

Food anticipatory activity and photic entrainment in food-restricted BALB/c mice

M.M. Holmes, R.E. Mistlberger*

Department of Psychology, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia V5A 1S6, Canada

Received 2 August 1999; received in revised form 30 August 1999; accepted 16 November 1999

Abstract

The BALB/c mouse was evaluated as a model for the study of entrainment of circadian rhythms by feeding schedules. Mice were housed in a 12:12-h light–dark (LD) environment with food available for 3–5 h/day (5 h before dark onset). Food anticipatory activity (FAA) rhythms were evident in all mice, ranging from robust in some to weak and variable in others. Advancing transients of the end of nocturnal activity were evident in many cases, culminating in a significant shortening of the main bout of nocturnal activity. Transients and contraction of nocturnal activity were not dependent on the expression of FAA. Following restricted feeding, nocturnal activity expanded by a series of delaying transients. On the first day of constant dark (DD) with ad libitum food access following restricted feeding in LD, the phase from which activity free-ran was advanced by comparison with control tests. Transients, compressed nocturnal activity, and advanced phase of free-run suggest that feeding schedules cause phase advancement of light-entrained rhythms in BALB/c mice. When restricted feeding was imposed in DD, several mice expressed robust FAA concurrent with a free-running activity component. In some cases, free-running rhythms entrained to feeding time, and in other cases, the period of the free run lengthened toward 24 h. These data show that restricted feeding in BALB/c mice can engage a circadian mechanism driving FAA rhythms and can also modulate the phase of photic entrainment, possibly by a direct entraining effect on the light-entrained rhythm. The BALB/c mouse strain, in several respects, appears to be a useful model for the study of scheduled feeding and circadian rhythms. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Circadian rhythms; Food restriction; Wheel running; Nonphotic entrainment

1. Introduction

The circadian timing system generates daily rhythms of behavior that permit organisms to anticipate predictable events in their environment, thus increasing the probability of locating essential resources (e.g., food and mates), avoiding predators, and ultimately increasing the likelihood of survival. For endogenously generated behavioral rhythms to serve these adaptive functions, they must be synchronized to local time. In mammals, the primary circadian pacemaker driving daily rhythms of behavior is located in the suprachiasmatic nuclei (SCN) of the hypothalamus [1]. For most species, daily light–dark (LD) cycles are the primary environmental stimulus (“zeitgeber”) for entrainment of the SCN pacemaker. Photic information is conveyed to the SCN by direct and indirect retinofugal pathways [1]. Because the SCN are retinorecipient and necessary for photic entrainment, they can be designated a light-entrainable pacemaker (LEP) [1].

In rats and a number of other mammalian species, circadian rhythms can also be synchronized by daily schedules of food availability. In most cases, feeding schedules appear to entrain only a component of activity that anticipates the daily feeding time. This component, known as food anticipatory activity (FAA), exhibits fundamental properties of a clock-controlled process, including limits to entrainment and persistence in the absence of the zeitgeber (i.e., during several cycles of total food deprivation; reviewed in [3]). This component also persists following complete SCN ablation, indicating that a separate, food-entrainable circadian pacemaker (FEP) exists elsewhere in the brain [4–7].

In a few species, most notably the Syrian hamster [8,9] and the marsupial kowari [10], feeding schedules more often appear to entrain or shift the entire circadian system. Phase control of circadian rhythms in these cases may be mediated by any of a number of nonphotic stimuli, including anticipatory locomotor activity, the arousing effect of food presentation, the ingestion of nutrients and water, or internal coupling with a FEP. In addition to exerting phase control on the SCN pacemaker, these nonphotic stimuli may also modulate the response of the SCN to photic input (e.g., [11–13]).

* Corresponding author. Tel. 604-291-3462; Fax: 604-291-3427.

E-mail address: mistlber@sfu.ca

Current address for M.M. Holmes: Department of Psychology, University of British Columbia, Vancouver, BC, Canada.

Historically, the laboratory rat, because of its adaptability to restricted feeding regimens, has been the species of choice in studies of food entrainment. However, mice are increasingly the species of choice in circadian rhythms research because of the development of new tools for molecular and genetic analyses of circadian mechanisms. The C57BL/6j strain has been most widely used for investigations at this level and has also proven to be a suitable strain for behavioral and physiological study (e.g., [14–17]). Although the C57BL/6j mouse has been used to demonstrate FAA in intact and SCN-ablated mice and to examine interactions between feeding schedules and light-entrained rhythms [11,12,14,16,18,19], we have generally found that the expression of FAA in this mouse strain is relatively weak when compared to FAA expression in rats ([16]; Mistlberger et al., unpublished observations). This may be because the LEP dominates circadian timing of behavior in this strain and exerts a relatively strong inhibition of activity during the rest phase of the circadian cycle.

The BALB/c mouse strain has also been used in genetic investigations of circadian rhythms (e.g., [20,21]) and has been described as having a circadian system comprised of a loosely coupled population of oscillators [22]. In preliminary studies, we have also found evidence suggestive of loose coupling within the BALB/c circadian system (e.g., inferred from rapid damping and instability of free-running rhythms in constant dark, DD). If separate food- and light-entrainable oscillators are also loosely coupled in this strain, the BALB/c mouse may be particularly well suited for investigations of the properties and mechanisms of FAA. The present study describes the effects of restricted feeding schedules on the activity rhythms of the BALB/c mouse in LD and DD. The results are consistent with the dual FEP–LEP model of the rodent circadian system and provide evidence for phase modulation of the LEP, in both LD and DD, by some aspect of restricted feeding schedules.

2. Methods—overview

2.1. Subjects

Adult male BALB/c mice were obtained from the University of British Columbia (Vancouver, British Columbia). All animals weighed approximately 20 g at the onset of the experiments. Mice were housed in a ventilated room with a 12:12 LD cycle (~250 lux) or in DD (0 lux). Animals had ad libitum access to food pellets (Lab Diet) and water at the beginning of each experiment.

2.2. Apparatus

Mice were housed individually in plastic cages (45 × 24 × 20 cm) with wire floors suspended over waste collection bins filled with wood shavings. All cages were equipped with running wheels (17 cm) and plastic tubing for environmental enrichment. Each wheel was connected to a microswitch that detected wheel revolutions. Wheel revolutions

were counted continuously by a microcomputer system (486 PC and Activity Counting System, Simon Fraser University) that summed and stored counts to disk at 10-min intervals. Data were then transferred to a Macintosh computer for display and analyses. To prevent unscheduled exposure to light, animal maintenance was performed using an infrared viewer during periods of darkness.

During restricted feeding schedules, powdered food (Lab Diet) was mixed with oil (25–30% by weight) to create a palatable, high-calorie diet. The food was placed in small glass containers set on the cage floor to maximize food availability and minimize spillage by the animals.

2.3. Data analysis

For visual inspection, activity data were displayed as actograms and average waveforms by the program *Circadia* (Behavioral Cybernetics, available from the corresponding author). The following parameters were quantified: mean daily activity, magnitude of FAA (percentage of total daily activity, excluding mealtime, occurring during the 3 h preceding mealtime), phase of nocturnal activity onset, mean duration of nocturnal activity (α), and period (τ) of rhythms in DD. Activity onset was defined as the first 10-min bin containing 50 wheel counts after an interval of 240 min in which activity did not exceed this level in any 10-min bin. For some animals that were less active, a lower threshold for activity counts was used. To measure τ in DD, a regression line was fitted by computer to activity onsets. The phase of nocturnal activity onset was expressed as the difference in minutes between the onset of the main bout of nocturnal activity and lights-off. An unmasked measure of nocturnal activity onset was obtained by measuring the difference in minutes between the onset of activity on the first day of DD and the mean activity onset on the last 4 days of LD. The duration of nocturnal α was calculated using the point on the average waveform at which activity rose above 50 wheel counts per 10 min and the point at which activity fell below that cutoff. For animals that required a lower threshold, the same criterion was used for both onset and offset of α duration. Mean differences across conditions were evaluated statistically where necessary by the Wilcoxon test for paired comparisons. Means are reported as $\pm$ SD.

