

Chapter 5

**Conflicting light and food Zeitgebers in the
expression of circadian rhythms in the common
vole (*Microtus arvalis*)**

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Submitted

Abstract

Light is the main entraining cue for the central circadian pacemaker in the SCN of the hypothalamus which governs circadian organisation of activity behaviour. Restricting food availability to certain parts of the day does not change entrainment of the SCN in the way light does, but it can affect timing of peripheral clocks and circadian organisation of behaviour. Here we have used two different protocols to investigate the combined effects of light and food availability on circadian organisation of behaviour in the common vole. In the first experiment we show that a 4 h phase advance in light does not lead to a phase shift in the circadian activity rhythm when a simultaneous 8:16 h no food-food cycle is not shifted. This effect is attenuated when voles are given access to a wheel. When food restriction shifts together with the light, the phase of activity readily reentrains to the new light phase. In the second experiment, voles were subjected to simultaneous light-dark cycle and food-fast cycles. This led to two distinct behavioural components. After effects of both light and food availability are seen when voles are released in constant light conditions and ad libitum food availability. The effects of food availability in both phase and period of light-entrained circadian activity show that food availability can be a very potent Zeitgeber, affecting circadian organisation of behaviour in voles.

Introduction

There is evidence that different organs in the mammalian body have their own circadian rhythmicity, and have to some extent different pathways for entrainment. The main circadian system entrains to light dark (LD) cycles. Light is perceived by the eye and is directly transmitted to the master circadian pacemaker in the suprachiasmatic nuclei of the hypothalamus (SCN). The timing of feeding (itself normally in phase with other behaviours that are under the control of the SCN) synchronizes the liver's circadian clock (Damiola *et al.*, 2000, Stokkan *et al.*, 2001), which possibly feeds back on to the SCN. Simultaneously, experimental feeding schedules employed elicit anticipatory activity, that is itself based on an endogenous oscillator (Stephan, 1979), possibly located elsewhere. Little is known on the expression of overt rhythmicity when the light and food Zeitgeber give conflicting information. In this paper we explore such conflicts in the circadian system of the common vole (*Microtus arvalis*).

Krieger *et al.*, (1977) reported circadian rhythms in plasma corticosteroid levels and body temperature in rats that were entrained by a regime of two hours of food per day. Since then, food availability has gained interest as a Zeitgeber for a non-SCN self-sustained oscillator system, which was originally demonstrated by Stephan (1979) in SCN lesioned rats. Typically, when an animal is subjected to a feeding schedules, a rise in locomotor activity is seen prior the restoration of the food availability. This Food Anticipatory Activity (FAA) is the main argument for an oscillator underling food-entrainment (for review see Mistlberger, 1994 and Stephan, 2002), the Food Entrainable Oscillator (FEO).

The concept of a peripheral circadian oscillator, capable of generating sustained rhythms, independent of the SCN, has been applied to many tissues and organs (Balsalobre *et al.*, 1998, Yamazaki *et al.*, 2000). In the liver a molecular mechanism similar to that of the SCN oscillates with a circadian period, phase lagging the SCN by a couple of hours (Lopez-Molina *et al.*, 1997, Yamazaki *et al.*, 2000). When food is restricted to a certain part of the day, the liver shifts its phase accordingly, while the SCN remains coupled to the light (Damiola *et al.*, 2000, Stokkan *et al.*, 2001). Such results indicate the independence and weights of Zeitgebers. When rats are entrained to two daily meals (showing two daily bouts of FAA), the liver rhythm shows entrainment to only a single daily meal. This suggests that the liver is not responsible for the FEO (Davidson *et al.*, 2003). The dorsomedial hypothalamus (DMH) has been suggested to act as an integrator region (Saper *et al.*, 2005) and as a possible site of the FEO. Lesions in the DMH greatly attenuate FAA in a dose responsive manner (Gooley *et al.*, 2006, but see contradictory results in Landry *et al.*, 2006). Also the rhythm in c-Fos expression in the DMH is coupled to food availability (Gooley *et al.*, 2006). Wherever the FEO may reside, it is capable of activating rhythms in the liver by food schedules in the absence of the SCN (Hara *et al.*, 2001).

In the common vole, an herbivorous rodent exhibiting a predominantly ultradian behavioural activity pattern specific questions arise about the impact of Zeitgebers on its behaviour. The vole does not show rhythms of clock genes in the liver under *ad libitum* food availability, but it does so when food availability is restricted to certain

parts of the day (Van der Veen *et al.*, 2006). Under both *ad libitum* and restricted food availability, the SCN has a circadian pattern of core clock gene expression, and is apparently not influenced by temporal food restriction. Differences in the liver rhythms as a result of food restriction thus seem independent of the SCN. In contrast, providing the vole with a running wheel generates strong circadian expression patterns both in activity and in liver clock gene expression, with a stable phase relationship with the SCN.

Although the natural food of voles (grass) does not vary in availability with time of day, experimental food schedules can act as a Zeitgeber for liver rhythms. In the lab both running wheel availability and food restriction enhance the circadian modulation of behaviour. Apparently, there is dual control over behavioural activity in the common vole. This raises the question how light and food restriction modulate the expression of circadian rhythmicity when providing conflicting information. Such conflicts have been investigated before in rats showing only a weak interaction between LEO en FEO (Stephan, 1986). We have approached this question in two different protocols. We first exposed common voles to a phase shift of the light dark cycle, while maintaining the food schedule. This turns out to attenuate the behavioural response to a phase advance, while running wheel availability adds to the weight of the light Zeitgeber. In a second experiment we provided LD cycles and feeding cycles with different Zeitgeber periods. Here the feeding cycle turned out to be the dominating Zeitgeber. Besides the food related circadian activity component, the protocol allowed the identification of a second component with a period intermediate between the periods of the two Zeitgebers.

Material and Methods

Animals

Male common voles (*Microtus arvalis*) born and raised in a breeding colony based on individuals trapped in the Netherlands (53°20'N: 6°16'E) were individually housed in translucent cages (Macrolon type 1; long). Water was available *ad libitum*, and when applicable, running wheels (Ø 14 cm) were made available in the home cage. None of the animals had experienced running wheels at the onset of the experiment. Cages were placed in a sound attenuated, temperature controlled room (22 ± 0.5 °C; 70% humidity; initial LD 12:12, 250-350 lux, depending on cage placement). Following entrainment to the LD 12:12 regime animals were kept entrained to a single light pulse (30 minutes) per 24 h (LD ½ :23½), leading to stable entrainment by phase delays, such that the pulse coincided with the beginning of the subjective night (DeCoursey, 1972, Pittendrigh & Daan, 1976). This single light pulse was chosen to minimize masking of activity by light (Aschoff, 1960, Mrosovsky, 1999). Half of the animals were housed with and half without a running wheel. Food availability and light/dark conditions varied with the experimental protocol. Principles of laboratory animal care (NIH publication No. 86-23, revised 5) were adhered to. All experiments were approved by the Animal Experimentation Committee of the University of Groningen (DEC No. 2809 and 2809-1).

