Circadian Food Anticipatory Activity Across the Seasons: The Relationship Between Feeding Schedules and Photoperiod in Mice

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Sarah C. Power

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Approval

Name:	**Sarah C Power**
Degree:	**Master of Arts**
Title:	**Circadian Food Anticipatory Activity Across the Seasons: The Relationship Between Feeding Schedules and Photoperiod in Mice**
Examining Committee:	**Chair: Timothy Racine** Professor

Ralph Mistlberger
Senior Supervisor
Professor

Neil Watson
Supervisor
Professor

Brian Prendergast
External Examiner
Professor
Department of Psychology
University of Chicago

Date Defended/Approved: December 14, 2018

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Abstract

Daily feeding schedules induce circadian rhythms of food anticipatory activity (FAA) by entrainment of circadian oscillators outside of the master light-entrainable pacemaker in the suprachiasmatic nucleus (SCN). Efforts to localize these food-entrainable oscillators (FEOs) and specify molecular mechanisms have been complicated by the wide range of non-circadian factors that can modulate expression of food-motivated behaviours. Here, we examine the effect of photoperiod (duration of the daily light period) on FAA induced in mice by restricting food to a 4h daily meal in the light period, the usual rest phase in nocturnal rodents. To express FAA in the light period, FEOs must compete with SCN clock outputs, which normally suppress activity and promote sleep at this time of day. Photoperiod modifies both the period (τ) and amplitude of the SCN pacemaker, as indicated by aftereffects of long and short days on τ and on the phase shift response to light pulses in constant dark (DD). Exposure to long days is thought to reduce SCN amplitude, and would be expected to permit greater FAA to a daytime meal. To test this prediction, mice were entrained to a 16h light:8h dark (L16) or L8 cycle, with or without running discs, and then maintained in DD for 2 weeks. Mice previously entrained to L16 exhibited a shorter τ and smaller phase shift to light in DD, confirming an effect of photoperiod on the SCN pacemaker. After re-entrainment to L16 or L8, food was restricted to the last 4h of the light period. FAA was enhanced in L16 in mice with running discs, but the difference was reversed in mice without running discs. Additional groups of mice were entrained to L18, L16, L12 or L8, and the 4h daily meal was centered in the light period. Prior to restricted feeding, photoperiod modified parameters of the light-entrained rhythms as expected. During restricted feeding, there was no systematic effect of photoperiod on FAA. After restricted feeding, an aftereffect of photoperiod on τ in DD was absent. Centering of daily mealtime in the light period may block the effect of photoperiod on the SCN pacemaker, and thereby eliminate the potential impact of day length on the expression of FAA to daytime meals.

Keywords: Photoperiod, Circadian Rhythms, Food-Anticipatory Activity, Suprachiasmatic Nucleus

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List of Acronyms

LD	Light-Dark
SCN	Suprachiasmatic Nucleus
FAA	Food Anticipatory Activity
FEO	Food-Entrainable Oscillator
DD	Dark-Dark
CT	Circadian Time
ZT	Zeitgeber Time

Glossary

Alpha (α)	Duration of the active phase of an organism.
Amplitude	Difference between the minimum and maximum value of a biological oscillation.
Entrainment	The process by which a biological oscillator is synchronized to an environmental rhythm such as the light-dark cycle.
Phase of entrainment (Φ_{LD})	The timing of nocturnal activity onset relative to lights-off.
Photoperiod	The duration of daylength (lights on).
Tau (τ)	The free running period of an organism in constant conditions.
Zeitgeber	German for 'time-giver', an entrainment signal.

Chapter 1.

Introduction

In mammals, circadian rhythms of behaviour and physiology can be entrained to local environmental time by daily light-dark (LD) cycles and by scheduled mealtimes. At the cellular and systems level, entrainment by LD cycles is well understood. Light is transduced by intrinsically photoreceptive retinal ganglion cells, which project to and depolarize circadian clock cells in the suprachiasmatic nucleus (SCN). Depolarization induces *period* gene expression, which resets transcription-translation feedback loops that comprise the gears of the cell autonomous circadian clock. SCN outputs drive circadian rhythms directly or by synchronizing circadian oscillators elsewhere in the brain and body thus earning it the title of master pacemaker (Golombek & Rosenstein, 2010; Mohawk, Green, & Takahashi, 2012).

By contrast, entrainment of circadian rhythms by scheduled mealtimes is not so well understood. When food is freely available, nocturnal rats and mice eat primarily at night, under control of the LD-entrained SCN pacemaker. If food availability is limited to a particular time of day or night, circadian oscillators in most peripheral organs and tissues, and many brain regions, shift to align with mealtime (Dibner, Schibler, & Albrecht, 2010; Mohawk et al., 2012). This is associated with a shift in the timing of physiological rhythms, and with the emergence of a daily rhythm of food anticipatory activity (FAA), even if the mealtime is in the middle of the light period, when nocturnal rodents normally sleep (Boulos & Terman, 1980; Mistlberger, 1994, 2011; Stephan, 2002). Resetting of peripheral clocks and emergence of FAA occurs with little or no shifting of the SCN pacemaker in LD, and is not impaired by SCN ablation or by housing in constant light or dark (Boulos & Terman, 1980; Damiola et al., 2000; Hara et al., 2001; Petersen, Patton, Parfyonov, & Mistlberger, 2014; Stephan, Swann, & Sisk, 1979; Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001). Formal properties of FAA are consistent with control by a circadian clock that is entrained by daily feeding schedules (Boulos & Terman, 1980; Mistlberger, 1994; Stephan, 2002), but the molecular basis of this clock, its location in the brain or body, and the feeding-related stimuli that entrain it, remain uncertain (Challet, Mendoza, Dardente, & Pévet, 2009; Mistlberger, 2011; Pendergast & Yamazaki, 2018).

Efforts to delineate these mechanisms are complicated by other factors that may affect the expression of food-motivated behaviour independently of circadian timekeeping. For example, FAA is increased by weight loss (Persons, Stephan, & Bays, 1993) and deficient leptin signaling (Mistlberger & Marchant, 1999; Ribeiro et al., 2011). Conversely, FAA may be weak or absent if feeding schedules do not produce weight loss, and is reduced by diet induced obesity, high fat foods and ghrelin deficiency (Patton & Mistlberger, 2013; Persons et al., 1993). It is increased by cool ambient temperature, and decreased in warm temperatures (unpublished observations). FAA timing and magnitude also vary with the behaviour measured, e.g., FAA is more robust when measured using running wheels compared to motion sensors, more precise when measured using an operant lever, and may be robust in activity directed toward a food bin while minimal in general cage activity (Dattolo et al., 2016; Flôres, Bettilyon, Jia, & Yamazaki, 2016; Mistlberger & Rusak, 1987; Petersen et al., 2014). FAA can be induced by a highly palatable daily snack (Ángeles-Castellanos, Salgado-Delgado, Rodríguez, Buijs, & Escobar, 2008; Hsu, Patton, Mistlberger, & Steele, 2010; Mistlberger & Rusak, 1988), shifted by a dopamine agonist (Smit, Patton, Michalik, Opiol, & Mistlberger, 2013), and attenuated or eliminated entirely (along with other motivated behaviors) by blocking dopamine transmission (Gallardo et al., 2014; Mistlberger & Mumby, 1992). There is also a likely interaction with genetic sex (Aguayo et al., 2018; Li et al., 2015; Michalik, Steele, & Mistlberger, 2015). Consequently, any neural or genetic manipulation that affects metabolic homeostasis, reward processing, or the ability to learn or perform operant behaviours, can be expected to affect expression of FAA, whether or not the manipulation has impinged directly on the neural or molecular substrate of food-entrainable circadian oscillators (FEOs) hypothesized to drive circadian rhythms of FAA.

Here, we examine the role of photoperiod as another ‘off-target’ factor that might affect expression of FAA in mice. Previously, we have shown that FAA induced in rats and mice when food is restricted to the middle of the daily 12h light period is enhanced when the full photoperiod is replaced by a skeleton photoperiod (two 30 min light periods simulating dawn and dusk) (Dantas-Ferreira et al., 2015; Patton et al., 2013). This is consistent with a ‘negative masking’ (i.e., suppression) effect of light on activity levels in nocturnal rodents (Mrosovsky, 1996). The duration of the light period (i.e., the photoperiod) may also be important. SCN neural activity exhibits a daily rhythm, with peak firing rates in the middle of the light period, which is the rest phase in nocturnal animals

(Meijer & Michel, 2015). Converging evidence indicates that in nocturnal species, SCN outputs during the day suppress activity, promote sleep, and oppose expression of FAA induced by a daytime meal (Acosta-Galvan et al., 2011; Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs, & Escobar, 2010; Landry, Simon, Webb, & Mistlberger, 2006; Mistlberger, 2005, 2006). Photoperiod may therefore be expected to modulate FAA duration or magnitude if it alters the amplitude of the SCN pacemaker and the relative strength of SCN control of behavior.