3. Experiment 1

3.1. Methods

Mice ($N = 6$) in this initial experiment were subjected to a series of lighting and feeding schedules to explore the response of their circadian system to photic and feeding zeitgebers alone and in combination. The sequence of conditions is illustrated in the actograms of Fig. 1. The mice were first recorded in a 12:12 LD cycle, and 6 h after dark onset on day 3, food pellets were removed to begin restricted feeding. During restricted feeding, food was provided each day 5 h prior to dark onset. To facilitate adaptation to time-

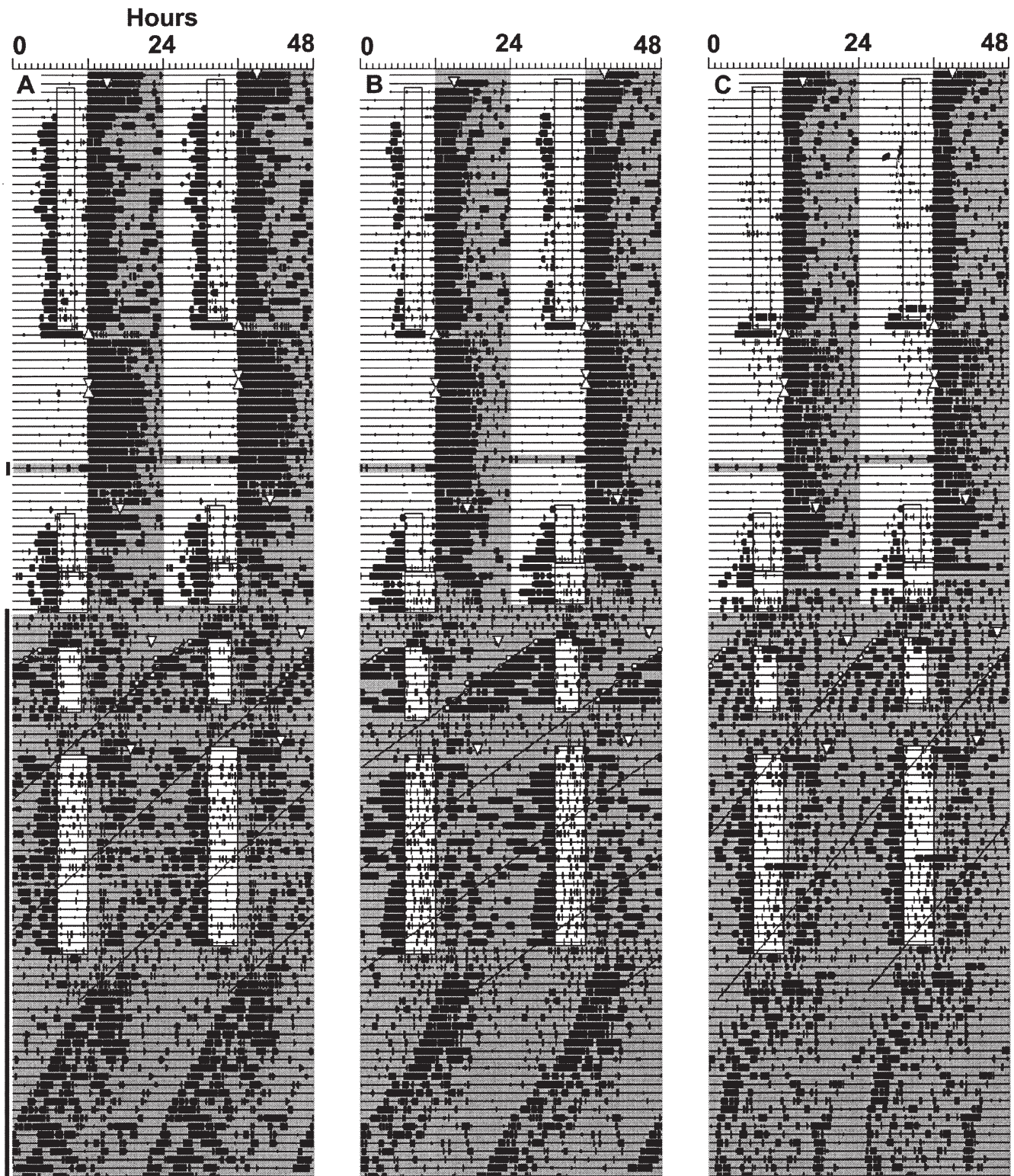


Fig. 1. Double-plotted actograms of wheel-running activity of three mice recorded during LD (12:12) and DD with periods of restricted and ad libitum feeding. Each line represents 2 consecutive days, plotted left to right. Consecutive days are also aligned vertically. Time bins during which activity occurred are denoted by vertical deflections. Shading represents periods of darkness, and the vertical boxes represent meal time during the restricted feeding schedules. These boxes remain unshaded during periods of DD to facilitate visibility, not to represent a lighting schedule. Black vertical bars to the left of the actograms indicate days when the mice were in DD. Inverted triangles mark times when food was removed, and upright triangles mark the completion of a food deprivation test. The regression line fit to activity onsets in DD (represented by opaque circles) is extrapolated forward to illustrate the difference between predicted and actual phase of the free run after the last restricted feeding schedule. Note that on the first 2 days of the first restricted feeding schedule, food was provided for 11 and 7 h, respectively, before being fixed at 3 h.

limited food access, meal duration was decreased from 11 h to 7 h to 3 h over successive days and then was fixed at 3 h for 27 days. To minimize stress, the mice were not weighed at regular intervals. On day 21, the caloric density of the food was reduced by replacing the oil chow with regular chow pellets. The pellets were available in hanging metal food bins connected to a circuit that produced a pulse with each contact. Mice were weighed at this time and again 4 days later. On the last day of restricted feeding, food was withheld until dark onset, after which the animals were provided with food ad libitum for 5 days. To determine if FAA would reappear at the prior scheduled feeding time if the mice were hungry, the mice were then deprived of food for 24 h starting at dark onset. After another 9 days of ad libitum feeding, lights were turned off for 24 h, beginning at the usual time of dark onset, to obtain an unmasked measure of the phase of the light-entrained activity rhythm. LD was resumed for another 5 days. Restricted feeding was then reinstated as above for 7 days. On day 8, meal duration was extended to 5 h when 3 mice exhibited weight loss. This schedule continued for 5 additional days. On the last day of restricted feeding, the lights were again turned off at the usual time of dark onset, and the animals remained in DD for the rest of the experiment. Food was provided ad libitum for 4 days to permit an assessment of the phase of the activity rhythm without masking from the LD and feeding schedules. Restricted feeding was then reinstated for 9 days, followed by ad libitum feeding for 4 days, again to assess phase. Restricted feeding resumed for 24 more days, using a 5-h meal duration. All animals had ad libitum food access in DD for a final 28 days.

3.2. Results and discussion

3.2.1. Wheel-running rhythms during food restriction and refeeding in LD

Wheel-running activity was almost exclusively nocturnal in LD prior to food restriction. When food availability was limited to 3 h, beginning 5 h before dark onset, all mice showed some evidence of diurnal wheel running in anticipation of feeding time. In five of six mice, FAA was evident by day 5 of food restriction (3.8 ± 0.8 days; e.g., Fig. 1a,b). One mouse exhibited significant FAA only during the last 3 days of the schedule (Fig. 1c). The duration and magnitude (percentage of total daily wheel revolutions) of FAA was variable between and, in some cases, within animals (Fig. 1a–c). Across animals, FAA duration ranged from 80–350 min (193 ± 100 min), and FAA magnitude ranged from 8–34.6% of total daily activity ($22.5 \pm 11.6\%$). In three of the mice (e.g., Fig. 1C), FAA was most robust during the last 5 days of food restriction, after the caloric density of the food was reduced. Body weights declined by $4.9 \pm 3.8\%$ on this normocaloric diet.