Table 1: Experimental groups in experiment 1

A1	N = 8	FR not phase shifted	Running wheel absent
A2	N = 6	FR not phase shifted	Running wheel present
B1	N = 8	FR phase shifted	Running wheel absent
B2	N = 6	FR phase shifted	Running wheel present
C1	N = 8	No FR	Running wheel absent
C2	N = 6	No FR	Running wheel present

Experiment 1: Reentrainment to a phase advance in light

Experiment 1 was designed to establish whether the behavioural response to a phase advance in the light of 4 h could be affected by the timing information contained in the Zeitgeber food. In addition to the primary question, the effect of running wheel access in this protocol was studied. After an initial period of 10 days entrainment to LD ½:23½, animals were assigned to one of six experimental groups (see table 1), with the provision that adjacent cages belonged to different groups. In experimental groups

Table 2: Experimental groups in experiment 2

D1 N = 8	FR started at activity onset	running wheel absent
D2 N = 8	FR started at activity onset	running wheel present
E1 N = 8	FR started 12 h after activity onset	running wheel absent
E2 N = 8	FR started 12 h after activity onset	running wheel present

A and B, for an episode of 10 days, food was taken away for 8 h directly following the light pulse. This eight hour food restriction (FR) would normally comprise about three feeding bouts of a vole's ultradian feeding cycle. Longer food restriction would lead to loss of body weight. At the onset of each food restriction, cage beddings were refreshed to prevent feeding on scattered food pellets. A control group C was not subjected to FR, but cage bedding was refreshed together with those of groups A and B.

After 10 days, the light pulse was advanced by 4 h in all experimental groups. Following the phase advance in light, FR either remained at the same clock time (group A) or was phase advanced together with the light (group B). In each of the groups A, B and C there were 14 voles, of which 8 (subgroups A1, B1, C1) had no running wheel, while 6 animals (subgroups A2, B2, C2) had a running wheel. Activity of all animals was recorded using Passive InfraRed (PIR) detectors above the cage; table 1 summarizes the treatment per group.

Experiment 2: Conflicting Zeitgeber periods of light and food

Experiment 2 was designed to assess whether different Zeitgeber periods of light and food availability interfere in the behavioural expression of the circadian rhythm. Again the availability of a running wheel was an additional factor controlled experimentally. Animals were assigned to one of six experimental groups (N = 8 per group, see table 2), with the provision that adjacent cages belonged to different groups. Following 10 days of entrainment to LD ½:23½ h, the Zeitgeber period was reduced from T=24 to T=23.5 h (LD ½:23). After 10 light cycles, all animals received food restriction for 8 h followed by 17 h of *ad libitum* food availability (T = 25 h). This food restriction was run though 16 cycles, after which *ad libitum* food was restored. Prior, during and after the food cycle, the light cycle of LD ½:23 remained enforced, resulting in the presentation of two Zeitgeber cycles with different periods. The first FR episode was either presented directly after lights off, i.e., at the beginning of the active period (group D), or 12 h later (group E). The food restriction cycle ended after 16 days, while the light cycle continued. Experiment 2 was again carried out with voles housed with and voles without a running wheel (table 2).

Information on the phase and period of a free running vole in constant conditions was obtained by extending the experiment for group D (D extended). Voles of group D were exposed again to the LD 12:12 h Zeitgeber following the first episode of conflicting Zeitgebers. The animals then again received a FR cycle with a period of 25 h for 7 cycles, the first cycle starting at the onset of activity. Hereafter animals were given *ad libitum* food and constant dim red light starting at a time at which the phase difference between the conflicting cycles was maximal.

Behavioural analysis

General activity was recorded using Passive Infrared (PIR) detection of movement in the cage. Movements detected in the cage were stored in our event recording system (ERS) summed in 2 minute intervals. Periodicity in activity during entrained and free running conditions was analyzed by periodogram analysis (Sokolove & Bushnell, 1978). Because of technical problems in the recording system, we had to skip data from in total 5 animals (D1; N = 1, E1; N=3 E2; N= 1).

In experiment 1 the circular centre of gravity (CG) of daily activity was used as a phase marker of the circadian rhythm (Kenagy, 1980, Hut *et al.*, 2000). Phase angle differences (ψ) between the CG and the LD cycle were used to express phase of entrainment.

Running CGs were calculated according to Mardia (1972). CGs were calculated over 24 hour periods starting every 2 minutes in the 20 day dataset. CGs were grouped per day using the criterion that a step of more than 1000 minutes between two CGs indicated a transition between days. Average CG and (linearized) standard deviations per day were calculated. The overall average CG during the first 10 days before the shift in light was calculated and the difference between the average CG per animal during this period and the overall average CG was established. To correct for the variation in phase angle difference between animals, we added to each CG the phase lead of the initial (first 10 day interval) CG of the individual relative to the interindividual mean initial CG. Data were expressed as average daily CG and SEM based on the linear standard deviations per vole.

In experiment 2, the data were smoothed by a 3-h running average to minimize ultradian interference in the periodogram analysis. The proportions of the variance explained by the different rhythmic components in behaviour were then assessed using an algorithm based on Hiddinga *et al.*, (1997). In short, as a starting point a profile of a behavioural component with a period of that of the FR cycle was established by calculating a circular average with a 25 hour period. This average profile was subtracted from the raw data, leaving a residual dataset with the behavioural activity that was not accounted for by the 25 h profile. Periodogram analysis was then used on the residual data to establish the period of the most dominant rhythmic component in the residual behav-

ournal dataset. A circular average with the period of this second rhythmic component was then subtracted from the raw data, leaving a residual dataset with the behavioural activity that was not accounted for by the second behavioural component. Periodogram analysis was then used on this residual data to refine the period of the first behavioural component (which was initially set at 25 h). This algorithm was iterated three times after which the periods of the components were stable. Thereupon the variance of both residual datasets (obtained by the subtraction of the final circular averages of the both components from the raw data) was expressed as the percentage of total variance. This leaves us with the period and the variance explained by the two most dominant circadian behavioural components. To compare phase of activity during experiment 2 and phase of activity in the subsequent free run period, smoothed activity (4 h) that exceeded average activity (24 h) was used as a phase marker. This method is insensitive to the differences in distribution of activity during the entrained and the free run episode.