Although mice commonly used in studies of circadian mechanisms (e.g., *Mus musculus*) do not exhibit photoperiodic regulation of reproduction or metabolism, there is ample evidence from behavioural, electrophysiological and gene expression studies that photoperiod does regulate the SCN pacemaker in this species. Mice housed in long days (e.g., 18h) exhibit a marked compression of the duration of nocturnal activity (α) that gradually dissipates in constant dark (DD). This is associated with a shorter free-running period (τ) (Pittendrigh & Daan, 1976a), and smaller phase shifts in response to light (Pittendrigh, Elliott, & Takamura, 1984; Refinetti, 2002; vanderLeest, Rohling, Michel, & Meijer, 2009). Mice in long days also show more rapid re-entrainment to a phase shift of the LD cycle (Ramkisoensing et al., 2014). Concurrent with the changes in behavioural rhythms, there is a change in the waveform of the circadian rhythms of SCN neural activity, characterized by a lower and broader mid-day peak in the population firing rate of SCN neurons (Buijink et al., 2016; Schaap et al., 2003; VanderLeest et al., 2007). Similar changes are evident in circadian rhythms of clock gene expression in mice (Inagaki, Honma, Ono, Tanahashi, & Honma, 2007; Naito, Watanabe, Tei, Yoshimura, & Ebihara, 2008), and in other species (Hazlerigg, Ebling, & Johnston, 2005; Sumová, Trávnícková, Peters, Schwartz, & Illnerová, 1995). These changes in circadian rhythm parameters at the behavioural and neuronal levels have been interpreted as reflecting an effect of photoperiod on phase relations among the multiple oscillators that comprise the SCN pacemaker (Evans & Gorman, 2016; Jagota, de la Iglesia, & Schwartz, 2000; Pittendrigh & Daan, 1976b; Refinetti, 2002). Analyses of the circadian rhythms of SCN firing rate indicate that under long days, there is a greater dispersion in the timing of the daily peak firing rate at the single neuron level, reflecting reduced synchrony among SCN clock cells (Brown & Piggins, 2009; VanderLeest et al., 2007). Given that firing rate represents neuronal output, dispersion of peak firing rates within a population of coupled SCN clock cells presumably translates into a lower amplitude, and thus weaker, population output in

the midday. This leads to a prediction that the ability of SCN output to oppose behavioural activity during the day should be reduced in mice entrained to long days, and that this will permit enhanced expression of FAA to a daytime meal.

Almost all studies of FAA in mice housed in LD have employed a standard 12-hour photoperiod. There is one report that FAA is enhanced in mice housed with running wheels under long days (18h) compared to mice in short days (6h) (Pendergast et al., 2009), although the sample size was limited (only 4 wildtype mice). Unlike mice, Syrian and Siberian hamsters are photoperiodic breeders, and are normally housed in long photoperiods (e.g., 14h) to prevent gonadal regression in response to shorter photoperiods. Siberian hamsters housed with running wheels exhibited enhanced FAA under short days compared to long days (Bradley & Prendergast, 2014). By contrast, Syrian hamsters housed with wheels showed no effect of photoperiod on FAA, while those housed without wheels showed reduced FAA in short days (Dantas-Ferreira et al., 2015). These mixed results may be attributable to differences in species, parameters of the feeding schedules, or housing conditions.

Current Research

Given that mice are currently the species of choice for neurogenetic studies of circadian mechanisms in mammals, we sought to substantiate the reported effect of photoperiod on FAA in this species, and to determine whether any effects where dependent on access to a running disc. We confirmed that exposure to long photoperiods compresses α, shortens τ in DD and reduces the magnitude of phase shifts to light pulses, in mice with free access to food. When food was restricted to a 4h daily meal beginning 4h before lights-off, there was an effect of photoperiod that depended on the availability of a running disc; FAA duration and magnitude (activity counts as a percent of total daily activity) were increased in long days (16h) in mice with running discs, but were decreased in long days in mice without discs, compared to mice in short days (8h). When mealtime was centered in the light period, photoperiod (8, 12, 16 or 18h) had no systematic effect on FAA parameters, measured with or without running discs. Also, the effect of photoperiod on τ in DD that was observed prior to food restriction was absent in DD following food restriction. These results indicate that in mice, photoperiod is not a major determinant of FAA when food is restricted to the middle of the light period, possibly

because mid-day feeding, or associated waking and locomotion, attenuates or blocks the effect of photoperiod on the SCN pacemaker.

Chapter 2.

Materials & Methods

2.1. Animals and Apparatus

Male, C57/BL6 mice (n=120, Charles River, Quebec) were acquired at 5 weeks of age. The mice were housed in standard clear plastic cages (20.5 cm x 14 cm x 36.5 cm) with enriched bedding and a plastic igloo house (11cm diameter, 5.7 cm tall; Igloo Fast-Trac, BioServ) with or without a horizontal running disc, depending on group assignment. The cages were housed in sound attenuated, ventilated cabinets (n=10 per cabinet), with programmable lighting provided by white LEDs (~15 lux measured from the floor of each cage). Room temperature was ~22°C. Locomotor activity was monitored using infrared motion sensors above each cage or magnetic sensors attached to the running discs. Activity was recorded continuously using the Clocklab data acquisition and analysis system (Actimetrics, Wilmette Illinois). All experimental procedures were approved by the University Animal Care Committee at Simon Fraser University (protocol #1106-P-09).

2.2. Photoperiod and Feeding Schedules

The mice were tested in 6 groups of 20 (Figure 2-1). Within each group, half of the mice had free access to a horizontal running disc. To quantify the effects of photoperiod on circadian rhythm parameters before, during and after restricted feeding, the following schedules were implemented. Mice in Groups 1 and 2 were entrained to light-dark (LD) cycles consisting of 16h (L16) and 8h (L8) of light, respectively, for 7 weeks. The LD cycles were then reduced to skeleton photoperiods consisting of 60 min of light twice daily, corresponding to the beginning (dawn) and end (dusk) of the light period, for 12 days (see section 2.3 for rationale). To confirm that these photoperiods affect the free-running period and the phase shift response to light, the LD cycle was then replaced by DD for 14 days. The mice were exposed to a 15-min light pulse on day 5 of DD. The mice were then re-entrained to full L16 or L8 photoperiods for 4 weeks with food available ad-libitum. Scheduled feeding was initiated by removing food at lights-off and returning food 20 h later, 4-h before lights-off (Zeitgeber Time (ZT) 8, where ZT12 is lights-off by convention). The daily mealtime was gradually reduced from 12h to 4h in 2h increments every two

days, with meal onset fixed at ZT8. This 'late-day' feeding schedule was maintained for 6 weeks.

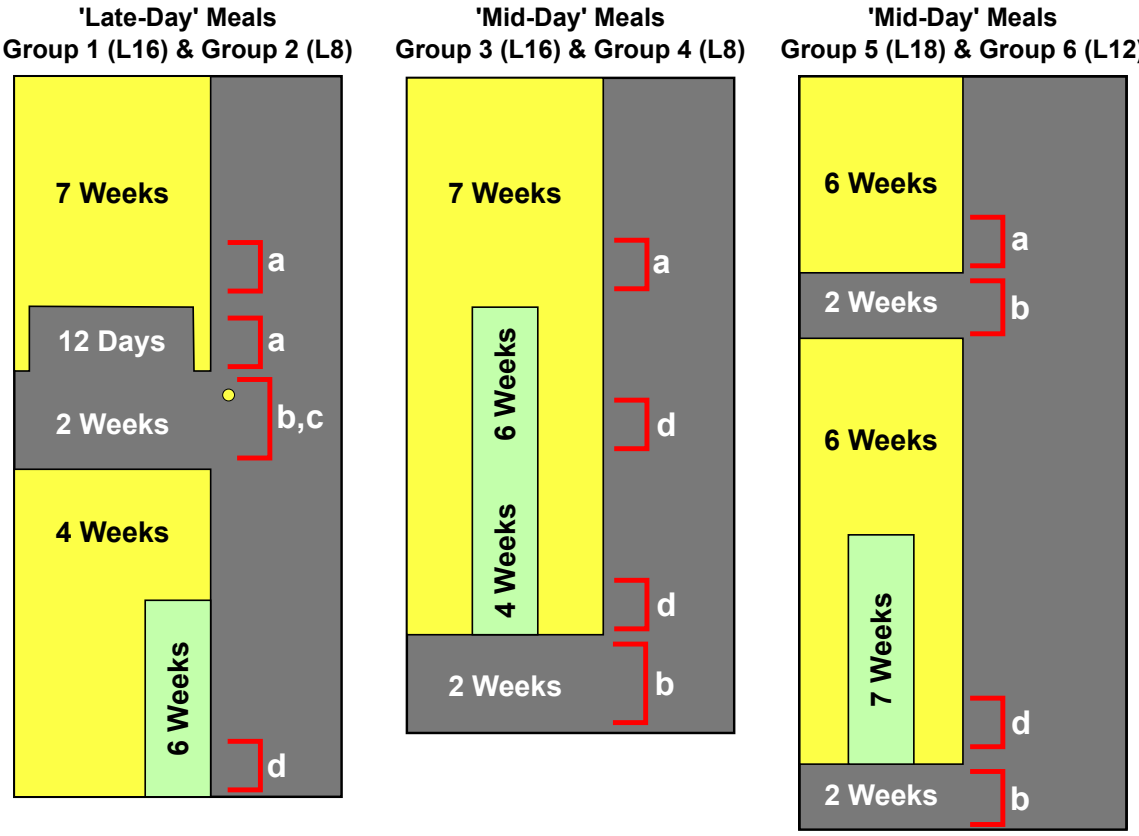

Figure 2.1 Timeline of lighting schedules and food availability in groups 1 & 2 (late-day meals) and groups 3-6 (mid-day meals).

