was a 1-h phase advance of the LEP. The phase advance of the nocturnal period of activity (assessed in DD) in calorie-restricted mice fed at ZT2 was similar in experiments 1 and 3. There was, however, only a 1-h phase advance of daily rhythm of activity (experiment 3) compared with the 2-h phase advance in a group similarly calorie restricted during experiment 1. As previously hypothesized (experiment 1), some positive masking effects of activity may be involved in the latter group (cf. Figs. 2D and Fig. 7A). The less marked effects in calorie-restricted mice fed at ZT2 during experiment 3 are unclear. In calorie-restricted mice fed at ZT10, there may be similar positive masking effects of activity (due to superimposed FAA in this case) on the onset of the daily nocturnal wheel-running activity. This suggests that the onset of the daily rhythm of activity under LD conditions cannot be used as a reliable phase marker of the underlying LEP.
In calorie-restricted mice previously fed at ZT2 and ZT22, a light pulse started at CT15 was able to phase shift not only the free-running nocturnal period of activity but also the free-running FAA. In the mice fed at ZT10 and ZT14, FAA was superimposed onto the onset of the daily rhythm of activity during calorie restriction; consequently, no free-running FAA was observed during the first days in DD. A light pulse applied at CT15 phase delays the free-running nocturnal period of activity and did not unmask any free-running FAA that would be insensitive to the phase-shifting effects of a light pulse (in the case where the FEP and LEP would be uncoupled). Accordingly, this experiment demonstrates that the FEP is strongly coupled to the LEP in mice.

**GENERAL DISCUSSION**

The main findings of these experiments are as follows. 1) In contrast to normocaloric feeding that does not affect the LEP, timed calorie restriction in C57BL/6J mice exposed to an LD cycle is capable of phase advancing the LEP with a phase-dependent magnitude. In addition, whatever the time of day at which limited food was given (i.e., ZT2, ZT10, ZT14, or ZT22), timed calorie restriction led to a phase advance of the LEP (experiment 3). A larger phase advance was observed in response to hypocaloric feeding in late afternoon (i.e., ZT10). That the phase shifts are in the same direction when hypocaloric food was given repeatedly during daytime (ZT2 and ZT10) or night (ZT14 and ZT22) indicates that the phase-shifting effects of timed calorie restriction are different from the two classical families of phase-response curves (PRC), namely, the PRC to light pulses in DD and the PRC to dark pulses in constant light and nonphotic factors (for review see Ref. 23). However, in the present study, calorie-restricted mice received daily photic (i.e., LD cycle) and nonphotic (i.e., hypocaloric feeding) temporal cues. This is in contrast to those studies in which single phase-shifting “pulses” in constant lighting conditions were used.

Nevertheless, our results indicate direct effects of timed calorie restriction on the circadian timing system. On the basis of studies that use food access for a daily limited time, the internal coupling between the FEP and the LEP is considered to be weak in most mammals. This has been seen in laboratory rats (16, 24–26), Golden hamsters (2), and C57 mice (1). Also, the rhythm of neuronal firing in the SCN pacemaker (i.e., LEP) is unchanged by restricted-feeding schedules (11). In accordance with this, the initial phase of the LEP in normocalorie-fed mice (without calorie restriction) transferred to DD and subsequently fed ad libitum is similar to that of control mice fed ad libitum throughout the study (experiment 1). By contrast, calorie restriction led to phase advances of the circadian activity rhythm after the mice were placed in DD. It is therefore possible that timed calorie restriction enhances the potency of food synchronization of the FEP, leading to a stronger coupling of the LEP to the FEP in calorie-restricted mice (i.e., the LEP being to some extent subordinate to the FEP) than in normocalorie-fed mice. This, however, seems unlikely, because the output of the FEP (i.e., FAA), which usually free runs in parallel with the nocturnal period of locomotor activity (experiments 1–3), can be shifted by a light pulse (experiment 3). These findings provide support for an opposite coupling strength between the LEP and the FEP in ad libitum feeding conditions (i.e., the LEP controls the period of the FEP). In hamsters also the FEP has been considered a secondary oscillator compared with the “master” LEP, such that the FAA is more robust when the SCN are destroyed or under constant bright light, a situation that may uncouple the LEP and FEP (2).