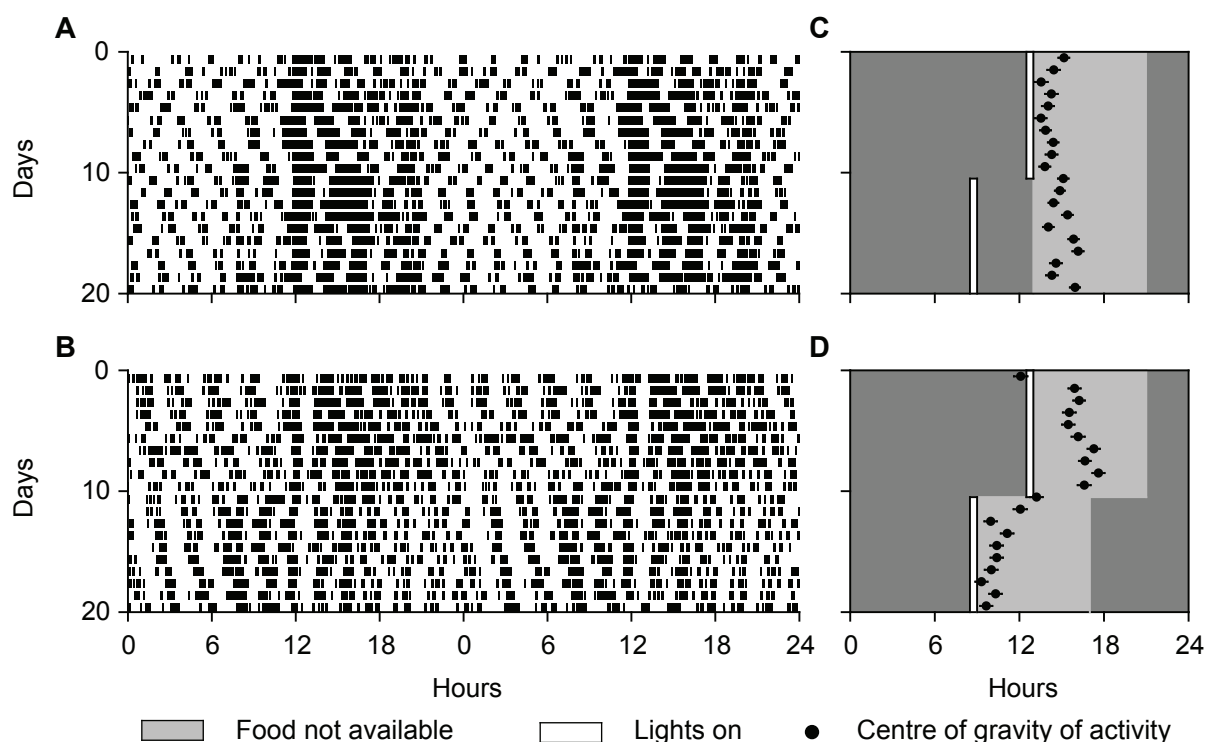


Figure 1

Activity records and centre of gravity for common voles housed without a running wheel. Panel A shows the double plotted actogram for a vole receiving a food restriction that is fixed in time. After the phase shift in light, centre of gravity (panel B) does not shift in accordance to the light. Activity (panel C) and centre of gravity (panel D) for a vole receiving food restriction that shifts with the light shows re-entrainment to the light pulse. Note the strong ultradian modulation of activity for both voles. Grey areas indicate when the lights are on and patterned areas indicate food absence.

Results

Experiment 1: Reentrainment to a phase advance in light

All voles initially entrained to the LD schedule of 0.5:23.5 h (fig. 1). Periodogram analysis showed stable entrainment to the light pulse with mean $\tau = 23.9$ h (SEM = 0.04 h; N = 42). For three of the sixteen animals that were housed without a running wheel, the circadian signal was insufficient to calculate reliable centres of gravity (CG) (A1; N = 2, C1; N = 1). 5 Other voles had circadian rhythms that broke free from entrainment following the phase advance in the light (A1; N = 1, B1; N = 3, C1, N = 1). These 8 animals were excluded from further analysis.

In figure 1 representative actograms are presented for animals housed without a running wheel, along with daily centres of gravity. Panel A shows an animal in group A1 receiving unshifted food restriction (FR), panel B an actogram of an animal from group B1 where the FR was advanced by four h, along with the light. Panels C and D show CGs for both animals. No shift in activity is observed in panel C following the shift in LD (group A1). The vole in figure 1B, D did re entrain after the 4 h shift. To minimize the effect of the interindividual variation in the phase angle difference ($\psi = \text{time of lights on} - \text{time of CG}$), the difference of the individual average CG from the group average CG ($\psi' = \text{mean } \psi - \text{group mean } \psi$) was subtracted from individual CGs. In this way, the average CG per animal during the first 10 days before the light was the same for each animal.

Table 3: Phase angle differences (\pm SEM) between light and CG pre- and post-light shift

		ψ pre-light shift	ψ post-light shift
<i>Without wheel</i>	A1	-2.75 ± 0.21 h	-7.23 ± 0.16 h
	B1	-2.92 ± 0.19 h	-2.75 ± 0.42 h
	C1	-2.40 ± 0.36 h	-5.33 ± 0.51 h
<i>With wheel</i>	A2	-1.40 ± 0.17 h	-4.24 ± 0.42 h
	B2	-1.55 ± 0.22 h	-0.53 ± 0.45 h
	C2	-1.50 ± 0.25 h	-1.92 ± 0.58 h

The three left panels in figure 2 show the individual courses of the CGs during the experiment and the middle three panels show the average uncorrected CGs. The three right-hand panels in figure 2 show these corrected CGs and the average CG for all animals during the last 5 days before the shift in light and day 6 though 10 following the light pulse are summarized in figure 3, in the left panels. The same protocol has been applied to animals housed with a running wheel and the corresponding CGs are shown on the right panels of figure 3. Table 3 shows the average phase angle differences of activity for pre and post light shift.