Lowercase letters represent sampling periods for analysis of circadian rhythm parameters. (a) Represents the 5-day sampling periods for alpha and phi calculations. (b) Periods of constant darkness used for tau calculations. (c) Phase shifts to light pulses. (d) FAA ratios and durations. Time of day is plotted left to right; duration of study is plotted top to bottom. Yellow shading represents time of lights on, grey shading time of lights off and green shading time of food availability.

Group 3 and 4 mice were also entrained to L16 and L8 photoperiods, respectively, with food available ad-libitum, for 7 weeks. Food availability was then reduced incrementally from 12 h/day to 4 h/day, but timed so that the target 4 h meal was centered in the light period (i.e., 'mid-day' feeding schedules, with meal onsets 6 h and 2 h after lights-on, respectively). After 10 weeks, food was provided ad-libitum and the LD cycle was replaced by DD for 2 weeks.

Group 5 and 6 mice were maintained on L18 and L12 schedules, respectively, for 6 weeks, followed by DD for 2 weeks. The mice were then re-entrained to L18 and L12

for 6 weeks, after which restricted feeding was initiated. Food availability was reduced incrementally from 12 h/day to 4 h/day, with the 4h meal centered in the light period (i.e., meal onsets 7 h and 4 h after lights-on, respectively). After 7 weeks, food was provided ad-libitum and the LD cycle was replaced by DD for 2 weeks.

2.3. Phase Shifts to Light

The magnitude of phase shifts to light pulses in DD varies with the prior photoperiod. This has been interpreted as reflecting an effect of photoperiod on the amplitude of the SCN pacemaker (Refinetti, 2002; vanderLeest et al., 2009). In these prior experiments, mice were housed in long or short full photoperiods (e.g., 8 or 16 continuous hours of light per day) and then released into DD to measure phase shifts to single light pulses. Mice in different photoperiods thus had a history of exposure to different amounts of light. This raises the possibility that smaller shifts to light pulses in mice previously exposed to long photoperiods might reflect a long-term adaptation effect. To confirm that prior photoperiod affects the magnitude of phase shift to a light pulse in DD, and strengthen the evidence that this is due to an effect on pacemaker amplitude, mice in Groups 1 and 2 were entrained to skeleton photoperiods prior to DD. Skeleton photoperiods limit light exposure to the beginning and end of the light period, simulating dawn and dusk. This amount of light is typically sufficient to maintain stable entrainment, while eliminating group differences in total daily light exposure (Challet, Poirel, Malan, & Pévet, 2003; Rosenwasser, Boulos, & Terman, 1983). In the present study, the skeleton photoperiod was maintained for 12 days before DD. On day 5 of DD, the mice were exposed to a 15-minute light pulse (300 lux) 3h after the beginning of the night of the previous LD cycle. This corresponded approximately to circadian time (CT) 15 (where CT12 is the onset of the daily active period, and corresponds to ~ZT12 in mice entrained to LD). Although each animal received the light pulse at the same external time, the actual CT of light exposure varied slightly depending on the period of the free-running rhythm. Post hoc analysis showed that despite these minor individual differences, the average CT of light exposure was equivalent in the long day and short day groups (t_{35} =0.47, p=0.64). Therefore, any group differences in phase shift magnitude were not due to differences in the phase at which the light pulses occurred. Activity was monitored for another 9 days in DD to determine the maximum phase delay produced by the pulse.

2.4. Analysis of Circadian Rhythm Parameters

Activity counts were exported from Clocklab in 10-min bins for plotting actograms (Circadia) and average waveforms (GraphPad Prism 7.0, GraphPad Software, Inc., La Jolla, CA). Circadian rhythm parameters were quantified using the Clocklab routine for automated detection of the beginning and ending of the daily active period (with manual adjustments for outliers). The α was calculated as the time between activity onsets and offsets averaged over the last 5 days of the LD cycle, prior to restricted feeding. The timing of nocturnal activity onset relative to lights-off (Φ_{LD}; the phase of entrainment) was calculated as the difference in minutes, where by convention onsets preceding lights-off were assigned positive values, and onsets following lights-off negative values. Free-running τ of activity rhythms in DD was quantified using the chi-square periodogram in Clocklab. The magnitude of the phase shift induced by a 15-minute bright light pulse in DD was calculated by comparing separate regression lines fit to activity onsets on the five days before the light pulse, and the seven days after the pulse. The time difference between the two regression lines extrapolated to the day after the light pulse is the amount of phase shift. FAA was quantified as a duration and a ratio. FAA duration represents the timing of FAA relative to mealtime, and was obtained by calculating the average onset of activity in minutes prior to mealtime. The FAA ratio was obtained by summing activity counts during the 3 hours before mealtime and dividing by total daily activity, excluding the 4-hour mealtime. All FAA parameters represent an average over 5 days during week 6 and, if applicable, week 10 of restricted feeding.

2.5. Inferential Statistics

The effects of photoperiod (L18, L16, L12, L8) and activity type (disc running or general activity) on circadian variables (α, Φ_{LD}, τ, phase shifts to light, FAA duration, FAA ratio) were evaluated using one-way repeated measures ANOVA, two-way ANOVA, or independent samples t-tests (Prism 7.0), as appropriate. Post-hoc tests maintained family-wise error rates at 5%. Group averages are reported ± S.E.M.

Chapter 3.

Results

3.1. Photoperiod modifies activity rhythm parameters in mice fed ad-libitum

Mice in groups 1 (L16) and 2 (L8) were first entrained to a full photoperiod which was then reduced to a skeleton photoperiod for 12 days prior to assessing τ and the phase shift response to a light pulse in DD. Visual inspection of the averaged waveforms indicates that under the full photoperiods, α duration was compressed in the L16 group compared to the L8 group (Figure 3.1), and that this effect was maintained in the skeleton photoperiod. Mice housed without running discs showed a more bimodal activity waveform compared to mice with running discs, with activity peaks occurring at both the beginning and the end of the night.

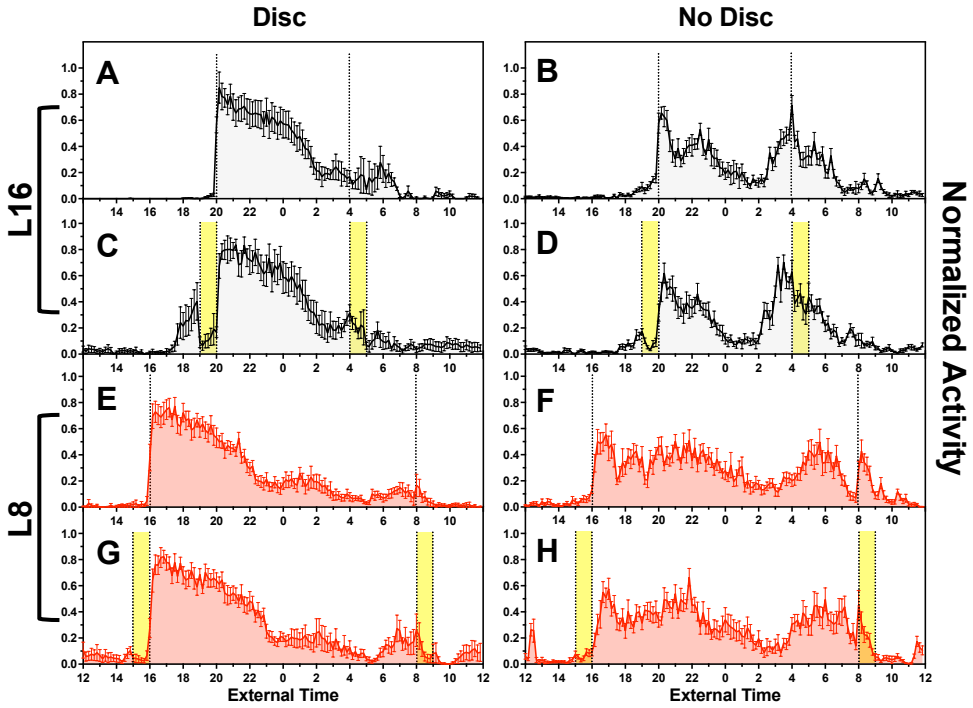

Figure 3.1 Averaged activity waveforms of mice housed in L16 (A-D, group 1) and L8 (E-H, group 2) with access to a running disc (left panel) or not (right panel).

(A,B) L16 full photoperiod, (C,D) L16 skeleton photoperiod, (E,F) L8 Full photoperiod and (G,H) L8 skeleton photoperiod. Locomotor activity was averaged over the last 5 days of the LD cycle, prior

to any food or lighting manipulations. To prevent overly active mice from skewing group averages, absolute activity in each of the 144 daily 10-min bins was first converted to a percentage of the highest daily activity count, then each bin averaged over 5 days for each animal. Waveforms represent the average of 7-10 animals in each condition. Dotted lines denote beginning and end of the daily dark cycle, yellow bars represent 1-hour of 'dawn' and 'dusk' in a skeleton photoperiod.

A two-way ANOVA of α duration in groups 1 (L16) and 2 (L8) confirmed a significant effect of photoperiod ($F_{1,31}$ = 32.77, p<0.0001), and disc availability ($F_{1,31}$ = 9.90, p=0.004) and no significant interaction ($F_{1,31}$ = 0.052, p=0.82). Post hoc tests showed that α duration was significantly shorter in L16 mice with (p=0.009) and without (p=0.0004) running discs (Table 1). Separate repeated measures one-way ANOVAs of α under the full photoperiod, skeleton photoperiod and first day of DD revealed no change across these lighting conditions in any group (L16 disc: $F_{2,12}$ = 3.12, p = 0.09, n=7; L16 no disc: $F_{2,18}$ = 0.48, p = 0.59, n=10; L8 disc: $F_{2,14}$ = 4.33, p=0.07, n=8; L8 no disc: $F_{2,18}$ = 1.76, p=0.22, n=10). Thus, the effects of full photoperiods on α persisted in the skeleton photoperiod and in DD. Three mice in Group 1 and two mice in Group 2 exhibited an unstable phase of entrainment to the skeleton photoperiod, and were therefore excluded from this analysis.