Concomitant with the emergence of diurnal FAA, the duration of the main bout of nocturnal wheel running, as defined in the methods, was reduced in five of six mice, from

546 ± 77 min prior to restriction to 385 ± 104 min during the last 5 days of food restriction ($z = 2.20$, $p = 0.027$; Fig. 1a–c, Fig. 2a,b). Visual inspection of the actograms revealed that contraction of this primary bout of activity was accomplished by a series of small shifts of the end of activity that were reminiscent of advancing transients; these shifts were completed in 6.5 ± 1.5 days.

Transient cycles may, in part, reflect our procedure of restricting the duration of food availability in steps over the first 3 days of the feeding schedule. However, as noted, nocturnal α duration did not stabilize, on average, for 6 days. An alternative interpretation of transient cycles is that day-time restricted feeding caused a phase advance of the LEP. This raises the possibility that FAA in these animals represents the onset of the daily active phase driven by the LEP, rather than the output of a separate FEP. If so, then one might predict that the duration of α , if measured from the onset of FAA to the end of the main bout of nocturnal activ-

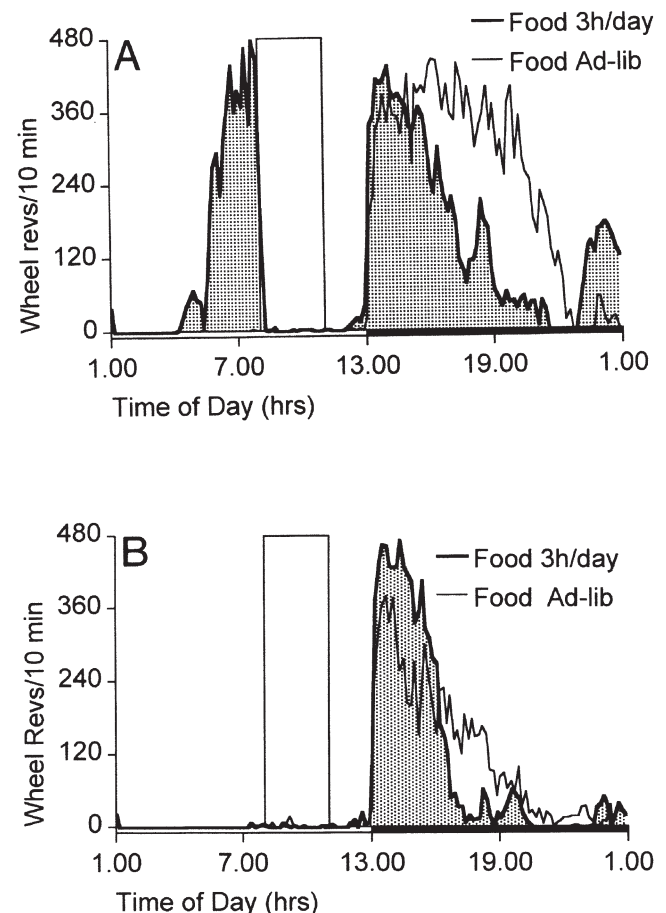


Fig. 2. Waveforms of wheel-running activity from representative mice in experiment 1. The bold line enclosing the shaded area represents the waveform of activity average over the last 10 days of restricted feeding, whereas the thin line represents activity averaged during ad libitum feeding. The vertical columns represent meal time during the restricted feeding schedule. The heavy bar along the abscissa indicates lights off. (A) A mouse exhibiting robust food anticipatory activity; corresponds to Fig. 1a. (B) A mouse exhibiting little food anticipatory activity; corresponds to Fig. 1c.

ity, would be similar to nocturnal α during ad libitum feeding and that there would be expansion of nocturnal α with delaying transients when ad libitum feeding was resumed. Only one of these predictions was correct. When α was measured from FAA onset, it was much longer (875 ± 194 min) during restricted feeding than during baseline ($z = 2.20$, $p = 0.028$ vs. ad libitum feeding prior to or following the feeding schedule). However, during the first week of recovery feeding, the main bout of nocturnal activity did gradually expand to 673 ± 183 min. In four of six mice, this was associated with delaying transients of the end of α for 6.25 ± 0.5 days (e.g., Fig. 1a–c).

When the animals were deprived of food for 24 h, little or no activity was apparent during the previous diurnal feeding time (e.g., Fig. 1a–c). The same was true when the lights were turned off for 24 h. In five of six mice, activity began within ~ 60 min of the usual time of dark onset, suggesting that the LEP was stably synchronized to LD after the apparent transient cycles were complete. One mouse showed several bouts of activity at intervals during the usual inactive phase, rendering any assessment of phase equivocal.

The advancing and delaying transients suggest that FAA might be mediated by a prominent (~ 8 h) advance of the phase of the LEP induced by daytime feeding. However, other features of the data detract from that interpretation. First, as noted, the main bout of α , if calculated to include FAA, was significantly expanded, rather than preserved, during restricted feeding. Second, during the feeding schedule, a minor bout of activity was evident on many days at the end of the dark period, in apparent anticipation of light onset (e.g., Figs. 1, 2). If this bout is taken as the end of nocturnal α , then no phase advance of the LEP is indicated. In addition, when feeding time was delayed by 6 h on the last day of food restriction, FAA was extended through the usual feeding time, but in all cases, it terminated 1–2 h before dark onset (e.g., Fig. 1). If FAA was the leading edge of LEP α , then one might expect activity to continue well beyond the usual feeding period.

During the second restricted feeding schedule in LD, FAA was evident in all six mice within 3 days, which was, on average, significantly earlier than during the first feeding schedule (1.66 ± 0.8 days, $p < 0.001$). FAA duration ranged from 170–320 min (246 ± 62 min), and FAA magnitude ranged from 25–52% of total daily activity ($35 \pm 12\%$). Advancing transients of the end of the main bout of nocturnal activity were also evident in some cases during the first 7 days when food was available for 3 h. However, nocturnal activity was more erratic and, in some cases, exhibited delaying transients when feeding time was extended to 5 h, ending at dark onset (e.g., Fig. 1b). Three mice were retired from the experiment at this point because of apparent weight loss.

During the first day of DD, with food available ad libitum, the main bout of activity in the remaining 3 mice began 196 ± 92 min before the usual time of dark onset (Fig. 1a–

c). This was on average 320 ± 126 min later than the onset of FAA during restricted feeding but 130 ± 100 min earlier than activity onset during the 24-h DD test prior to the second food restriction schedule. This strengthens the evidence, based on the pattern of transients, that the light-entrained activity rhythm was advanced in phase by diurnal food restriction. In addition, the low level of wheel-running activity at the usual time of FAA on the first day of ad libitum food access in DD suggests that this bout of activity is highly state (i.e., hunger) dependent.

3.2.2. Wheel-running rhythms during food restriction in DD

The three mice recorded in DD for 72 days displayed strong FAA when food was restricted to 5 h/day. FAA duration and magnitude in DD appeared equal to or greater than FAA during LD, in part because of superposition of FAA with activity previously occurring in the dark period. Initially, FAA and a free-running activity component appeared concurrently (Fig. 1). Following extended time on the feeding schedule, the free-running component became more difficult to track. A regression line was fitted to the free-running activity component during the first week of restricted feeding (Fig. 1) and was extrapolated forward to provide an estimation of the free-run phase assuming no entrainment of the LEP. The phase of the free-run that emerged following the last day of restricted feeding was very similar in all three mice, and in all cases, it differed by at least 4 h from the prediction derived via extrapolation. In addition, the τ was longer after food restriction. These data suggest that at least under some conditions, the LEP in BALB/c mice can be entrained by a daily feeding schedule.