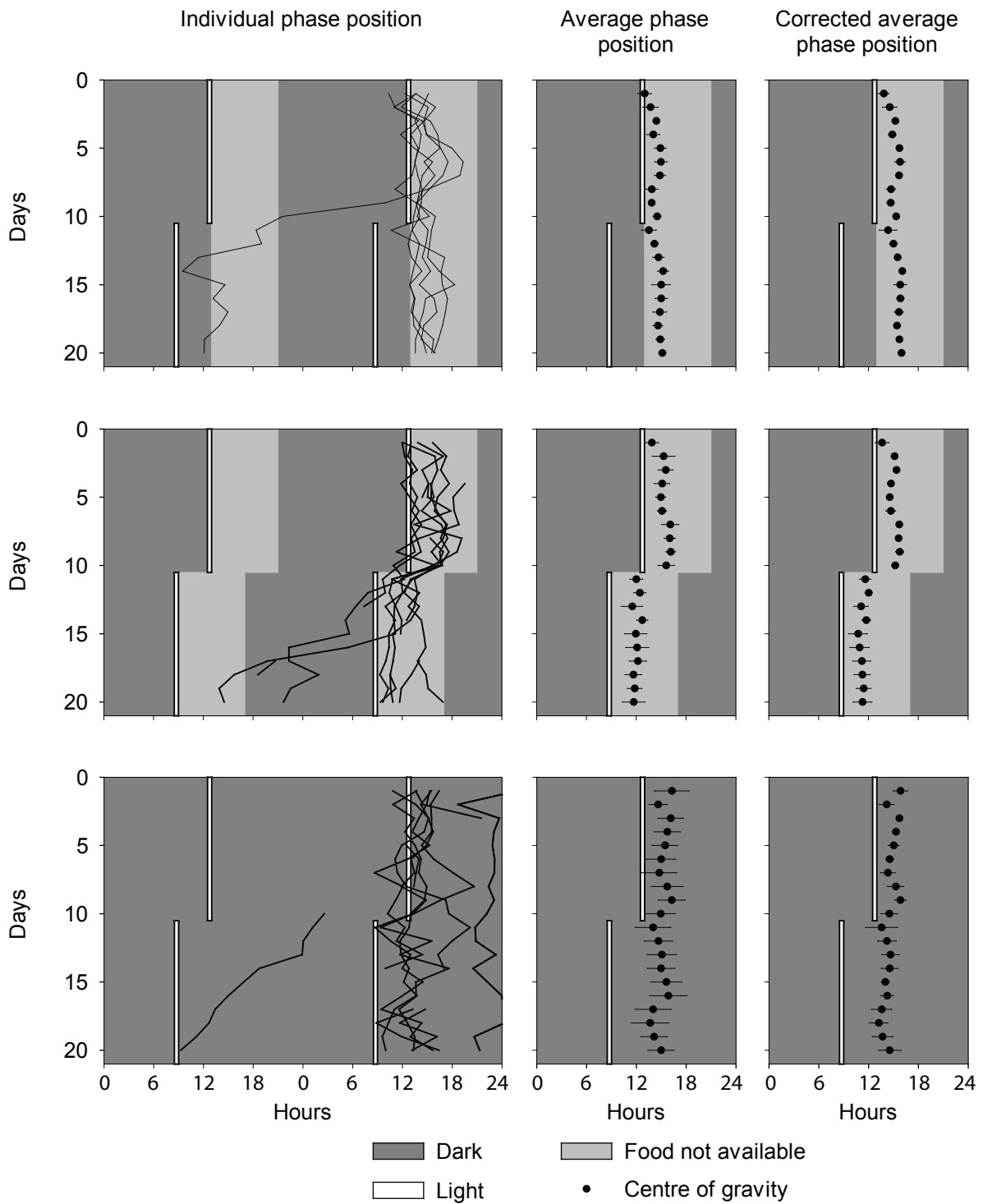


Figure 2

Individual-, average absolute- and average relative centres of gravity (CG) for animals receiving fixed food restriction (top row), animals receiving shifting food restriction (middle row) and animals with ad libitum food availability (bottom row). Individual daily centres of gravity (panels A, D & G) show large inter-individual variation, resulting in large SEM for the averages (panels B, E & H). Correction for inter-individual variation is accomplished through normalizing individual centres of gravity to the average for the whole group, resulting in relative CG (panels C, F & I).

Average uncorrected CG of activity of animals housed without a running wheel (groups A1, B1 and C1) did not differ between during the 10 days before the shift in the light (Kruskal-Wallis; $p > 0.05$) and CGs corrected for interindividual variation were used to calculate ψ . The average ψ of the activity of the voles in groups A1, B1 and C1 together, during the last 5 days before the shift in light was -2.55 h (SEM = 0.28 h), and these phase angle differences did not differ between the groups. After the shift in the light, animals in group A1, receiving unshifted FR show an average increase of 4.48 h, while for animals in group B1, receiving shifted FR, this difference is only -0.17 h. Animals from group C1, that had *ad libitum* food availability showed an intermediate average change of ψ of 2.93 h, in between the animals receiving FR.

Phases of activity, when compared between before and after the shift in the light within and between the groups A1, B1 and C1, were different after the phase advance in the light (two way repeated measures ANOVA; $p < 0.01$ for group, $p < 0.05$ for before/after and $p < 0.05$ for interaction). Pairwise multiple comparisons (Holm-Sidak