Table 1 **Group averages for Alpha, Tau and Phase of entrainment in a full photoperiod prior to light or food manipulations. Values are mean ± SEM (N).**

	Group		α	τ	Φ_{LD}
Disc	5	L18	7.44 ± 0.43 (9)	23.07 ± 0.17 (9)	25.8 ± 9.18 (9)
	3	L16	10.76 ± 0.40 (10)	---	35.11 ± 6.03 (10)
	1	L16	9.18 ± 0.63 (7)	23.27 ± 0.15 (7)	17.13 ± 15.92 (7)
	6	L12	11.55 ± 0.45 (10)	23.45 ± 0.07 (10)	3.16 ± 2.6 (10)
	4	L8	14.20 ± 0.63 (10)	---	-2.60 ± 12.95 (10)
	2	L8	13.71 ± 1.26 (8)	23.68 ± 0.06 (9)	-6.01 ± 2.27 (8)
No Disc	5	L18	7.72 ± 0.22 (10)	23.59 ± 0.03 (10)	16.56 ± 5.21 (10)
	3	L16	10.6 ± 0.17 (10)	---	47.16 ± 5.23 (10)
	1	L16	11.75 ± 0.29 (10)	23.68 ± 0.04 (10)	36.54 ± 8.03 (10)
	6	L12	11.95 ± 0.33 (10)	23.78 ± 0.03 (10)	-1.20 ± 2.84 (10)
	4	L8	15.73 ± 0.27 (10)	---	-22.67 ± 4.74 (10)
	2	L8	15.92 ± 0.64 (10)	23.95 ± 0.04 (10)	-2.47 ± 3.16 (10)

Mice in groups 3-6 were entrained to L18, L16, L12 or L8 full photoperiods, with or without access to a running disc, prior to restricted feeding. Visual inspection of the averaged waveforms indicates that α was compressed as photoperiod lengthened, regardless of disc availability (Figure 3.2). Two-way ANOVA revealed a marginal effect of disc availability ($F_{1,71}$ = 3.56, p=0.06), a predicted strong effect of photoperiod ($F_{3,71}$=123.2, p<0.001), and no significant interaction ($F_{3,71}$ = 1.77, p=0.16). The longer the photoperiod, the shorter the duration of the nocturnal activity period (Table 1, Figure 3.3A).

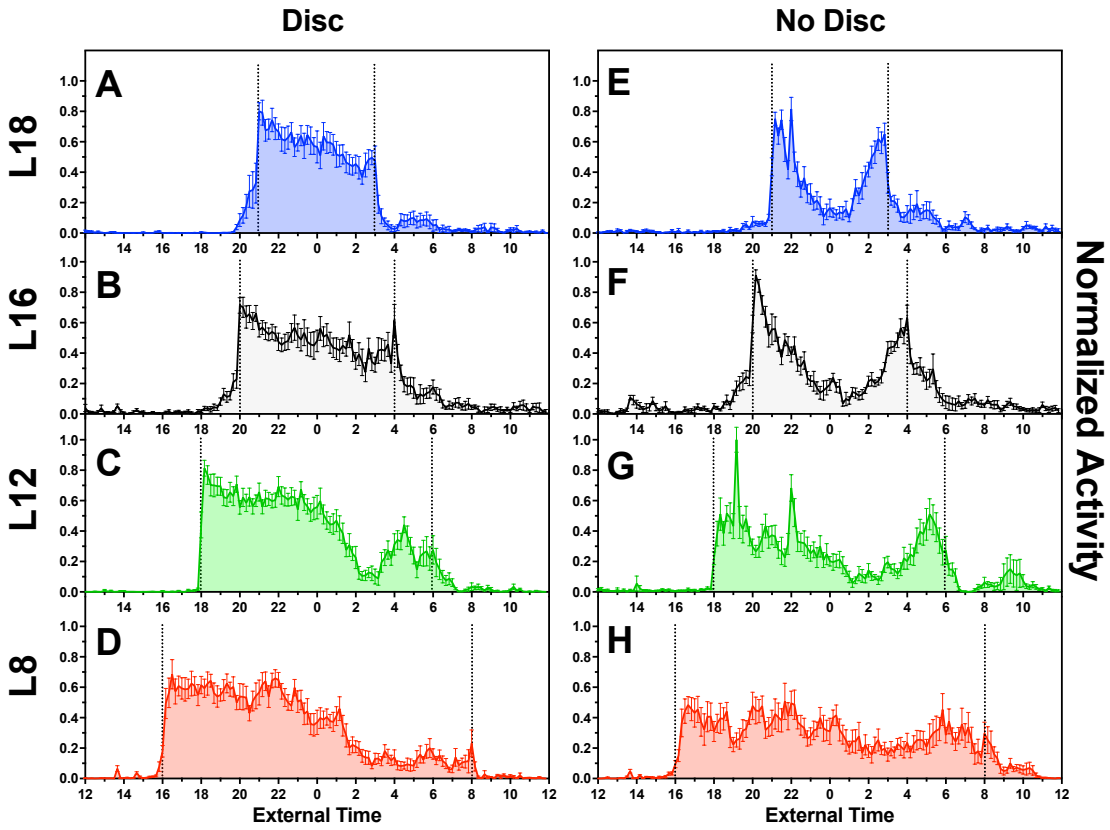

Figure 3.2 Averaged activity waveforms of mice housed in L18 (A, E), L16 (B, F), L12 (C,G) and L8 (D,H) with access to a running disc (left panel) or not (right panel).

Locomotor activity was averaged over the last 5 days of the LD cycle, prior to any food or lighting manipulations. To prevent overly active mice from skewing group averages, absolute activity in each of the 144 daily 10-min bins was first converted to a percentage of the highest daily activity count, then each bin averaged over 5 days for each animal. Waveforms represent the average of 9 or 10 animals in each condition. Dotted lines denote beginning and end of the daily dark cycle.

Two-way ANOVA of Φ_{LD} revealed no effect of disc availability ($F_{1,71}$ = 2.74, p=0.10), a significant effect of photoperiod ($F_{3,71}$ =41.52, p<0.001) and a significant interaction ($F_{3,71}$ =3.47, p=0.02). The main effect of photoperiod indicates an overall trend for earlier activity onsets in longer photoperiods (Table 1; Figure 3.3B). Post hoc tests reveal that the interaction was likely driven largely by early risers in the L16 group without running discs.

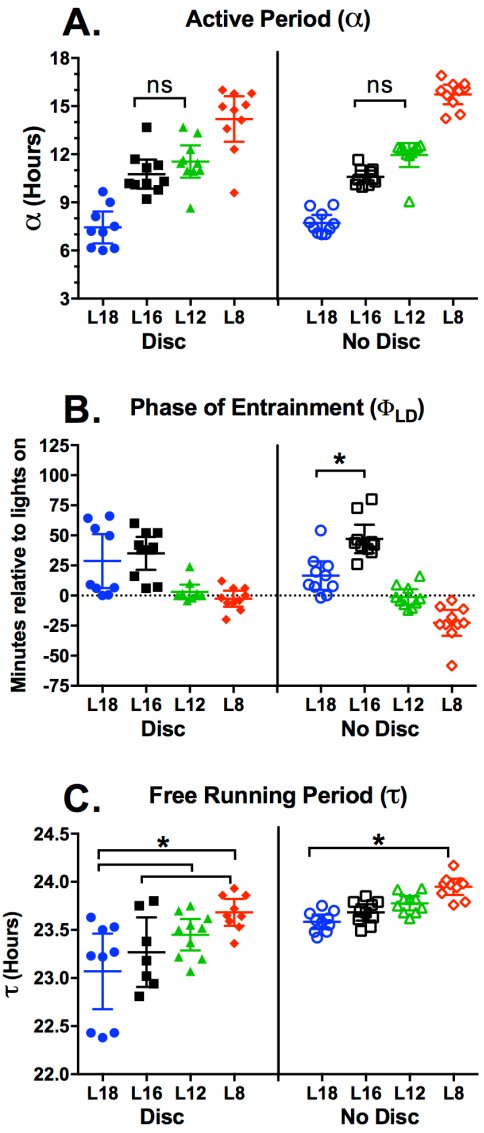

Figure 3.3 Individual scores for α (A), Φ_{LD} (B) and τ (C).
(A) Average active period, in hours for mice housed in a light-dark cycle. (B) Average activity onsets relative to lights off, positive values represent onsets prior to lights off and negative values represent onsets after lights off. (C) Free-running period of mice in DD prior to restricted feeding. Lines represent mean + 95% confidence intervals.