3.2.3. Food bin activity during and after restricted feeding in DD

Food-bin activity was also recorded in three mice during food restriction and ad libitum food access in DD. Inspection of the actograms (Fig. 3a–c) and average waveforms (Fig. 3d–f) revealed that during the first week of ad libitum feeding, following the last feeding schedule, the mice exhibited a concentration of food bin activity during the hours previously designated as mealtime. Activity during this 5-h block, averaged over the first 5 days of ad libitum food access, accounted for 24.9–29.6% ($27.0 \pm 2.4\%$) of total daily food bin activity. If the 2 h preceding this were included (the hours when FAA was maximal during food restriction), the magnitude increased to 31–40% ($34.2 \pm 5.1\%$). Wheel-running activity was generally low at this time. These data suggest that there is a persisting rhythm of feeding-related activity, with a peak at the prior mealtime, that persists for at least 5 days following a time-limited feeding schedule.

4. Experiment 2

In Experiment 1, LD-entrained activity rhythms exhibited several characteristics suggestive of a phase advance during restricted feeding. Two of the most salient features were the apparent phase advance of the end of the main bout

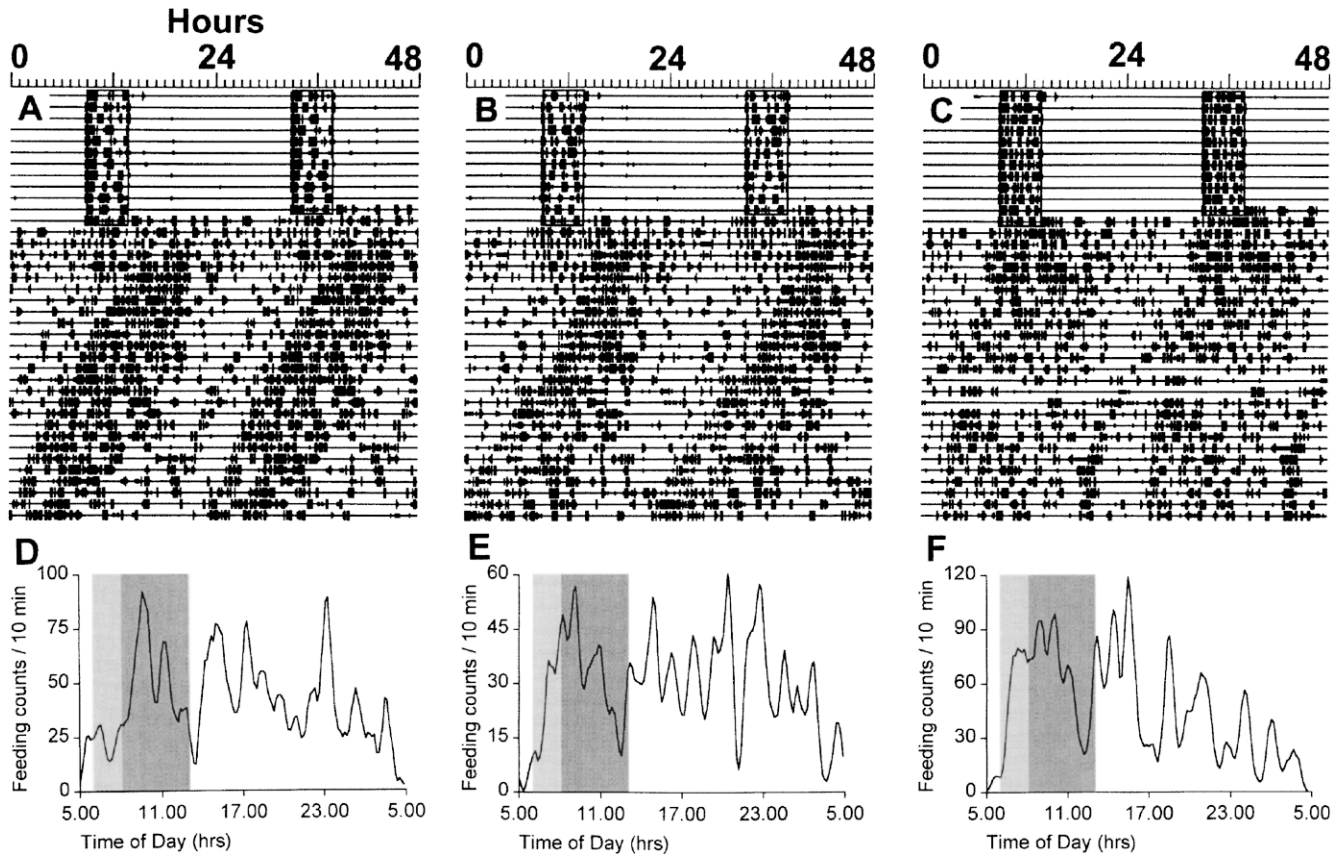


Fig. 3. Actograms (A, B, and C) and corresponding average waveforms (D, E, and F) of food-bin activity during the last 12 days of restricted feeding (denoted by the vertical column) in DD, and subsequent ad libitum food access (Experiment 1). The waveforms represent activity averaged over the first 5 days of ad libitum food access. Dark shading represents the 5-h period equivalent to the meal time during food restriction. Lighter shading represents the 2-h period prior to meal time that corresponded to the time of robust FAA during food restriction.

of nocturnal activity and the advanced phase from which rhythms free-ran on the first day of DD immediately after food restriction in LD. However, the mice were recorded in LD for only 2 days prior to restricted feeding, and only three mice were included in the DD phase assessment. Although all six mice appeared to be stably entrained to LD prior to food restriction (i.e., wheel activity was strictly nocturnal), a replication study was conducted to confirm that restricted daytime feeding can advance nocturnal activity in phase in mice that have been recorded in LD for a more extended period. This also provided the opportunity to repeat the DD phase assessment test on a larger sample.

4.1. Methods

Mice ($N = 8$) were housed singly in plastic cages with-out running wheels for at least 24 days in a 12:12 LD cycle. They were then transferred to wheel cages at the time of dark onset, and LD entrainment was assessed for at least 14 days. Food was removed, and a daytime restricted-feeding schedule was initiated, using the graduated meal duration procedure described for Experiment 1, with meal onset beginning 5 h before dark onset. To assess whether phase changes are dependent on wheel running in anticipation of

food, two mice were not provided with running wheels, and contact drinkometers were used to measure activity phase and duration. All animals were sacrificed after 11 days, when all had demonstrated stable adjustment to the feeding schedule (i.e., no more transients were evident).

A second group of mice ($N = 10$) were entrained to a 12:12 LD cycle for 33 days, at which time the lights were turned off for a 48-h DD test to assess the phase angle of LD entrainment without the masking effects of light. Following another 23 days in LD, mice were exposed to a 24-h DD test. Following 3 more days of LD, restricted feeding was initiated. The feeding schedule was maintained for 11 days. The lights were then turned off at the usual time of dark onset and remained off for 18 days, during which the mice had ad libitum access to food.