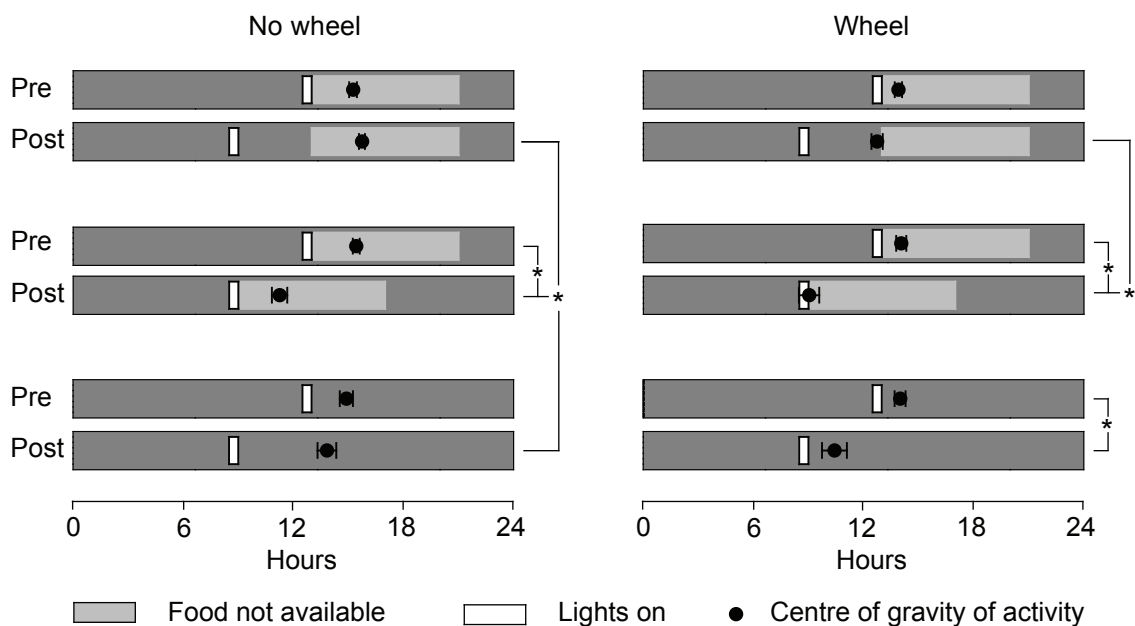


Figure 3

Relative centres of gravity shown in blocks of pre- and post-light shift averages, based on the last 5 days of each ten day period. Dark grey bars indicate light out, with a white block representing the dusk light pulse. Light grey areas depict the position of the 8 hour period when food was absent. Cages were cleaned on time points as indicated by the arrows. Asterisks indicate significant differences. Block A, B and C represent pre- and post-light shift averages of voles housed without a running wheel. Animals receiving stable food restriction (A) or *ad libitum* food availability (C) do not show significant phase shift after the phase advance in light. Animals receiving a shifting food restriction (B) show a significant phase advance. Post-light shift, all three phase positions differ, where animals receiving no food restriction show the intermediate phase. Blocks D, E & F represent voles housed with a running wheel. Animals receiving a fixed food restriction (D) do not show a significant phase change after the light has phase advanced. Both animals receiving shifting food restriction (E) and animals receiving *ad libitum* food availability (F) show significant post-light shift phase advances.

Table 4: Behavioural components identified in behavioural activity

	Ultradian component	F component		L component	
		Period AVG \pm SEM	% Of smoothed AVG \pm SEM	Period (h) AVG \pm SEM	% Of smoothed AVG \pm SEM
D1	26.9 \pm 4.6%	25.07 \pm 0.02	35.0 \pm 4.8%	24.30 \pm 0.32	12.2 \pm 1.8%
D2	30.8 \pm 3.8%	25.03 \pm 0.02	31.2 \pm 6.5%	24.00 \pm 0.24	15.2 \pm 1.2%
E1	21.3 \pm 2.6%	25.01 \pm 0.01	32.9 \pm 9.5%	24.40 \pm 0.44	15.3 \pm 4.2%
E2	30.1 \pm 2.6%	25.06 \pm 0.02	23.8 \pm 4.5%	23.91 \pm 0.20	12.5 \pm 2.2%

method) shows that only the animals exposed to a phase shift in food restriction (group B) show a significant advance following the shift in the light (Holm-Sidak; $p < 0.001$). Although the phase of activity of the animals not receiving FR (group C1) does not differ significantly before and after the phase advance in the light, post light shift comparison indicates that CGs of activity of all three groups differ (Holm-Sidak; $p < 0.05$ at least). The CG of activity of the animals in group C1 is intermediate to that of A1 and B1, indicating that the CG of activity of non FR voles is, as a result of a shift in the light, different from that of animals receiving FR that remains at the same time (group A1).

For animals housed with a running wheel, average uncorrected CG of activity did not differ between the groups during the 10 days before the shift in the light (Kruskal-Wallis; $p > 0.05$), and the average ψ between light and CG of activity was -1.48 h (SEM = 0.02 h). The CG of activity is significantly earlier than that of animals without a running wheel and is not dependent on feeding conditions (two way repeated measures ANOVA; $p < 0.001$ for housing conditions). A significant effect of the phase shift in light is seen on the CG of the activity position (two way ANOVA; $p < 0.001$). Pairwise multiple comparisons show that post light shift, both the animals exposed to a phase shift in food restriction (group B2) and the animals receiving no food restriction (groups C2), but not the animals in group A2 showed a significant advance in the CGs (Holm-Sidak; $p < 0.005$, see table 3 and figure 3 right panels).

Experiment 2: Conflicting Zeitgeber periods of light and food

For further investigation of the combined effects of temporal food restriction, light information and running wheel availability on phase of entrainment, we applied a protocol of conflicting Zeitgebers. The results often yield reason to distinguish two components in activity. Either there are two activity peaks in a profile, or/and there are two periods distinguished simultaneously by periodogram analysis. We call these two components L and F. We presume that these are reflecting the light-entrainable oscillator (LEO) and the food-entrainable oscillator (FEO), respectively.

Figure 4, upper left panel, shows a double plotted actogram of animal #399, housed with a running wheel and receiving the first food restriction cycle after lights off (group D1). The upper right panel of figure 4 shows the same data after smoothing; note that the