3.2. Photoperiod modifies τ and phase shifts to light in DD prior to scheduled feeding

An effect of photoperiod on τ was evaluated using mice tested in DD prior to restricted feeding, which includes groups 1 (L16), 2 (L8), 5 (L18) and 6 (L12). Two-way ANOVA of τ revealed a main effect of disc availability ($F_{1,67}$ = 42.92, p<0.001) and photoperiod ($F_{3,67}$ = 13.02, p<0.001) and no significant interaction ($F_{3,67}$ = 0.90, p=0.45). As predicted, τ was shorter in mice previously housed in longer photoperiods. Also consistent with prior reports (Edgar, Kilduff, Martin, & Dement, 1991; Edgar, Martin, & Dement, 1991; Mistlberger, Bossert, Holmes, & Marchant, 1998), τ was shorter in mice with running discs, an effect that was statistically significant in the L12, L16 and L18 groups (p<0.05, Sidak post-hoc tests) (Table 1; Figure 3.3C).

The effect of photoperiod on phase shifts to light pulses was evaluated in groups 1 (L16) and 2 (L8) in DD after 12 days of entrainment to skeleton photoperiods. On day 5 of DD, the mice in these groups were exposed to a 15-min light pulse at ~CT15. This induced a phase delay shift of the free running rhythm in all mice, with a range of 23-174 minutes in the L16 group, and 61–305 minutes in the L8 group (Figure 3.4). Consistent with an earlier study (Mistlberger & Holmes, 2000), shifts did not differ between mice with and without running discs in either photoperiod (L8: t_{18} = 0.83, p=0.42; L16: t_{15} = 0.67, p=0.51), therefore the groups were pooled by photoperiod. As predicted, a larger phase delay was observed in the short (L8) photoperiod group (-127 ± 11 min) compared to the long (L16) photoperiod group (-99 ± 11 min, t_{35} = 1.70, p=0.049, one-tailed).

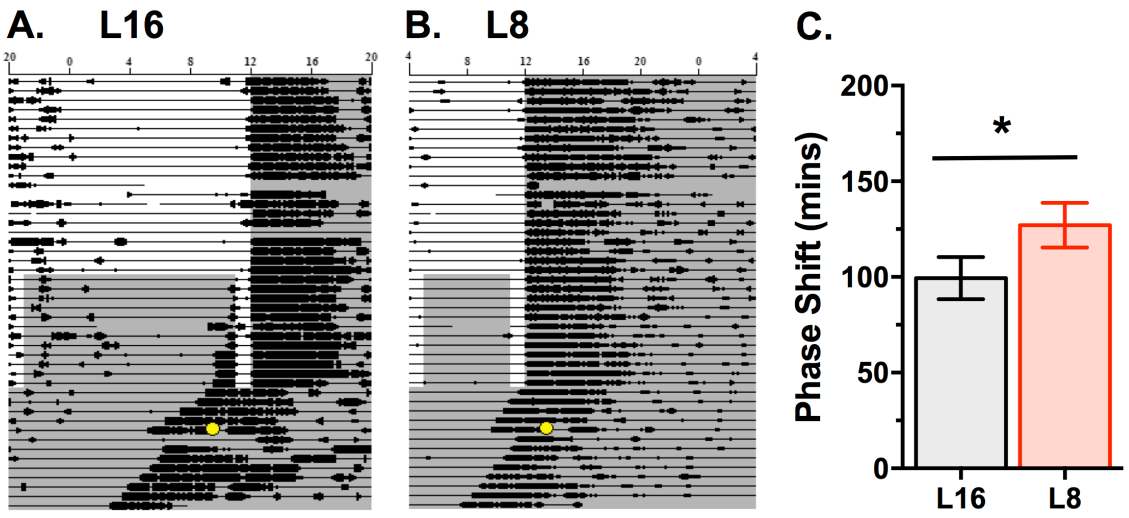

Figure 3.4 Phase delays of locomotor activity to a 15-minute bright light pulse at CT15.

(A), (B) Representative actograms of disc running in a mouse in L16 (B) and L8 (C). Each line represents 24-hours of recording, with time in 10 min activity bins plotted from left to right, and consecutive days aligned vertically. A 15min, 300lux light pulse is represented as a yellow circle on DD day 5. (C) Average phase delay (± SEM), in minutes to the light pulse.

3.3. Photoperiod interacts with running disc availability to modulate FAA in mice on late-day feeding schedule

After 12 days in DD to measure phase shifts to light pulses, mice in Groups 1 (L16) and 2 (L8) were re-entrained to LD, and then limited to a 4h daily meal late in the light period (ZT8-12). All mice in both photoperiods exhibited robust FAA regardless of disc availability (e.g., Figure 3.5A-D). Inspection of the average waveforms for the last week of restricted feeding reveals an unexpected interaction between photoperiod and activity type (Figure 3.6A,B). For FAA ratio, there was no main effect of photoperiod ($F_{1,33}$ = 0.21, p=0.64) or activity type ($F_{1,33}$ = 0.36, p=0.55) but the interaction was significant ($F_{1,33}$ = 11.73, p=0.002). Post-hoc tests (Sidak multiple comparison test) indicate that in mice with running discs, there was a trend for FAA ratios to be greater in the L16 group compared to the L8 group (p=0.08). In mice without a running disc, the difference between photoperiods was significant, but in the reverse direction; FAA ratio was greater in the L8 group (p=0.02) (Figure 3.6C).

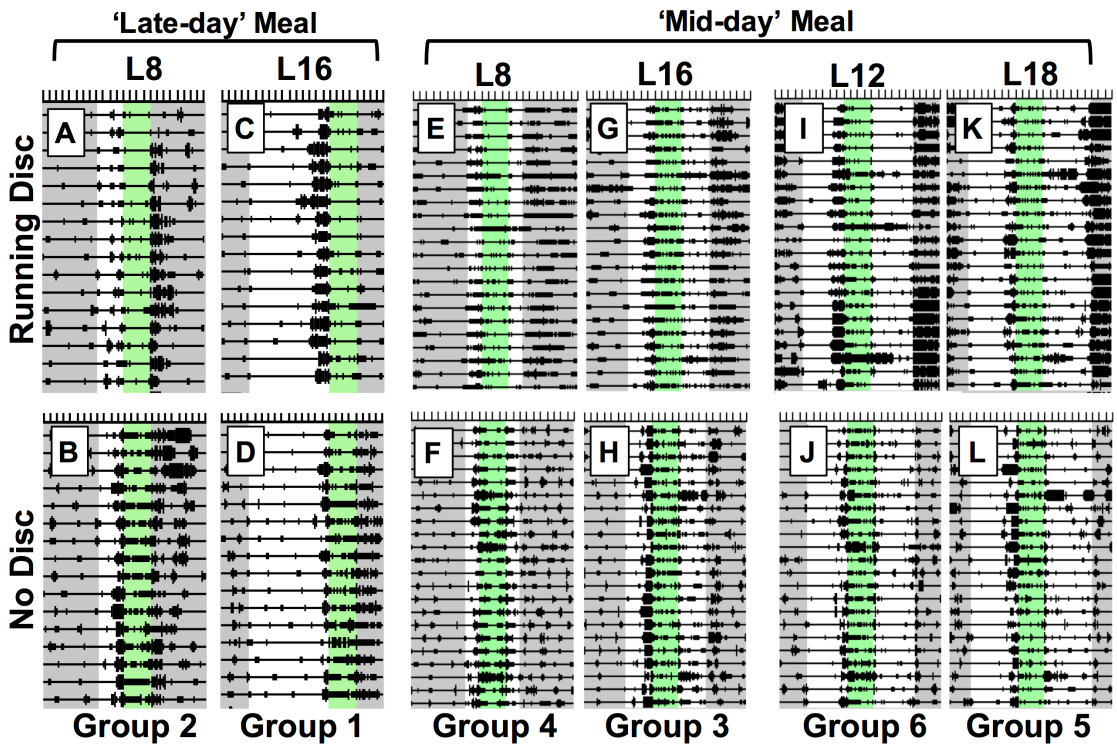

Figure 3.5 Representative actograms of locomotor activity in mice fed a 'late-day' meal (A-D) or a 'mid-day' meal (E-L).

Each line represents 24-hours of recording, with time in 10 min activity bins plotted from left to right, and consecutive days aligned vertically. Green shading indicates the 4-hour meal availability and grey shading time of lights off.

For FAA duration, there was again no significant main effect of photoperiod ($F_{1,33}$ = 1.22, p=0.28), but there was a significant effect of activity type ($F_{1,33}$ = 16.36, p=0.0003) and a significant interaction ($F_{1,33}$ = 5.94, p=0.02). Post hoc tests revealed that in mice with running discs, FAA duration was longer in the L16 group (p=0.03), whereas in mice without running discs, FAA duration was longer in the L8 group, although the difference did not reach significance (p>0.05) (Figure 3.6D). Overall, FAA duration was greater in mice housed with a running disc.