4.3. Results and Discussion

Among the first group of mice, seven of eight exhibited FAA within 2–4 days of restricted daytime feeding. FAA was quite robust in the wheel-running measure (mean magnitude = $45 \pm 20\%$, duration = 255 ± 94 min, $N = 6$; Fig. 4a) but was of much lower magnitude in the drinking measure in one animal (magnitude = 4.7%, duration = 50 min;

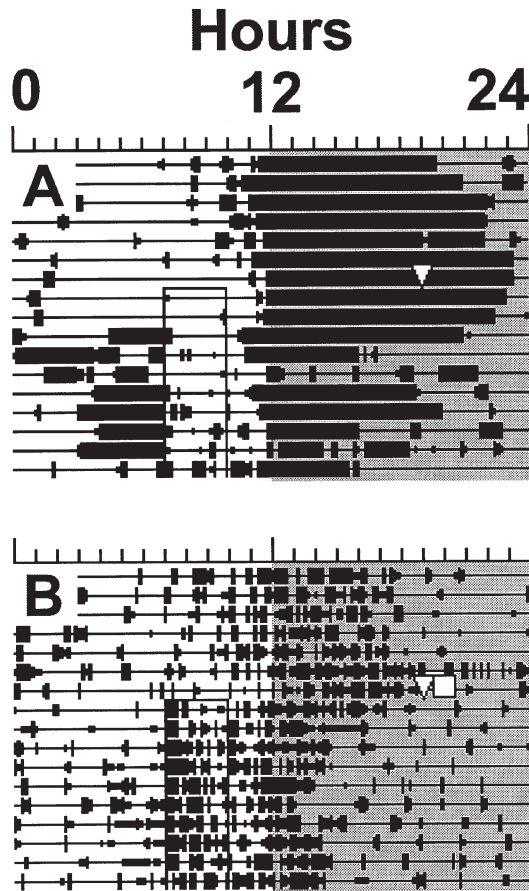


Fig. 4. Single plotted actograms from two representative mice in LD (shading represents lights off) from Experiment 2. Plotting conventions are as in Fig. 1. (A) Wheel running of a mouse with free access to a running wheel. (B) Drinking activity of a mouse with its wheel locked during food restriction. The square indicates time of wheel lock, and the inverted triangle indicates the time that food was removed.

Fig. 4b), and was absent in the other animal. This is consistent with studies showing that there is relatively little anticipatory drinking prior to scheduled feeding in rats [20]. The main bout of nocturnal activity was shorter during restricted feeding, compared with the case with ad libitum feeding, in six of eight cases ($-18\% \pm 17\%$, $z = -2.10$, $p < 0.04$, $n = 8$). Advancing transients were evident in some cases but were not clear in others. Both of the mice for which drinking activity was recorded also showed a clear contraction of α during food restriction (Fig. 4b).

With the exception of one animal, the second group of mice ($n = 10$) also exhibited robust FAA within the first few days of restricted feeding (Fig. 5). Among animals that expressed FAA, the duration ranged from 50–300 min (166 ± 83 min), and the magnitude ranged from 4.1%–61.4% of total daily activity ($20 \pm 20\%$). The mean duration of the main bout of nocturnal activity was shorter during the last 5 days of restricted feeding in 8 of 10 animals ($-5 \pm 18\%$, $z = -2.19$, $p < 0.03$). Advancing transients of the end of this activity bout were again evident in some mice (e.g., Fig. 5a).

During the two DD tests prior to food restriction, the assessment of phase of activity onset was problematic for several animals. In 4 cases, large bouts of activity were evident in the middle of the subjective day (the hours corresponding to the light portion of the former LD cycle) during both tests (e.g., Fig. 5c). In 3 cases, large bouts were evident during one of the two DD tests (e.g., Fig. 5b). In the other three cases, activity onset occurred within ~ 90 min of the usual time of nocturnal activity onset on both DD tests (e.g., Fig. 5a). For comparison with DD after restricted feeding, the phase of activity onset was defined as the first bout of activity in DD that continued into what was formerly the lights-off period. Using this criterion, 9 of 10 mice (Fig. 5c the exception) exhibited activity onset on at least one DD test that was within ~ 90 min of the onset of nocturnal activity during LD. On the first day of DD, with ad libitum food access, after restricted feeding in LD, activity onset occurred on average 314 ± 82 min in advance of the usual time of lights off. This was 210 ± 81 min in advance of activity onset during the DD tests prior to food restriction ($z = 2.80$, $p < 0.005$).

5. Experiment 3

The results of Experiment 2 bolster the evidence obtained from Experiment 1 that diurnal feeding induces FAA and advances the apparent phase of LD entrainment. The results of Experiment 1 also indicated that FAA and a free-running rhythm can be expressed simultaneously but that the free-running rhythm may eventually entrain to the feeding schedule. However, that experiment did not include an assessment of the stability of τ in DD with food provided ad libitum. Therefore, Experiment 3 was conducted to permit a comparison of the stability of τ in DD during food restriction and food ad libitum.

5.1. Methods

Five mice from Experiment 2 and five mice matched for age and lighting history but naive to restricted feeding were housed in LD 12:12 for 22 days and then in DD. On day 23 of DD, the mice were subjected to restricted feeding for 15 days, as described for Experiment 2. Food was then provided ad libitum for the rest of the experiment. After 28 days, the mice were re-entrained to LD 12:12 for 19 days and then released again into DD for 73 days.

5.2. Results and Discussion

5.2.1. Activity onset on the first day of DD following ad libitum feeding in LD

On the first day of DD, after entrainment to LD with food available ad libitum, wheel-running rhythms free ran from a phase of activity onset that was 97 ± 38 min in advance of dark onset. This is similar to the mean phase of activity onset recorded on 1 or 2 days of DD during ad libitum feeding in Experiment 2, and it is 217 min later than the