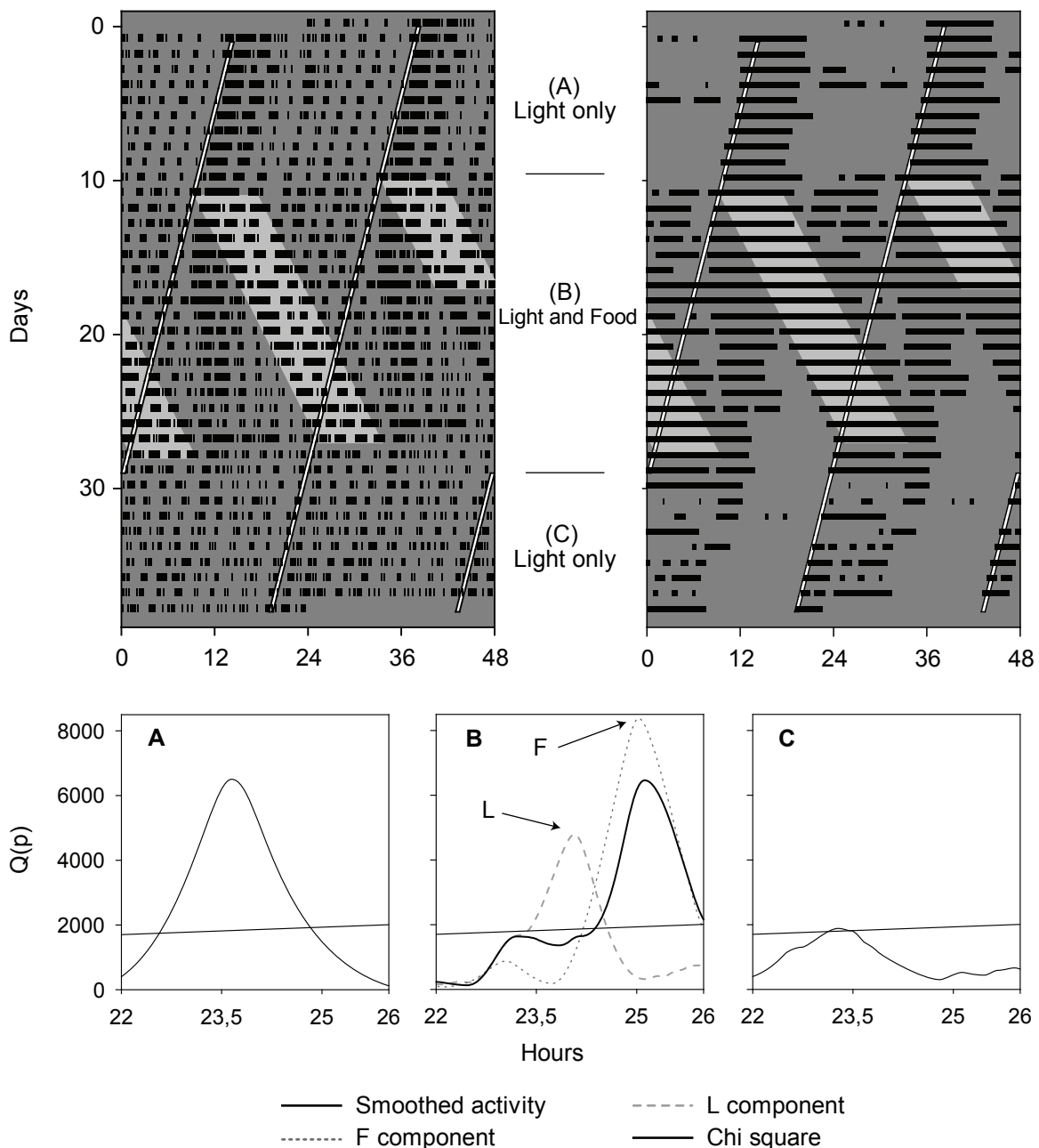


Figure 4

Double plotted actogram of overall activity (left) and smoothed overall activity (right) of a vole prior, during and after the desynchrony of food and light Zeitgebers. During the actual desynchrony (days 11 to 28 on y-axis) the light grey areas indicate the 8 hour episode in which food was restricted starting every 25 h. Data was smoothed with a 3 hour window to minimize ultradian interference in the periodogram analysis. Animals were entrained to a single light pulse every $23\frac{1}{2}$ h during days 1 to 11, the corresponding periodogram in the bottom panel A shows a large peak at $23\frac{1}{2}$, indicating entrainment. For the episode of the desynchrony of light and food Zeitgebers, 3 periodograms are shown in panel B. The solid line is the periodogram on the smoothed data during the protocol and peaks at 25.1 h. The dotted line is the periodogram for the food-entrained component which also peaks at 25.0 h. The striped line indicates the alternative behavioural component and peaks at 24.1 h. Following desynchrony, the periodogram analysis on activity data of days 29 to 38 is shown in the bottom panel C. Here animals are entrained with a $23\frac{1}{2}$ period, corresponding to that of the light, albeit with a decreased zenith.

circadian pattern remains while the ultradian pattern is reduced. The reduction of the total variance caused by the ultradian smoothing ($(\text{Variance}_{\text{smooth}} / \text{Variance}_{\text{raw}}) * 100\%$) is given in table 4. This reduction of the total variance caused by the ultradian smoothing was neither different between animals housed with or without a running wheel, nor did the start point of food restriction have an effect on this reduction in variance (two way ANOVA; $P_s > 0.05$).

Passive infrared detection of overall activity showed on average 8376 ± 1133 grid movements/day per vole before the start of the protocol of conflicting Zeitgebers and 10361 ± 999 grid movements/day during the protocol. The activity shown during the protocol of conflicting Zeitgebers is significantly higher than during initial entrainment to a single light pulse (paired t-test, $p < 0.05$).

Using a deconvolution method based on Hiddinga *et al.*, (1997), the F and L component in the overall activity were established for the animals going through the full episode of 16 FR cycles (groups D and E). Of these components, both the period and the percentage of the total variance explained by these components were established (table 4, F and L component). The bottom three panels of figure 4 illustrate the periodograms of the activity data before (A), during (B) and after (C) the desynchrony protocol belonging to the activity records shown of animal #399 (group D).