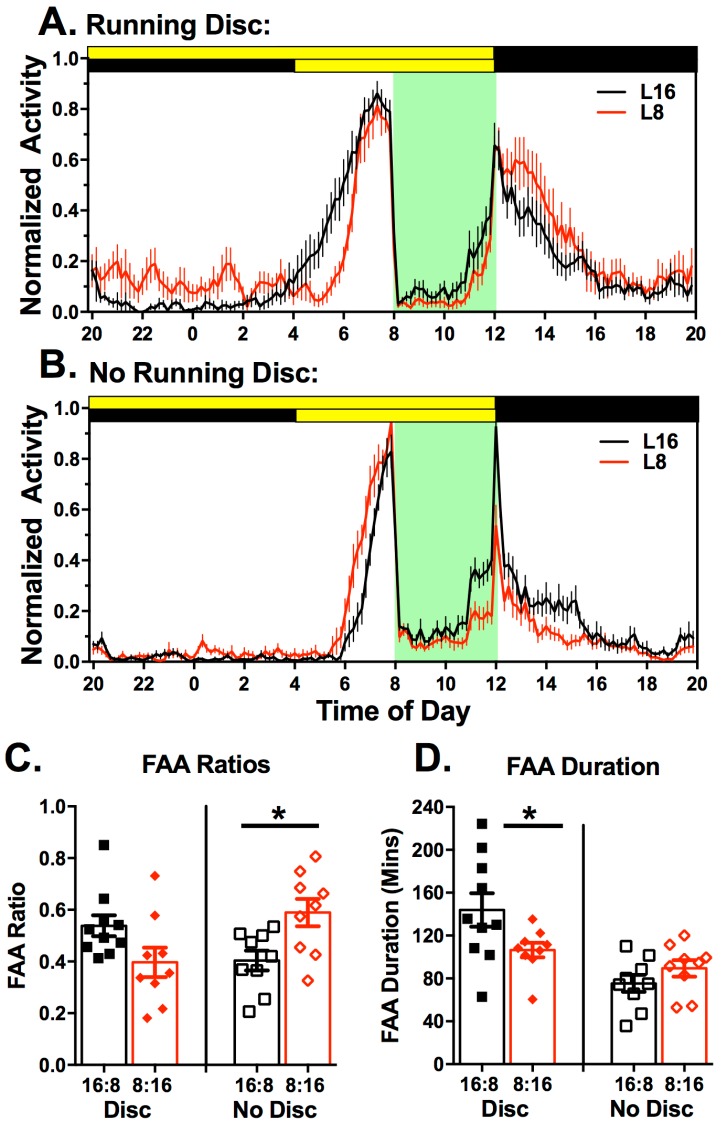

Figure 3.6 Food anticipatory activity in mice given a 4-hour meal prior to lights off.

Average locomotor activity waveforms for mice with (A) running discs and (B) no running discs. Activity data for individual mice were first normalized and then averaged across the last 5 days of restricted feeding. Group means are plotted ± SEM. Mealtimes are denoted by green shading. Yellow bars represent time of light on. Average FAA ratios (C) and FAA durations (D) in mice housed in L16 (black) and L8 (red). Significance at <0.05 denoted by an asterisk, Sidak post-hoc multiple comparisons test.

3.4. No effect of photoperiod on FAA in mice on mid-day feeding schedule

Groups 1 and 2 mice were fed during the last 4h of the daily light period. To determine whether the unexpected interaction between photoperiod and activity type would generalize to other mealtimes in the light period, mice in groups 3 and 4 were entrained to L8 or L16, with or without running discs, and then restricted to a 4h daily meal centered in the light period. To examine additional photoperiods, mice in groups 5 and 6 were entrained to L12 or L18, and 4h daily meals were also centered in the light period.

All mice exhibited robust FAA regardless of photoperiod or disc availability (e.g., Figure 3.5E-L; Figure 3.7A-D). Two-way ANOVA of FAA ratios evaluated at week 6 of restricted feeding revealed no main effect of photoperiod ($F_{3,71}$ = 1.33, p=0.27), but a significant main effect of activity type ($F_{1,71}$ = 10.69, p<0.002) and a significant interaction ($F_{3,71}$ = 6.56, p=0.005). FAA ratios tended to be higher in mice housed without running discs, except in the L8 and L16 photoperiods (Table 2, Figure 3.7C).

Table 2 Group averages for FAA ratio and duration in mice fed a 4-hour meal centered in the light period. Values are mean ± SEM (N).

		FAA Ratio		FAA Duration (mins)	
		Week 6	Week 10	Week 6	Week 10
Disc	**L18**	0.21 ± 0.04	---	109.80 ± 8.96	---
	L16	0.23 ± 0.04	0.20 ± 0.04	111.10 ± 9.39	97.63 ± 8.78
	L12	0.12 ± 0.02	---	70.93 ± 6.68	---
	L8	0.23 ± 0.03	0.25 ± 0.02	101.80 ± 9.28	101.80 ± 7.28
No Disc	**L18**	0.35 ± 0.03	---	75.33 ± 5.77	---
	L16	0.19 ± 0.03	0.40 ± 0.05 **	70.90 ± 3.90	77.84 ± 8.14
	L12	0.36 ± 0.05	---	72.61 ± 4.52	---
	L8	0.23 ± 0.04	0.24 ± 0.04	78.49 ± 7.87	71.20 ± 8.33

** Indicates significant difference between week 6 and week 10 values (p>0.05), paired samples t-test.

Two-way ANOVA of FAA duration evaluated at week 6 of restricted feeding revealed a main effect of photoperiod ($F_{3,71}$ = 3.63, p=0.02) and activity type ($F_{1,71}$ = 21.81, p=<0.0001) and a significant interaction ($F_{3,71}$ = 3.26, p=0.03). The main effect of photoperiod and the interaction appear to be driven by a significantly shorter FAA duration in the L12 mice housed with running discs, compared to the L18 (p=0.003), L16 (p=0.001) and L8 (p=0.02) groups with discs (Figure 3.7D). FAA duration across photoperiods was longer in mice housed with a disc compared to those without a disc.

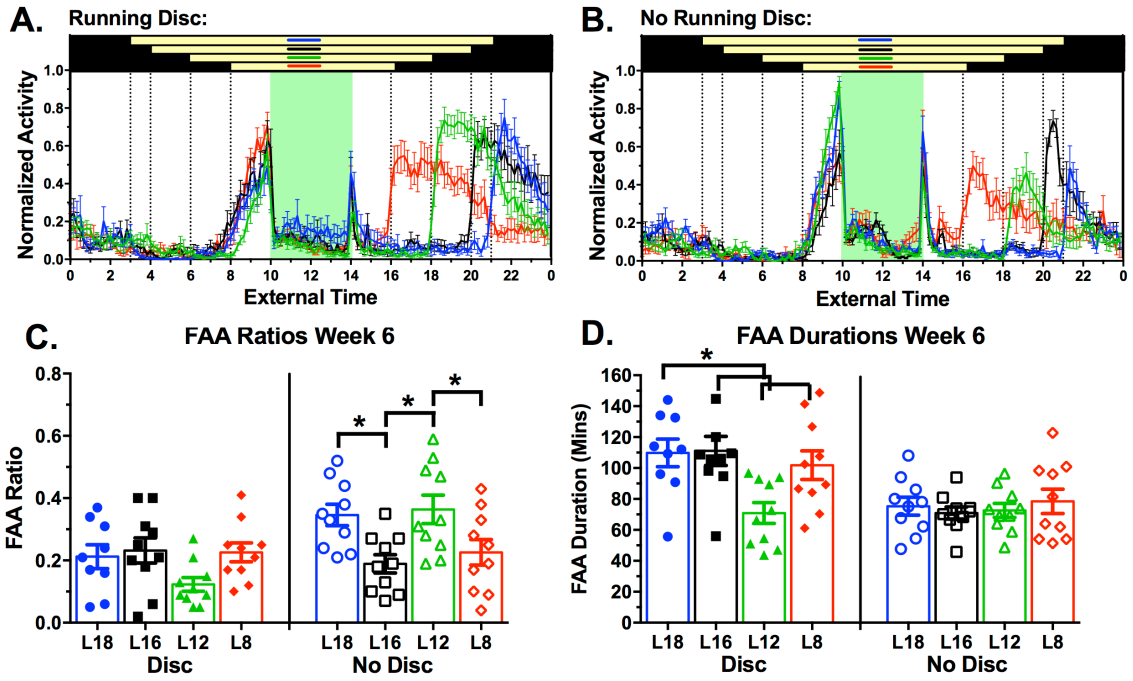

Figure 3.7 Food anticipatory activity in mice fed a 4-hour meal centered in the light period.

Average locomotor activity waveforms for mice with (A) running discs and (B) no running discs. Activity data for individual mice were first normalized and then averaged across 5 days of restricted feeding in week 6. Group means are plotted ± SEM. Mealtimes are denoted by green shading. Yellow bars represent time of light on. Average FAA ratios (C) and FAA durations (D) in mice housed in L18 (blue), L16 (black), L12 (green) and L8 (red). Significance at <0.05 denoted by an asterisk, Sidak post-hoc multiple comparisons test.

To determine if photoperiod effects on FAA, such as observed in groups 1 and 2, might emerge with longer exposure to restricted feeding, groups 3 (L16) and 4 (L8) were maintained on restricted feeding for an additional 4 weeks. A within-subject's comparison of FAA ratios between week 6 and week 10 (Figure 3.8A) revealed no differences in mice housed with running discs (L16: $t_9 = 0.81$, p=0.44; L8: $t_9 = 0.63$, p=0.54). In mice without running discs, FAA ratios were significantly lower in week 6 compared to week 10 in the L16 group ($t_9 = 4.30$, p=0.002) but not in the L8 group ($t_9 = 0.41$, p=0.69). FAA duration (Figure 3.8B) did not differ between weeks 6 and 10 in any group (L16 disc: $t_9 = 0.92$, p=0.38; L16 no disc: $t_9 = 1.03$, p=0.33; L8 disc: $t_9 = 0.007$, p=0.99; L8 no disc: $t_9 = 0.76$, p=0.47) (Table 2).