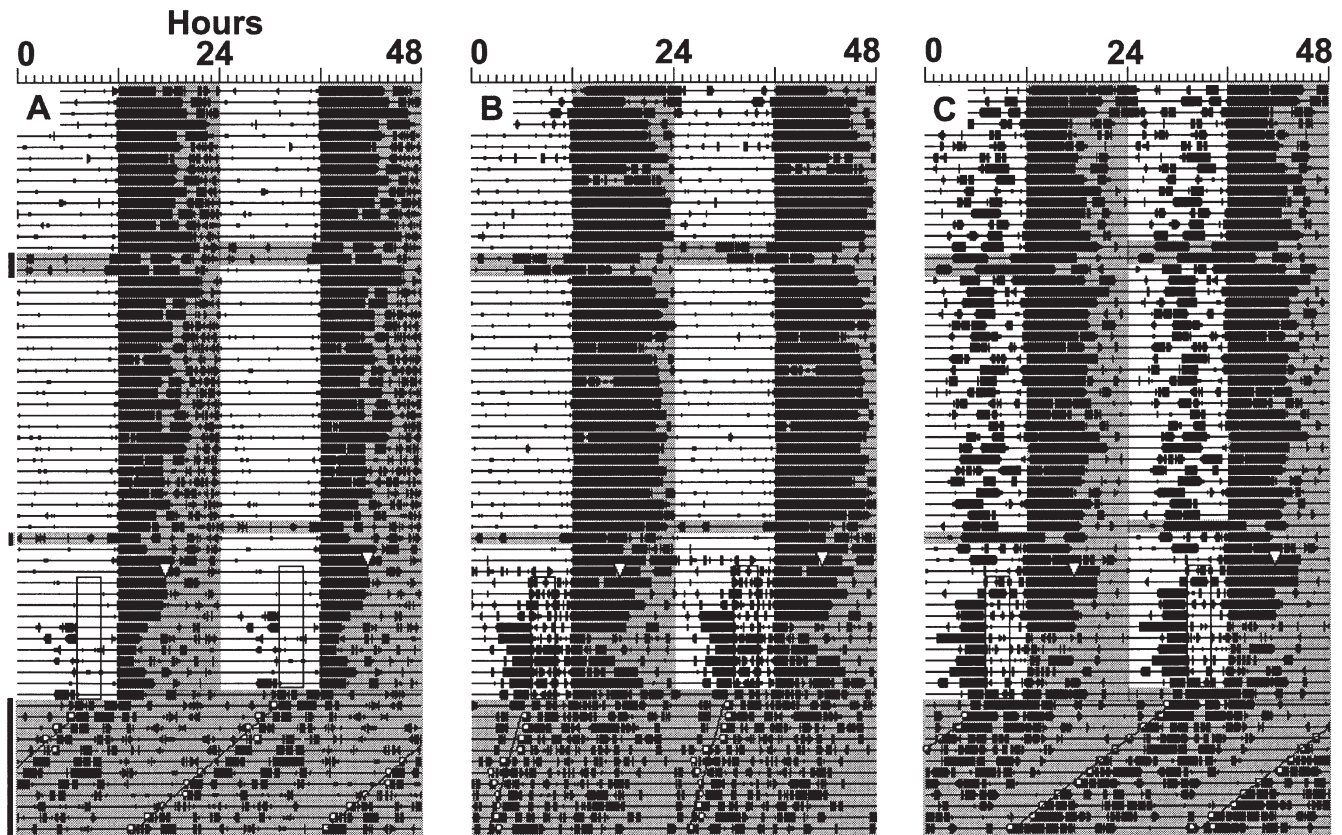


Fig. 5. Double-plotted actograms of wheel-running activity of three representative mice during LD (12:12) and DD from Experiment 3. Plotting conventions as in Fig. 1.

mean phase of activity onset of the first day of DD following daytime restricted feeding in Experiment 2 ($p < 0.01$).

5.2.2. Wheel-running rhythms during food restriction in DD

Free-running τ did not differ between mice naive to food restriction and mice previously food restricted, so results are presented only for pooled data. During the first 3 weeks in DD, all of the mice free-ran with $\tau < 24$ h (days 7–21 mean = 22.77 ± 0.59 h; Fig. 6). During the 15 days of food restriction, there was a clear lengthening of τ in 7 of 9 cases (data for one animal was lost because of switch failure; mean $\tau = 23.38 \pm 0.42$ h, $z = -2.38$, $p = 0.017$; Fig. 7a,b). However, in all cases, τ was estimated to remain < 24 h, indicating that free-running rhythms did not stably entrain to the restricted feeding schedule within 15 days in any animal. When ad libitum feeding was resumed, seven of nine animals exhibited a shortening of τ , although group mean τ remained longer than prior to food restriction (23.27 ± 0.14 h, Fig. 6, 7a,b).

During the first 3 weeks of the second DD test, following reentrainment to LD, all mice again exhibited $\tau < 24$ h. Although there was a nonsignificant group trend for longer τ over the 72 days of DD, τ did not lengthen precipitously during weeks 4–5, which correspond to the weeks of restricted feeding during the first DD test (Fig. 6). Consistent with earlier studies of BALB/c mice [23], apparently spon-

taneous phase or τ changes were common during ad-lib food access (e.g., last 3 weeks of Figs. 7a,b).

During restricted feeding, all of the mice exhibited wheel running in anticipation of mealtime. In some cases this was not readily distinguishable from the free-running bout of activity, but in other cases, a 24-h rhythm of FAA was evident concurrent with a free-running component of activity (Fig. 7).

6. General discussion

6.1. Food anticipation in the BALB/c mouse

The studies reported here show that the BALB/c mouse is a good model for physiological and molecular genetic studies of entrainment by feeding schedules in murid rodents. Most BALB/c mice adapted well to restricted feeding and remained healthy and active when food was restricted to as little as 3 h per day. In three separate experiments involving a total of 29 mice, FAA was evident on at least some days of restricted feeding in all mice recorded in running wheels and in one of two mice in which only drinking was measured. By comparison with C57BL/6j mice studied in our lab, we were struck by the rapid induction, high magnitude, and stability of diurnal FAA in many of the BALB/c mice. This may be related to weak coupling among oscillators of the circadian system in these animals, which might

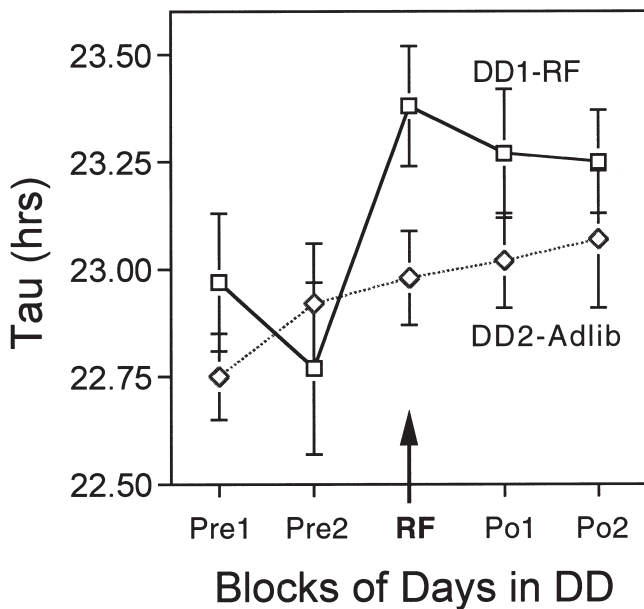


Fig. 6. Average ($\pm$ SD) circadian period in 14-day blocks of time during DD with food restriction (DD1-RF) or ad libitum food availability (DD2-Adlib) in mice from Experiment 2. Pre1 and Pre2 correspond to weeks 1–2 and 2–3, prior to restricted feeding. RF corresponds to the last 14 days of food restriction. Po1 and Po2 correspond to the first 14 and last 14 days after restricted feeding.

permit a more ready dissociation of food- and light-entrainable pacemakers when mice are exposed to feeding and photic zeitgebers simultaneously. Although we have no direct evidence of this, the rapid disorganization or phase lability of free-running rhythms in many BALB/c mice in DD is consistent with a hypothesis of weak coupling among oscillators comprising their circadian system (unpublished observations, see also [22]). Circadian organization in DD is generally much more stable in the C57BL/6j strain (e.g., [15–17,23]).

Although the duration of FAA showed low day-to-day variability in some animals, it was quite variable in other cases. Variability may be related to the caloric density of the diet and body weight. When pellet chow was substituted for oil chow in Experiment 1, mice that were only sporadically active in anticipation of feeding time became more active. This was associated with a modest reduction of body weight ($\sim 5\%$), suggesting that FAA in this mouse strain may be strongly gated by a factor related to energy reserves. Careful control of caloric intake and body weight during restricted feeding may reduce group variability in FAA expression (see also [19]).

In rats, Syrian hamsters, and the C57BL/6j mouse, lesion studies have established that the LEP located within the SCN is not necessary for the expression of FAA during scheduled feeding [3,8,16,24,25]. There are several reasons to believe that FAA in the BALB/c mouse is likely also not dependent on the LEP. First, although nocturnal activity appeared to be phase advanced during restricted feeding, this advance was, by one estimate of phase (the end of the main

bout of nocturnal activity), ~ 5 h too small to account for the timing of FAA (unless it is assumed that α of the LEP was expanded by that amount during food restriction). Second, on the first day of DD following restricted feeding in LD, activity free-ran from a phase that was 2–3 h delayed compared with the phase of FAA onset during food restriction. Finally, during the first week of restricted feeding in DD, a free-running activity component with $\tau < 24$ h was expressed concurrently with a FAA component with $\tau \sim 24$ h. Although these results are consistent with the two pacemaker (FEP: LEP) model of the rodent circadian system, SCN lesion studies are nonetheless necessary to confirm the existence of an FEP outside of the SCN in the BALB/c mouse.