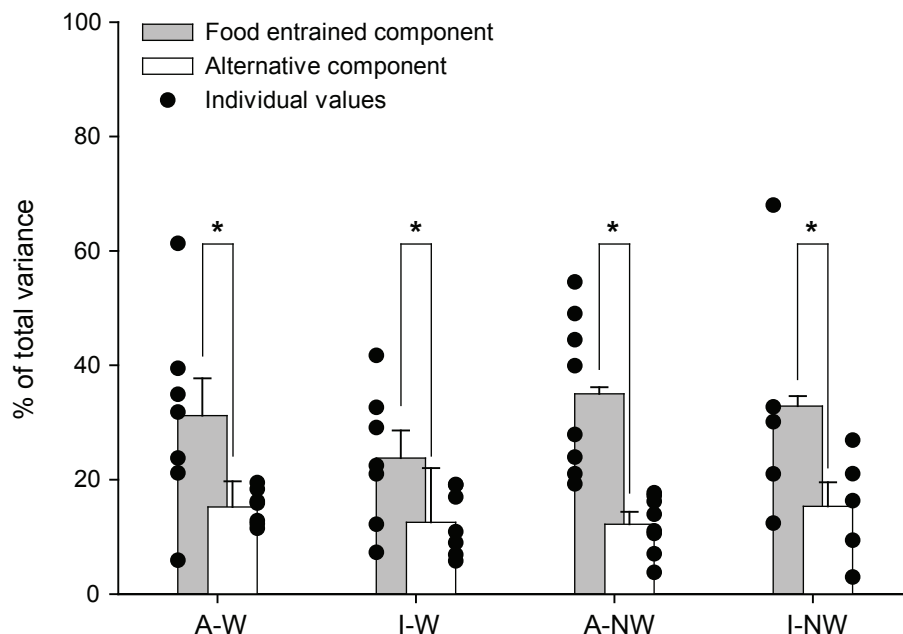


Figure 5

Amounts of total variance explained by the food-entrained (grey) and alternative (white) components during desynchrony between food restriction (25 h cycle) and a light pulse (23½ h cycle). Errors bars show SEM and individual values are denoted by black circles, for explanation of the group acronyms on the x-axis, see table 2 in the methods). For all groups either housed with or without a running wheel and the first food restriction started in the behavioural inactive or active period the amount of total variance explained by the food-entrained component is significantly more than that explained by the alternative component. Asterisks indicate significant differences.

Figure 4 (A and C) shows that both before and after the episode of FR, the animals entrain to the light cycle with a period of 23.5 h. During the period that the animals were subjected to conflicting Zeitgeber periods of light and FR, a F component with a period close to that of the FR (figure 4B, dotted line) is considerably stronger (higher dQp) than the period of the L component (figure 4B, dashed line). The F component also explains a significantly larger portion of the total variance than the L component does (table 4, figure 5, Mann-Whitney Rank Sum Test; $p < 0.001$). The F component on average has a period of 25.05 h (SEM = 0.01 h, see table 4 for group values), and the

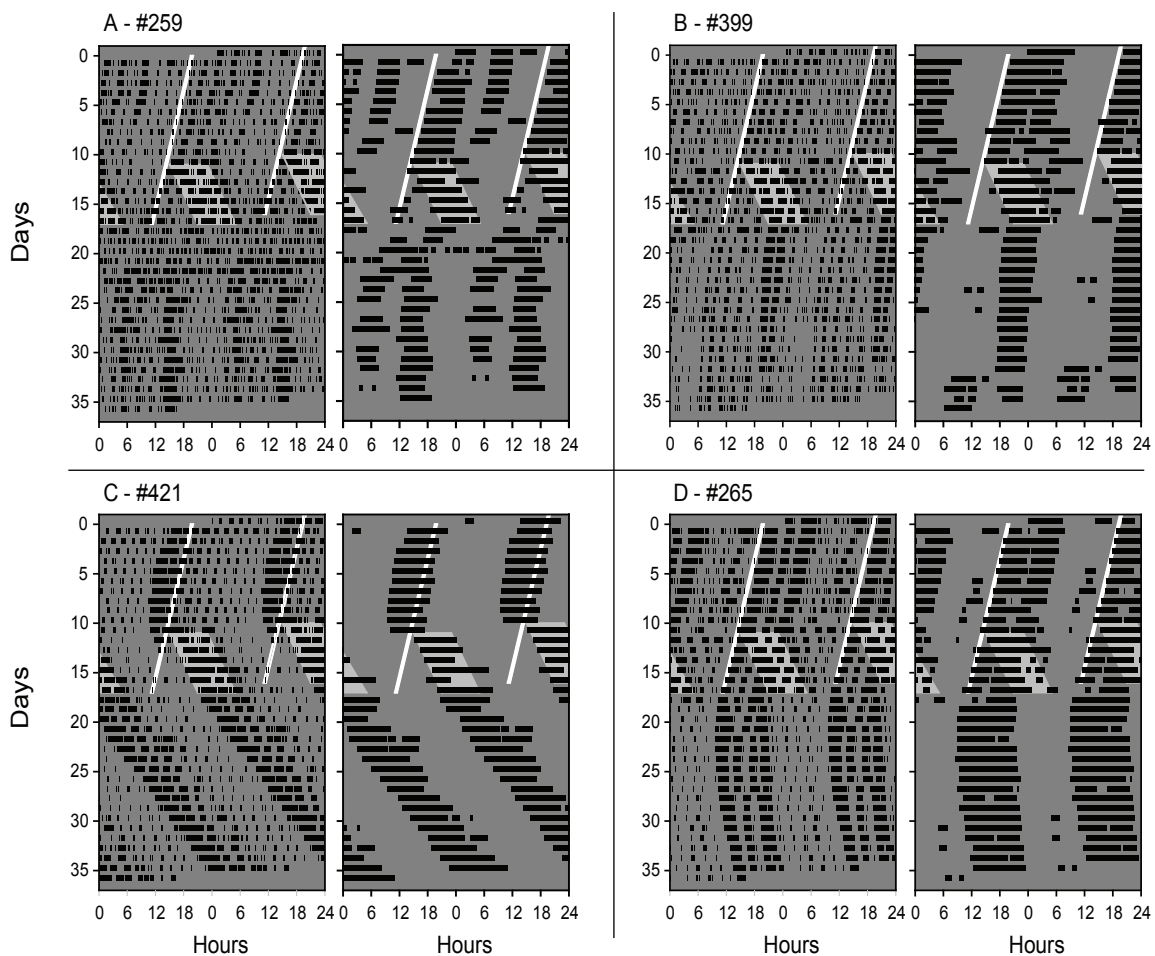


Figure 6

Four example actograms showing different free running activity patterns (left) and the above average activity (right) patterns. Panel A show and animal (#259) that exhibits a bimodal activity pattern during the desynchrony of food and light Zeitgebers and thereafter in free run. Panel B shown an actogram of animal #399 showing peak activity during free run that at first is in the phase of the food-entrained component and later switches to the phase of presumably the alternative component. Panel C shows and actogram of animal #421 that during free run is active in the phase corresponding to the food-entrained component and panel D show animal #265, active with a phase originates from the light-entrained component from the preceding light-entrained component. White line indicates the light pulse and the light grey area depicts the 8 hour episode in which food was not available.

length of the period is not different between groups of voles for whom FR started in the active/inactive period (D versus E) and/or voles having a running wheel or not (1 versus 2) (two way ANOVA; $P_s > 0.05$). The L component averages at a period of 24.14 h (SEM = 0.14 h) and explains a significantly smaller portion of the total variance than the F component. Also for this L component, the period does not differ between groups with or without a running wheel (D versus E) and/or different start times of the protocol (1 versus 2) (two way ANOVA; $P_s > 0.05$).