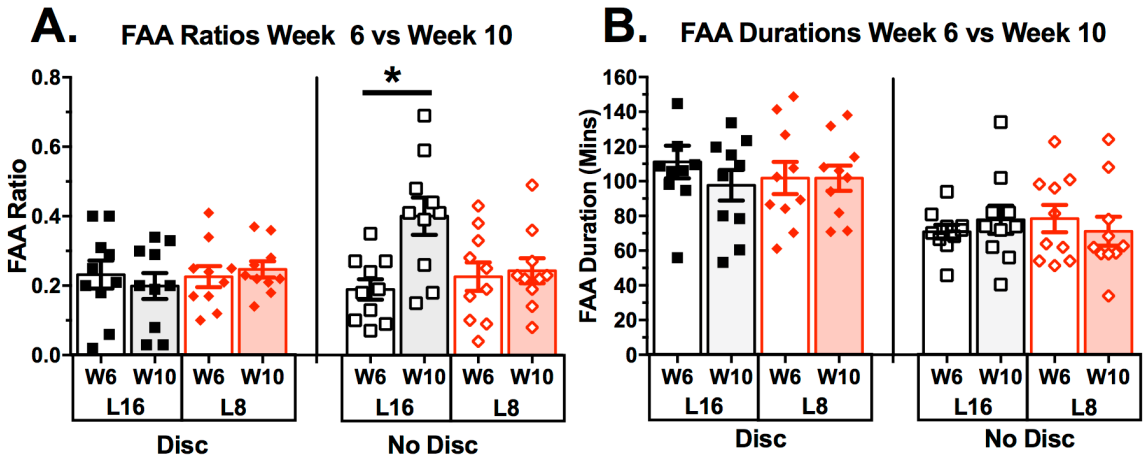

Figure 3.8 Food anticipatory activity during week 6 and week 10 in mice fed a 4-hour meal centered in the light period.

Group mean (± SEM) average FAA ratios (A) and FAA durations (B) in mice housed in L16 (black squares) and L8 (red diamonds) housed with (closed symbols) and without (open symbols) running discs. Week 6 (W6) and week 10 (W10). Significance at <0.05 denoted by an asterisk, between subjects t-test.

3.5. No effect of photoperiod on τ in DD following mid-day restricted feeding

To assess whether restricted feeding might alter the impact of photoperiod on parameters of the LD entrained SCN pacemaker, mice fed in the mid-day (Groups 3-6) were placed in DD coincident with resumption of ad-libitum food access. Two-way ANOVA of τ revealed no effect of photoperiod ($F_{3,71}$ = 2.01, p=0.12), a significant main effect of activity type ($F_{1,71}$ = 40.18, p< 0.0001) and a significant interaction ($F_{3,71}$ = 2.81, p=0.045) (Figure 3.9). Thus, unlike τ measured prior to restricted feeding (Figure 3.3C), τ measured after restricted feeding did not vary with photoperiod, although it was shorter overall in mice housed with a running disc.

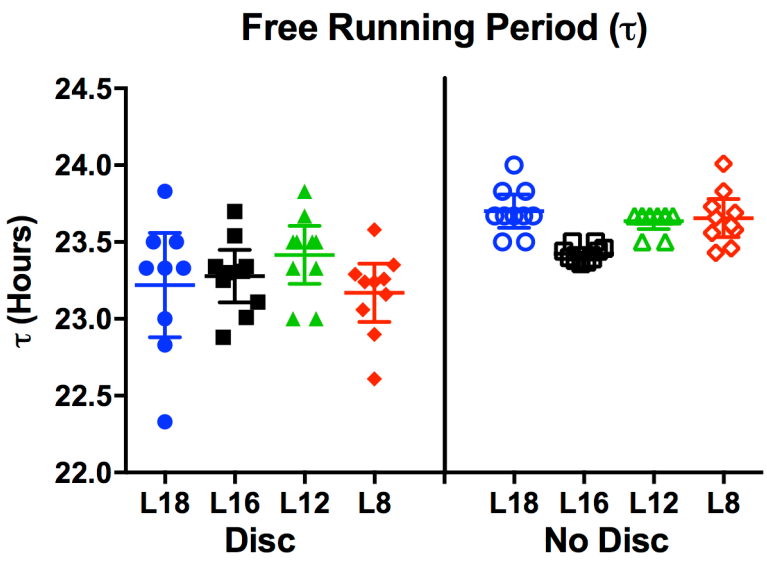

Figure 3.9 Free running period of mice released into DD following mid-day restricted feeding.
Lines represent mean ± 95% confidence intervals.

3.6. FAA is enhanced prior to late-day meals, compared to mid-day meals

To determine whether mealtime within the light period affected FAA magnitude independently of photoperiod, FAA in mice fed in the late-day was compared to FAA in mice fed in the mid-day, under the L16 photoperiod (groups 1 and 3) and L8 photoperiod (groups 2 and 4). Visual inspection of the average waves clearly reveals increased FAA to late-day meals (Figure 3.10A-D). Quantitatively, FAA ratios were greater in mice fed late-day meals compared to mid-day meals when housed with (L16: t_{18} = 5.36, p<0.001; L8: t_{17} = 2.71, p=0.02) or without running discs (L16: t_{17} = 4.93, p=0.0003; L8: t_{17} = 5.49, p<0.0001). FAA duration was longer in late day-fed mice in L16 with running discs compared to their mid-day fed counterparts, but did not reach significance (t_{18} = 1.80, p=0.09). Other differences in FAA duration between mid- and late-day groups were not statistically significant (L8 Disc: t_{17} = 0.40, p=0.70; L16 No Disc: t_{17} = 0.52, p=0.61; L8 No Disc: t_{17} = 0.99, p=0.33) (Figure 3.10E,F).

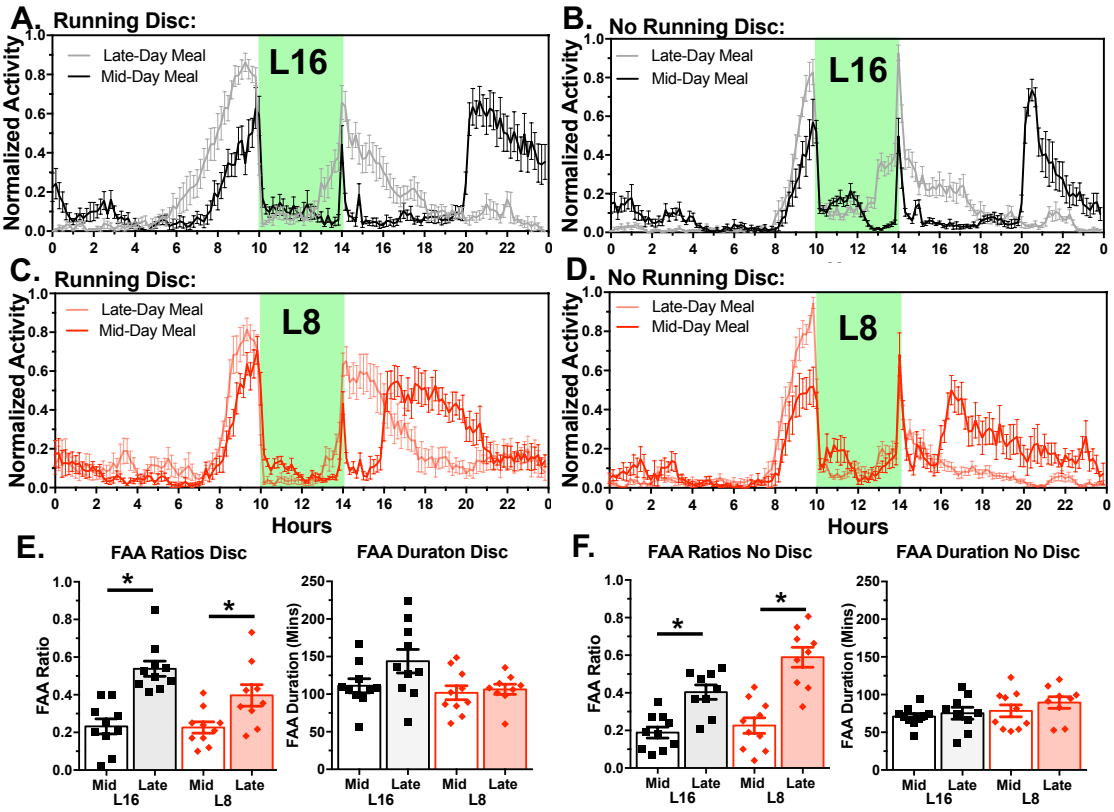

Figure 3.10 Food anticipatory activity in mice fed a 4-hour meal scheduled late-day or mid-day

Average locomotor activity waveforms for mice entrained to L16 (A,B) or L8 (C,D) and fed either in the late-day (heavier curves) or midday (lighter curves). Activity data for individual mice were first normalized and then averaged across 5 days of restricted feeding in week 6. Group means are plotted ± SEM. Mealtimes are denoted by green shading. Group mean FAA ratios and FAA durations in mice housed with (E) and without (F) running discs are plotted ± SEM.

Chapter 4.

Discussion

The daily rhythm of FAA that emerges when food is limited to a fixed time of day is controlled by a circadian timing mechanism with formal properties of an entrainable oscillator. The duration and magnitude of FAA is also affected by environmental, metabolic and hedonic factors independent of circadian timing, including light, temperature, access to a running wheel, body weight, leptin and ghrelin sensitivity, food palatability and fat content, and genetic sex. The objective of the present study was to determine whether FAA is also modified by photoperiod (the duration of the daily light period).