One interesting characteristic of FAA in rats is that its expression is gated by some physiological consequence of the availability of food. When food is provided ad libitum, FAA usually dissipates within a few days, if not within one circadian cycle (e.g., [3–5,25–28]). However, this apparent rapid damping appears to be more analogous to masking, because when rats are subsequently food deprived days or even weeks later, FAA reappears (i.e., is unmasked) at approximately the previous scheduled mealtime [26,28]. This persistence of FAA in the absence of a feeding zeitgeber (i.e., covertly during ad libitum food access and overtly during food deprivation) is compelling evidence that FAA is regulated by a self-sustaining pacemaker.

To obtain similar evidence for persisting FAA in the BALB/c mouse, six mice in Experiment 1 were food deprived for 24 h, beginning at dark onset, 1 week after the first restricted feeding schedule was completed. However, none of the mice exhibited significant activity at the prior feeding time. This may be because the hypothetical FEP in BALB/c mice is more labile in phase during ad-lib feeding, by comparison with the rat FEP, or because the food deprivation was not sufficiently long to unmask a persisting FEP rhythm (mice do not tolerate extended food deprivation). Evidence in favor of the latter explanation is that during the second feeding schedule in Experiment 1, FAA emerged in significantly fewer days than during the first feeding schedule, suggesting that there was some preservation of the phase of the FEP during ad-lib feeding. In addition, during the first 4–6 days of ad libitum food access following the last feeding schedule in Experiment 1, there was a high concentration of feeding activity at the previous scheduled mealtime. In one case, this bout of food-bin activity appeared to persist in parallel with the main bout of activity for the remaining weeks in DD. Similar evidence for long-term persistence and for coupling of FEP activity to the LEP during ad libitum feeding in C57BL/6j mice has also been recently reported [19].

6.2. Modulation of LEP phase during restricted feeding in LD

During restricted feeding in LD, the end of the main bout of nocturnal wheel-running activity appeared to be phase advanced. On the first day of DD, the onset of this activity

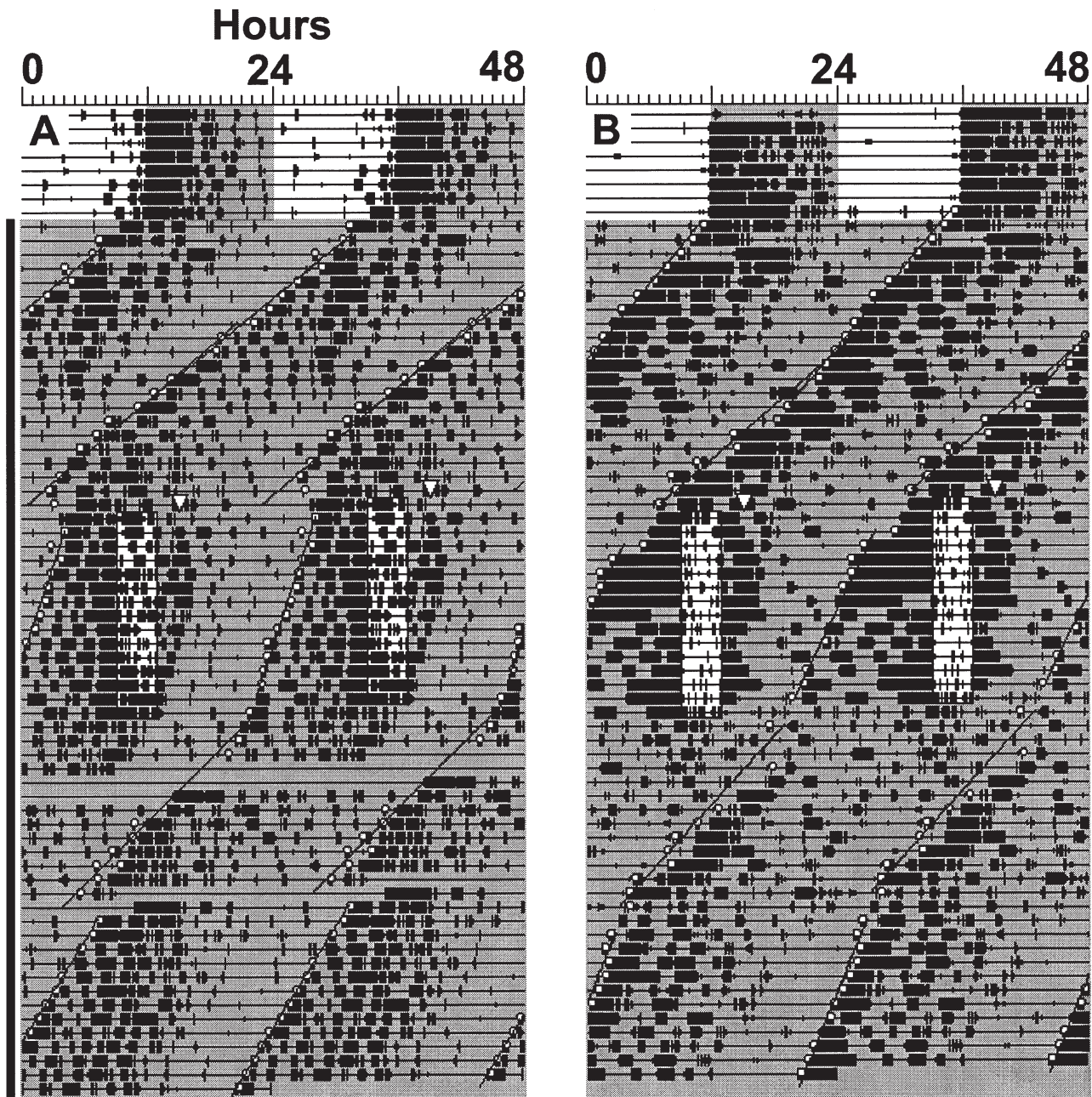


Fig. 7. Double-plotted actograms of the wheel-running activity of two mice, recorded during LD (12:12) and DD, with food restricted to 4 h/day (in DD, the unshaded vertical column denotes mealtime, not light). Format is the same as Fig. 1. Regression lines were fit to the onsets of daily free-running activity to estimate circadian period.

bout was also revealed to be advanced. While this study was in progress, similar evidence for a phase advance of nocturnal activity during restricted daytime feeding was reported for the C57BL/6j mouse [19]. Earlier studies of rats [27,29], hamsters [30], and some marsupials (e.g., [10,31]) have noted similar effects.

There are several possible mechanisms by which feeding schedules might alter the phase angle of LD-entrained activity rhythms. Previous studies have shown that mice are readily entrained by a daily bout of forced or voluntary run-

ning [2,15]. This raises the possibility that the LEP is phase advanced by the wheel running or arousal that is associated with food anticipation and feeding each day. Although arousal cannot be ruled out, anticipatory wheel running does not appear to be necessary because phase advances of nocturnal activity were evident in mice that ran little prior to mealtime or that did not have access to a running wheel. A similar conclusion was reached with respect to phase advances in food-restricted C57BL/6j mice [19].

A second possible mechanism by which the LEP might

be phase advanced during restricted feeding is by alteration of its sensitivity to photic input. Evidence that changes in glucose metabolism can alter the phase response curve to light has been reported recently; in the C57BL/6j mouse, phase delays and advances to light pulses were attenuated by glucoprivation induced by injections of 2DG or insulin or by 30 h of food deprivation [11,12]. To induce an advance of the LD-entrained phase, the phase-delaying action of evening light would have to be attenuated or the phase-advancing action of morning light would have to be accentuated. However, our animals ingested a large quantity of food within 2 h of dark onset each day. The animals were thus likely to be hyperglycemic or normoglycemic during the evening light exposure and to be hypoglycemic during the morning light exposure, 12–14 h later. This pattern of glucose variation should, if anything, accentuate phase delays and attenuate phase advances of the LEP, resulting in a net delay of the LD-entrained phase. It therefore seems unlikely that altered glucose metabolism was responsible for the modulations of the phase of nocturnal activity during restricted feeding in LD.