It is conceivable that timed calorie restriction affects the SCN. In agreement with this view is the shortening of the free-running period (τ) after long-term restricted feeding (4). Thus the phase advance of the photic entrained nocturnal pattern of activity observed in calorie-restricted mice could also be explained by a shortening of τ. However, no significant differences in τ were detected among the nutritional groups during the first 10 days of ad libitum food in DD (experiments 1 and 2). Another explanation for the advance of the photic entrained nocturnal activity period and the subsequent phase advance of the free-running activity rhythm is that timed calorie restriction alters the PRC to light. The temporal window for light-induced phase delays may have been reduced and/or the phase-advance region of the photic PRC may have been extended in calorie-restricted mice. This hypothesis is supported by the fact that Fos expression in response to light pulses is modified in calorie-restricted rats fed at ZT2 in comparison with controls fed ad libitum (5).

Timed calorie restriction induces behavioral changes, including increased expression of FAA (see experiment 1), and metabolic modifications, such as loss of body mass due to mobilization of energy stores (experiment 1). Both changes may play a role in the regulation of circadian rhythms (18, 29). When applied during the inactive period, stimuli as different as a cage change or an injection of benzodiazepines can induce wheel-running activity in hamsters. These stimuli are also capable of phase shifting the circadian rhythm of activity in hamsters. Because no phase shifts are observed when hamsters are prevented from running, stimulated activity is considered to be the main cause of the phase shifts through physical activity feedback to the SCN pacemaker (18, 30). As a first step in investigating the mechanisms that affect the circadian regulation during calorie restriction, experiment 2 was designed to test whether strong expression of FAA participates in
the phase advance of the LEP through locomotor activity feedback. Immobility of the calorie-restricted mice during the 2-h period in which they would have normally expressed FAA does not eliminate the phase advance of the nocturnal period of locomotor activity. This result demonstrates that the phase-shifting effect of timed calorie restriction is independent of activity feedback to the SCN through FAA. Because hypocaloric food was provided at ZT2 in experiments 1 and 2, it is still possible that the larger phase advance of the LEP, when hypocaloric diet was given at ZT10, was due to a feedback of FAA at that time (experiment 3).

Apart from the possible effect of activity feedback, hormonal and/or metabolic changes associated with timed calorie restriction also have to be taken into account. Food restriction induces a peak of plasma corticosterone before feeding (6, 19). This daily increase in circulating corticosterone may have participated in the phase advance of the LEP in calorie-restricted mice. Stress-induced immobilization also induces an increase in plasma corticosterone (8). The combined effect of immobilization and calorie restriction did not lead to a larger circadian phase advance (experiment 2) than that induced by timed calorie restriction alone (experiment 1). Moreover, immobilized mice fed ad libitum did not display any phase advance of circadian activity rhythm (experiment 2) in accordance with a previous study performed under DD conditions (unpublished data of Menaker et al. in Ref. 3). It is therefore unlikely that the daily peak of plasma corticosterone before feeding plays a major role in the phase advance of the LEP in calorie-restricted mice.

Another hormone, pineal melatonin, has been shown to have phase-resetting properties of the SCN clock in rodents (3). However, this hormone could not be involved in the present study, because C57 mice do not synthesize melatonin (9). As previously suggested, metabolic consequences of timed calorie restriction may be capable of interacting directly, or indirectly, with the photic entrainment of the SCN (5). Among these could be changes in hormones involved in the regulation of glucose, such as insulin or glucagon, or in glyceremia itself.

Functional properties of FAA. In accordance with previous studies using food access for a limited time each day (1, 14), normocalorie-fed and calorie-restricted mice express FAA before the expected time of food supply. When mice were fed ad libitum in DD after calorie restriction or normocaloric feeding under LD conditions, a free run of FAA starting at the circadian phase of restricted feeding was observed with a period similar to that of the LEP. This suggests a coupling between the FEP and LEP in mice. Meal-fed rats kept under LD conditions express a feeding-associated component of drinking. When rats are fed ad libitum after enucleation, this component free runs with the same period as the circadian rhythm of drinking (17). This finding is in agreement with our data.