In the groups D1 and D2, animals were again subjected to conflicting Zeitgeber periods of light and food, but the protocol was discontinued after 7 FR cycles and the animals were released in dim red light and *ad libitum* food availability. During free run, the voles showed an average period of 23.73 h (SEM = 0.15 h), which is not different to that F component in groups D and E (t-test $p > 0.05$). Inspection of the actograms, with emphasis on above average expression of activity (when the 4 h average exceeded the 24 h average) revealed differences in phase of activity. The phase of activity between subjects could be assigned to four categories of which examples are shown in figure 6. Figure 6A shows the actogram of animal #259. Both the raw data (on the left) and the above average activity (on the right) indicate a bimodal activity distribution. Whether these are based on different components oscillators cannot be said definitely. Animal #399 (figure 6B) also shows two components in activity. Here the peak first is associated with the F component, but gradually the L component takes over, while the F component attenuates. In the bottom two panels of figure 6, animal #421 (figure 6C) appears to be free running with a period and phase corresponding with the F component. Vole #265 runs free with a period and phase corresponding with the L component. In many animals two phases of activity are visible at least in the beginning, and both of these components free run with the similar period.

Discussion

The reentrainment of circadian activity following a 4 h phase advance of the LD cycle in voles was strongly influenced by food restriction (FR). When the daily 8 hour FR episode remained at the same time of day, no behavioural reentrainment to the phase shifted light was seen after 6 to 10 days after the phase shift in light. When FR was phase advanced together with the LD, the daily phase of activity almost immediately advanced with light. Voles receiving no FR show intermediate phase positions in the same time span of 6-10 days, i.e. they took longer to phase shift. When provided with a running wheel, the reentrainment to the new LD was more pronounced than without a wheel for animals not receiving FR. In voles, the speed of reentrainment is increased by running wheel availability. This is comparable to the effect that activity induced by a novel running wheel increases speed of reentrainment to a phase advance in LD (Mrosovsky & Salmon, 1987, Chidambaram *et al.*, 2004). In our study, the presence of a running wheel did not contain any timing information, but did have an enhancing effect in reentrainment. When the FR shifted together with the light, the phase advance in behaviour was more pronounced in the group with wheels. The wheel did not affect the pattern when FR was not shifted, and no reentrainment following the LD phase shift was seen.

We suggest that under the present protocol the temporal information contained within the food restriction was the decisive Zeitgeber in determining entrained phase of activity in the common vole. This result on the relative strength of the two conflicting Zeitgebers most likely depends on the protocol. A brief light pulse per cycle as we employed in this study in voles may be sufficient for circadian entrainment, but is a weak Zeitgeber compared to a daily 8-h food deprivation. The phasing of the FR cycle may have had an effect on its relative strength. Kalsbeek *et al.* (2000) have shown that 2 hour food availability during the light phase attenuates reentrainment of pineal melatonin release to an 8 hour phase advance in light in rats. The food availability in Kalsbeek's study was given during the light phase, we restricted food availability to the dark phase. The absolute timing of food availability may not be the key factor, but phase locking the FEO to a certain time of day attenuates reentrainment to a new light phase.

Under conflicting Zeitgebers of LD (25 h) and FR (23.5 h) cycles we observed one component entrained to the FR, the F component. The deconvolution analysis confirmed the presence of another component, the L component with distinct periods and a significant contribution to the total variance. The behavioural F component explaining most of the variance had a period around 25 h; the L component had a period close to 24 h, similar to the period of the free running rhythm when the animals were released in constant dim red light and *ad libitum* food availability. Thus the FR acted as a Zeitgeber and entrained behavioural activity, but light could no longer entrain. Rats can entrain to multiple Zeitgebers (for reviews see Mistlberger, 1994, Stephan, 2002). In the voles we see two components, one of which runs free. In rats, a free running component in activity besides a food-entrained component has also been reported in experiments where no light cue was available (Edmunds & Adler, 1977). The LD cycle

in our vole study apparently was insufficiently strong to entrain the L component.

When released in constant conditions following entrainment to desynchronized Zeitgebers, voles showed different behavioural responses. Animals either had a bimodal circadian activity distribution, a continuation of the distribution seen during the entrainment by two Zeitgebers, or only a single component, either originating from the light or food-entrained activity. Some voles in free running conditions initially showed a dominant F component, and later a dominant peak based on the prior light-entrained component (figure 6B). A food-entrained component in behaviour is known to disappear rather quickly in *ad libitum* conditions (Stephan *et al.*, 1979). Coleman *et al.* (1982) reported that in rats, several weeks after a FR protocol an activity bout was still discerned at the phase of the former feeding time. When those same rats were given episodes of *ad libitum* food availability this component in behaviour disappeared, only to re-appear in a subsequent episodes of food deprivation, although shifting towards and eventually merging with the activity component belonging to the former LD. This seems similar to our voles that persist their F component and eventually shift to the L component. The F component is not masked by food availability. The circadian periods observed during free run in experiment 2 do not differ from those of the non FR component in experiment 1.

In this study, we challenged the food and light-entrainable components of the vole circadian system by adding timing information both to food by temporally restricting the availability and to behaviour by providing running wheels (Gerkema *et al.*, 1993). The integration mechanism of these Zeitgebers is complex. Which physiological or behavioural oscillators and which integrative centres are involved remains to be answered. Although the presence of a food-entrainable oscillator is evident, there is still uncertainty about the substrate for such an oscillator. It is unlikely to be in the liver (Davidson *et al.*, 2003). The dorsomedial hypothalamus does seem to be involved in food-entrained rhythms (Saper *et al.*, 2005, Gooley *et al.*, 2006, Landry *et al.*, 2006). While rhythms in liver clock genes are always expressed in mice and rats (Damiola *et al.*, 2000, Stokkan *et al.*, 2001), in voles they are only present under food restriction cycles or when provided with a running wheel (Van der Veen *et al.*, 2006). In all cases, the SCN is unaffected by FR, unless it is the FR is also restricting the amount of food consumption (Mendoza *et al.*, 2005). Thus, although temporal food restriction is a potent Zeitgeber, it does not act at the level of the central pacemaker, but at the level of behaviour and peripheral clocks. Here we have shown that food availability cycles can affect both phase and period of circadian activity under entrained conditions. There are after-effects of FR under free running conditions in the phasing of activity, and occasionally in period (fig. 6D). This supports the conclusion that food availability can in principle be a very potent Zeitgeber to the extent that it may override the effect of the LD cycle, even in such animals with non-periodic natural food as voles.