Photoperiod modifies activity rhythm parameters and phase shifts to light in mice fed ad-libitum

Previous studies have shown that photoperiod affects parameters of circadian activity rhythms regulated by the light-entrained SCN pacemaker (Naito et al., 2008; Pittendrigh & Daan, 1976b). Here we confirm that exposure to long days is associated with compression of the active phase, advanced phase of entrainment to the LD cycle, shorter τ in constant conditions and smaller phase shifts in response to a light pulse, in mice housed with or without access to a running disc. By entraining mice to skeleton photoperiods prior to DD, we are able to rule out differences in total daily light exposure as an alternative explanation for smaller phase shifts in the long-day group. To our knowledge this has not been demonstrated previously. These effects have been interpreted as evidence that entrainment to long days reduces SCN amplitude at the network level, by increasing dispersion of peak firing rates within the population of SCN clock cells (Brown & Piggins, 2009; Buijink et al., 2016; VanderLeest et al., 2007)

FAA to late-day meals reveals an unexpected interaction between photoperiod and disc availability

Given the evidence that SCN outputs during the day actively supress locomotion and promote sleep in nocturnal mammals (Mistlberger, 2005), a reduction in the amplitude

of the SCN output rhythm would be expected to enhance FAA in mice fed in the light period. Taken together with the evidence that SCN amplitude is decreased in long days, this leads to the prediction that FAA should be enhanced in mice entrained to long days compared to mice in short days. Consistent with this prediction, there is one report, albeit based on a very small sample (four wildtype mice), that exposure to long days enhances the magnitude of FAA in mice, measured by wheel running (Pendergast et al., 2009). We sought to substantiate this finding, and determine whether availability of a running device matters, as found in a study of Syrian hamsters (Dantas-Ferreira et al., 2015).

Our first approach was to compare FAA in L16 and L8, with food availability restricted to the last 4h of the light period. Photoperiod did affect parameters of FAA in these groups but the direction of the effect varied by activity type. The predicted effect was evident only in mice housed with running discs; among those mice, the group entrained to L16 exhibited enhanced FAA duration and magnitude compared to the group entrained to L8. Unexpectedly, among mice housed *without* running discs, the group entrained to L16 exhibited a *reduced* FAA duration and magnitude compared to the group entrained to L8.

It is not clear how this result should be interpreted. Photoperiod affected α, τ, Φ_{LD} and the magnitude of phase shifts to light pulses equally in mice housed with and without a running disc, which implies an equivalent effect on pacemaker period and amplitude. Without an obvious explanation, we decided to determine whether this effect would generalize to other times of day during the light period. We chose to center mealtimes in the light period, when the circadian rhythm of SCN neural activity normally peaks (Houben, Coomans, & Meijer, 2014), and any differences in SCN amplitude between long and short days should be maximal.

FAA to mid-day meals reveals no effect of photoperiod

Contrary to prediction, when meals were centered in the light period, we observed no consistent effect of photoperiod on FAA. In mice with running discs, FAA duration and magnitude were statistically equivalent in the L8, 16 and 18 groups. The one 'outlier' was the L12 group, which showed a shorter FAA duration and lower FAA ratio. However, neither FAA duration nor ratio exhibited a linear trend across photoperiod. In mice without running discs, FAA duration was statistically equivalent in all 4 photoperiods, while FAA

ratio was lower in the L8 and L16 groups compared to the L18 and L12 groups. Overall, mice with running discs had lower FAA ratios and longer FAA durations compared to mice housed without running discs. As there was no consistent (i.e., linear) effect of photoperiod on FAA, we conclude that FAA is modulated by disc access but not photoperiod.

If SCN amplitude is altered by photoperiod, and if SCN outputs oppose the expression of activity during the light period, then photoperiod should modulate FAA, particularly to meals centered mid-day. The absence of evidence for modulation of FAA by photoperiod when meals are centered in the light period could be explained by an effect of the feeding schedules on SCN response to photoperiod. Notably, the effect of photoperiod on free-running τ that was evident in DD prior to food restriction was absent in DD following food restriction. This suggests that limiting food access to the middle of the light period may attenuate or block the effect of photoperiod on SCN parameters in mice, thereby eliminating any potential secondary effects on FAA. Indeed, in Syrian hamsters, wheel running induced during the rest phase acutely decreases expression of the clock gene *per2* in the SCN (Maywood & Mrosovsky, 2001; Maywood, Mrosovsky, Field, & Hastings, 1999; Yannielli, McKinley Brewer, & Harrington, 2002), while in mice, daytime activity supresses SCN firing rates (Oosterhout et al., 2012). Two studies have assessed neural activity in the SCN during restricted feeding. One study used mice and observed suppression of neural activity in the SCN in 3 of 7 freely moving mice during the FAA window (Dattolo et al., 2016). The other study used rats and did not report a change in neural activity during mealtime, but also did not analyze the data specifically for this (Inouye, 1982). In both of these studies, the mealtimes were 'late-day' rather than centered in the light period. Whether or not daytime restricted feeding modifies the dispersion of SCN neuronal peaks seen in long photoperiods remains to be assessed.

Single-cell electrical recordings of SCN neurons from mice exposed to different day lengths reveal that the amplitude and width of firing of individual neurons does not vary, only their phase distribution (Brown & Piggins, 2009; VanderLeest et al., 2007). This raises the possibility that it is individual cell amplitude that determines the influence of SCN output on behavioural state, and not population output. This seems unlikely, as firing of neurons in synchrony should yield a higher, stronger output signal (e.g., analogous to electrical coupling of neuroendocrine cells to drive high amplitude pulses of hormone release). Analysis of FAA in a mouse model that has reduced single-cell amplitude

because of a mutation in the 'after-hours' (Guilding et al., 2013) gene might be useful to address this question, if the reduction in amplitude at the cellular level is not also associated with dispersion of phase at the population level.

The relationship between SCN synchrony and amplitude

Conventional models view the SCN as a Van-der-pol limit-cycle oscillator. One property of limit-cycle oscillators is that the magnitude of phase shift in response to a stimulus (e.g. light) of a given strength will increase as the amplitude of the oscillation decreases, because the phase displacement will represent a larger fraction of the radius of the circle (Jewett, Forger, & Kronauer, 1999; Jewett & Kronauer, 1998). Constant light damps out circadian rhythms of behaviour and firing rate (Ohta, Yamazaki, & McMahon, 2005), and long photoperiods are thought to also reduce the amplitude of the SCN rhythmicity. Two studies, however, reported that mice in longer photoperiods actually display smaller phase shifts to light compared to short photoperiods (Refinetti, 2002; vanderLeest et al., 2009), a result we also obtained. This is in the opposite direction to the prediction that lower SCN amplitude in long days should result in larger phase shifts to light. vanderLeest et al. (2009) argue that the SCN response to phase shifting stimuli is a network property. They found that in long days, SCN clock cells are more dispersed in phase, while in short days they are more tightly synchronized. From this network perspective, the prediction reverses. Following entrainment to a short day photoperiod, when phase synchrony among SCN clock cells is increased, a 15-min pulse of light would activate more SCN neurons at similar phases, resulting in more coherent, and therefore larger aggregate phase shift.

An alternative explanation for smaller phase shifts after entrainment to long photoperiods, is that the retina becomes desensitized to light and is therefore less responsive to light input for some period of time in DD. Our results do not support this explanation. To prevent differences in total light exposure under long and short days, the mice were entrained to skeleton photoperiods prior to DD, and phase shifts to a 15-min light pulse were larger in the short day group. Phase delays ranged from 23-174 minutes in the L16 group, compared to 61-305 minutes in the L8 group. While significant, our results show a smaller difference between groups than those previously reported (Refinetti, 2002; vanderLeest et al., 2009). There are two possible explanations for our smaller effect. First, an entire cabinet was pulsed at the same external time of day (n=10

per cabinet), meaning that individual circadian time varied somewhat between each mouse. Second, the after-effects of photoperiod on population synchrony among SCN neurons last for approximately four days in DD (VanderLeest et al., 2007). We chose to administer light pulses on day five of DD to increase the number of days with which to fit a regression line to predict activity onset on the day after the pulse. Differences in phase shift magnitude between long and short day groups might have been greater if light pulses had been applied on day 3 or 4 of DD.

FAA varies with meal time independent of photoperiod

Although FAA did not vary systematically with photoperiod, it was affected by the timing of food access in the light period. In mice fed late in the light period, FAA accounted for a significantly greater percentage of total daily activity. There was also at least a trend for FAA to begin earlier in these mice, although this was mostly evident in one group (mice in L16 with running discs). The enhanced FAA ratio in mice fed during the last 4h of the light period likely reflects at least in part an effect of satiety on the expression of activity, which normally peaks at lights-off. It is also possible that near the end of the light period, homeostatic sleep drive is lower or SCN facilitation of sleep is weaker. All three of these factors may contribute.

Conclusions

Contrary to predictions and limited prior evidence, the results of this study do not support a general proposition (Pendergast et al., 2009) that long-day photoperiods are optimal for observing robust daytime FAA in mice. Failure to observe an effect of photoperiod on FAA parameters to mid-day meals may be because feeding at this time of day blocks the effect of photoperiod on SCN pacemaker properties. Meals scheduled at the end of the subjective day may have less impact on these properties. It is perhaps not so surprising that FAA under mid-day restricted feeding does not consistently vary by photoperiod as it represents an inherently adaptive mechanism. The ability to forage efficiently when food is scarce is critical for survival at all times of year.

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