By contrast, in most other studies in rats and hamsters, there is a fading of FAA during ad libitum refeeding. This has been interpreted as an oscillator damping or a rapid extinction process (16). After a period of ad libitum food access, however, FAA may reappear during intervals of food deprivation. Results in intact rats kept in DD have been inconsistent with respect to the endogenous period of the FEP. In keeping with our results, previous studies have shown that FAA during fasting periods may be expressed at the circadian time of the previous restricted feeding. That is, the endogenous periods of FEP and LEP are similar and free run in parallel (22). In other studies, however, FAA during fasting was observed to reappear at the actual end of previous restricted feeding, suggesting that the endogenous period of the FEP was close to 24 h (7). The free running of FAA that we observed in calorie-restricted and normocalorie-fed mice transferred to DD can be used as an experimental tool to study the properties of the FEP in free-feeding conditions (i.e., without intervals of forced fasting).

The present study provides a clear experimental demonstration that FAA can be phase shifted by a pulse of light in mammals. This indicates a direct coupling of the FEP to the LEP in C57 mice (experiment 3). Although a direct effect of light on the putative FEP cannot be fully excluded, the similar free-running periods of circadian activity rhythm and FAA and their light-induced phase delays suggest that the period of the FEP can be controlled by the LEP in mice. The shorter light-induced phase delay of FAA indicates complex interactions within the circadian timing system (experiment 3). In contrast to the light-induced phase shifts that we studied in free-feeding conditions, another study (25) used a 2-h light pulse (applied in early or late subjective night) in rats subjected to restricted feeding under DD. FAA was not phase shifted clearly and remained synchronized to the time of food access, a result interpreted as a weak coupling of the LEP and FEP in rats. Phase shifting the LD cycle, however, is able to reset the FEP in rats (20, 22). This result and ours support the hypothesis that the SCN, or LEP, in rodents is the master pacemaker that would influence other pacemakers such as the FEP. Such a view is consistent with the classical model of a multioscillator circadian system (21). In this framework, a circadian pacemaker, located in the mammalian retina (28), has to be taken into account, in that it may gate the photic cues before they, directly or indirectly, reach the LEP. Whether timed calorie restriction under LD conditions may affect the retina pacemaker is not known.

In conclusion, the hypometabolic state in response to chronic calorie restriction can lead to a phase advance of the LEP ranging from 1 to 3 h according to the time of day (i.e., ZT2, ZT10, ZT14, and ZT22) at which hypocaloric food is supplied. This unusual pattern of phase-shifting effects (under LD conditions) compared with the PRC to light and classical nonphotic factors (determined in DD) suggests that calorie restriction may affect the photic resetting mechanisms by different types of nonphotic stimuli.
The central role of the SCN of the hypothalamus in the generation of circadian rhythms was discovered 25 years ago. Since then, understanding the mechanisms by which the biologic clock in the SCN is synchronized to environmental signals has been an active field of research. Although the LD cycle is clearly a major synchronizer of circadian rhythms, other environmental factors can also influence circadian timing. The results shown here provided evidence that food synchronization in calorie-restricted mice may compete with photic entrainment, involving mechanisms independent of locomotor activity feedback. It is hypothesized that metabolic factors associated with reduced food availability may participate in the phase-shifting effects of calorie restriction on the circadian clock.

We are grateful to Sue Olson for helpful assistance. Portions of this work were presented in abstract form at the Annual Meeting of the Society for Neurosciences, New Orleans, LA, October 1997 (Soc. Neurosci. Abstr. 23: 1326, 1997). This work was supported by National Institute on Aging Grants PO1-AG-11412 and RO1-AG-02972 (F. W. Turek) and by a Postdoctoral Fellowship from the Fyssen Foundation (E. Challet).

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Received 23 October 1997; accepted in final form 2 February 1998.

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