6.3. Entrainment of the LEP by restricted feeding in DD

A third mechanism by which food restriction might alter the phase angle of photic entrainment is by a zeitgeber effect of timed daily feeding on the LEP. This could be due to a direct effect of some correlate of food intake on the LEP, or due to coupling forces from a separate FEP (e.g., [5–7]). To test this hypothesis, we subjected a total of 13 mice to restricted feeding in DD. The first three mice subjected to restricted feeding for 24 days in DD exhibited apparent synchrony to the feeding schedule, whereas most of the second group of 10 mice exhibited modulations of τ consistent with the actions of a zeitgeber. It is likely that free-running activity rhythms in at least some of these latter mice would eventually have been entrained by more extended exposure to the feeding cycle.

In the three mice of Experiment 1 that appeared to entrain to scheduled feeding in DD, activity on the first day of ad libitum food access began 2–5 h after the onset of FAA on the previous day. Assuming that this represents the phase angle at which the LEP entrained to the feeding schedule, and given that $\tau < 24$ h in DD, this suggests that the LEP is phase delayed by food intake during the mid-to-late portion of the rest phase (subjective day) of the cycle. From this, we can predict that a 3–5 h meal, after 24 h of food deprivation, should induce a phase delay of free-running rhythms if scheduled late in the mouse's rest phase. Paradoxically, this is the approximate phase at which feeding was scheduled during LD, when the LEP appeared to be phase advanced by restricted feeding. This suggests that there is a transition in the phase response curve from advances to delays that occurs sometime late in the rest phase of the LEP. It is not inconceivable that the shape of the phase response curve to scheduled feeding in LD is altered slightly when the LEP is

released from photic entrainment. From this and other work in rats, mice, and hamsters, we are only beginning to appreciate the complexity of the behavioral outcomes that are possible when animals are exposed to photic and nonphotic zeitgebers in combination.

Acknowledgments

Supported by operating grants (R.E.M.) and by a Research Scholarship (M.M.H.) from the Natural Science and Engineering Research Council, Canada.

References

- [1] Klein DC, Moore RY, Reppert SM, editors. *Suprachiasmatic Nucleus: the Mind's Clock*. New York: Oxford University Press Inc., 1991.
- [2] Edgar DM, Dement WC. Regularly scheduled voluntary exercise synchronizes the mouse circadian clock. *Am J Physiol* 1992;261:R928–33.
- [3] Mistlberger RE. Circadian food-anticipatory activity: Formal models and physiological mechanisms. *Neurosci Biobehav Rev* 1994;18:171–95.
- [4] Boulos Z, Rosenwasser AM, Terman M. Feeding schedules and the circadian organization of behaviour in the rat. *Behav Brain Res* 1980;1:39–65.
- [5] Stephan FK. The role of period and phase in interactions between feeding and light-entrainable circadian rhythms. *Physiol Behav* 1986;36:151–8.
- [6] Stephan FK. Coupling between feeding- and light-entrainable circadian pacemakers in the rat. *Physiol Behav* 1986;38:537–44.
- [7] Stephan FK. Interaction between light and feeding-entrainable circadian rhythms in the rat. *Physiol Behav* 1986;38:127–33.
- [8] Abe H, Rusak B. Anticipatory activity and entrainment of circadian rhythms in Syrian hamsters exposed to restricted palatable diets. *Am J Physiol* 1992;263:R116–24.
- [9] Mistlberger RE. Effects of scheduled food and water access on circadian rhythms of hamsters in constant light, dark, and light-dark. *Physiol Behav* 1993;53:509–16.
- [10] Kennedy GA, Coleman DJ, Armstrong SM. Restricted feeding entrains circadian wheel-running activity rhythms of the kowari. *Am J Physiol* 1991;261:R819–27.
- [11] Challet E, Bernard DJ, Turek FW. Gold-thioglucose-induced hypothalamic lesions inhibit metabolic modulation of light-induced circadian phase shifts in mice. *Brain Res* 1999;824:18–27.
- [12] Challet E, Losee-Olsen S, Turek FW. Reduced glucose availability attenuates circadian responses to light in mice. *Am J Physiol* 1999;276:R1063–70.
- [13] Mistlberger RE, Antle MC. Behavioral inhibition of light-induced circadian phase resetting is phase and serotonin dependent. *Brain Res* 1998;786:31–8.
- [14] Abe H, Kida M, Tsuji K, Mano T. Feeding cycles entrain circadian rhythms of locomotor activity in CS mice but not in C57BL/6j mice. *Physiol Behav* 1989;45:397–401.
- [15] Marchant EG, Mistlberger RE. Entrainment and phase shifting of circadian rhythms in mice by forced treadmill running. *Physiol Behav* 1996;60:657–63.
- [16] Marchant EG, Mistlberger RE. Anticipation and entrainment to feeding time in intact and SCN-ablated C57BL/6j mice. *Brain Res* 1997;765:273–82.
- [17] Schwartz WJ, Zimmerman P. Circadian timekeeping in BALB/c and C57BL/6 inbred mouse strains. *J Neurosci* 1990;10:3685–94.
- [18] Challet E, Bernard DJ, Turek FW. Lesions of glucose-responsive

- neurons impair synchronizing effects of calorie restriction in mice. *Brain Res* 1998;801:244–50.
- [19] Challet E, Solberg LC, Turek FW. Entrainment in calorie-restricted mice: conflicting zeitgebers and free-running conditions. *Am J Physiol* 1998;274:R1751–61.
- [20] Akiyama M, Kouzu Y, Takahashi S, Wakamatsu H, Moriya T, Maetani M, Watanabe S, Tei H, Sakaki Y, Shibata S. Inhibition of light- or glutamate-induced mPer1 expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms. *J Neurosci* 1999;19:1115–21.
- [21] Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, Sakaki Y. Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. *Nature* 1997;389:512–16.
- [22] Rosenwasser AM. Circadian activity rhythms in BALB/c mice: a weakly-coupled circadian system? *J Interdiscipl Cycle Res* 1990;21:91–96.
- [23] Pittendrigh CS, Daan S. A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. *J Comp Physiol* 1976;106:333–55.
- [24] Mistlberger RE. Nonphotic entrainment of circadian activity rhythms in suprachiasmatic nuclei ablated hamsters. *Behav Neurosci* 1992;102:192–202.
- [25] Stephan FK, Swann JM, Sisk CL. Anticipation of 24-hr feeding schedules in rats with lesions of the suprachiasmatic nucleus. *Behav Neural Biol* 1979;25:346–63.
- [26] Coleman GJ, Harper S, Clarke JD, Armstrong S. Evidence for a separate meal-associated oscillator in the rat. *Physiol Behav* 1982;29:107–15.
- [27] Mistlberger RE, Marchant EG. Computational and entrainment models of circadian food anticipatory activity: evidence from non-24-hr feeding schedules. *Behav Neurosci* 1995;109:790–98.
- [28] Rosenwasser AM, Pelchat RJ, Adler NT. Memory for feeding time: possible dependence on coupled circadian oscillators. *Physiol Behav* 1984;32:25–30.
- [29] Challet E, Pevet P, Vivien-Roels B, Malan A. Phase-advanced daily rhythms of melatonin, body temperature, and locomotor activity in food-restricted rats fed during daytime. *J Biol Rhythms* 1997;12:65–79.
- [30] Mistlberger RE. Scheduled daily exercise or feeding alters the phase of photic entrainment in Syrian hamsters. *Physiol Behav* 1991;50:1257–60.
- [31] Kennedy GA, Coleman DJ, Armstrong SM. The effect of restricted feeding on the wheel-running activity rhythms of the predatory marsupial *Dasyurus viverrinus*. *J Comp Physiol* 1990;166:607–18.