Circadian Properties of Food Anticipatory Activity Re-Examined: Entrainment limits and scalar timing in operant and general activity

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Abstract

Rats display anticipatory activity (FAA) to daily feeding opportunities. Early chronobiological research indicated that feeding schedules deviating from 24-hours failed to elicit FAA, suggesting that a circadian oscillator mediates this behavior. More recent work using operant measures of anticipation, uncommon to chronobiology, has reported that rats can anticipate non-circadian intervals and that this anticipation shows hallmarks of interval timing rather than oscillator control. To test whether operant and non-operant behaviors may be controlled by different timing mechanisms, we re-examined formal properties of FAA using lever pressing and motion sensors. Rats maintained in a 12:12 light-dark cycle or in constant light failed to show anticipation to meals at 18h intervals, but robustly anticipated meals at 24h intervals. FAA failed to scale with intervals between light changes and mealtime, violating the hallmark property of interval timing. These results support a food-entrained oscillator model of FAA and fail to provide evidence that cue-dependent interval timing contributes to FAA.

Keywords: circadian rhythms; food anticipatory activity; interval timing; scalar property

Acknowledgements

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List of Acronyms

FAA	food-anticipatory activity
FD	food deprived
FEO	food-entrainable oscillator
FI	fixed-interval
LD	light-dark
LEO	light-entrainable oscillator
RF	restricted food
SCN	suprachiasmatic nucleus
ZT	zeitgeber time

Chapter 1.

Introduction

The ability to anticipate, rather than merely react to, predictable biologically important environmental events is an important element of survival for all organisms. Due to this importance, organisms have evolved multiple timing mechanisms to ensure that adaptive responses come at appropriate times. Certain environmental events are synchronized with major geophysical changes that have such stable periodicities (e.g.; 24h or circadian) that organisms have evolved the ability to anticipate these events through rhythmic changes in behavior and physiology. Other events are not synchronized to a natural periodicity and the mechanisms timing them, much like a stopwatch, must be able to begin and end at arbitrary times. Likewise, research into how animals time events in their environment has proceeded along two distinct traditions: comparative cognition for the study of shorter arbitrary intervals in the seconds-to-minutes range and chronobiology for intervals approximating the 24h solar day.

1.1. Chronobiology

The circadian system is a complex regulatory mechanism that provides organisms with the means to adapt to and prepare for daily events necessary for survival within a particular environment, allowing the organism to anticipate regularly occurring environmental changes. Even in the absence of external time cues, endogenous circadian oscillators maintain near 24h rhythmic variations in a number of physiological and cellular processes, including temperature, hormone release, and metabolism (Ungar & Halberg, 1962; Hastings, O'Neill, & Maywood, 2007; Bass & Takahashi, 2010). In mammals these rhythms are coordinated by a master circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, whose activity is

synchronized, or entrained, by recurrent environmental signals (zeitgebers), of which light is the most salient (Stephan & Zucker, 1972). This light-entrained oscillatory system (LEO) allows the animal to take full advantage of a particular temporal niche by coordinating activity rhythms with the daily light-dark (LD) cycle (Hut, Kronfeld-Schor, van der Vinne, & De la Iglesia, 2012). Oscillations within individual SCN neurons are generated by a complex, auto-regulatory, transcriptional/translational feedback loop involving a set of so-called "clock genes" (Lowrey & Takahashi, 2000; Ko, 2006). At its core, the positive elements of this feedback loop, CLOCK and BMAL1 heterodimers, activate transcription of the negative elements, *Period* and *Cryptochrome* genes. The protein products of these genes form dimers that cross back into the nucleus and inhibit their own activation by blocking the CLOCK/BMAL1 dimers. Degradation of PER/CRY relieves this inhibition allowing the cycle to begin again every ~24h. Accessory loops involving RORA and Rev-Erbα, and post-translational modifications, such as phosphorylation, set the period and amplitude of these rhythms (Lim & Allada, 2013; Lowrey & Takahashi, 2000). Nighttime light exposure causes glutamate release in the SCN, causing the activation of *Period* genes, resulting in a time-dependent shift in their expression. Ultimately, changes in SCN output in response to light exposure result in a shift in activity onset (phase shift), with light exposure in the early night delaying, and exposure in the early morning advancing subsequent activity rhythms (Golombek & Rosenstein, 2010).

Although light provides the strongest entraining signal to the SCN, other, non-photic inputs are also capable of modifying activity, particularly in the absence of a light-dark cycle (Challet & Pévet, 2003; Mrosovsky, 1996). Of these non-photic inputs, food is remarkable in that it is capable of influencing the circadian system in two distinct ways. Some animals, including Syrian hamsters and certain strains of mice, will synchronize their daily activity rhythms around access to food when housed in constant lighting conditions. This 'food entrainment' occurs by directly altering SCN activity (Abe, Honma, & Honma, 2006; Castillo, 2004). In other animals, including C57BL mice and Norway rats, temporally limited food access is capable of generating a separate activity rhythm that is independent of the SCN and can be in direct conflict with the daily light-dark cycle (Bolles & Stokes, 1965; Stephan, 2002). When access to food is restricted to a few hours occurring at the same time each day, over the course of several days these

animals come to exhibit an increase in general locomotor and food-oriented behavior in the hours preceding food presentation (Bolles & Stokes, 1965). This food-anticipatory activity (FAA) typically begins 2-3h prior to and comes to peak at mealtime (Stephan, 2002). Early chronobiological studies found that this daily rhythmic increase in activity exhibits a set of formal properties that are characteristic of the outputs of entrained endogenous oscillators with circadian periodicities (for a thorough review see Mistlberger, 1994). First, FAA develops normally in constant environmental conditions and persists for multiple days when feedings are withheld (Boulos, Rosenwasser, & Terman, 1980). A major feature of a circadian oscillator is its self-sustaining nature, which ensures that periodic output will continue even after cessation of the entraining stimulus. Second, when mealtime is abruptly shifted, FAA does not immediately reset, but rather, shifts gradually, with the animal often displaying transient bouts of activity as FAA moves to the new feeding time (Stephan, 1984; Stephan, 1992). These transients are due to limits in the ability of an oscillator to shift in a single cycle, thus requiring multiple cycles to synchronize fully to the new time (Mistlberger, 1994). Furthermore, FAA was thought incapable of developing to meals scheduled at long, but non-circadian intervals (that is, outside of the 22-28h range) (Bolles & Stokes, 1965; Boulos et al., 1980). Oscillators are restricted in the range of intervals to which they are capable of entraining. These limits to entrainment reflect the limit in an oscillator's ability to phase shift to a daily zeitgeber and to deviate from its intrinsic periodicity. Finally, FAA appears in food-restricted animals whose normal daily activity rhythms have been eliminated due to SCN ablation or constant exposure to bright light, indicating that the oscillatory system involved in generating FAA is independent of the light-controlled SCN circadian oscillator (Boulos et al., 1980).

To account for the properties of FAA, chronobiologists hypothesize the existence of a food-entrainable oscillator (FEO) that is similar to, yet distinct from, the light-entrainable oscillator (LEO) of the SCN (Boulos & Terman, 1980; Mistlberger, 1994; Stephan, 2002). Under most circumstances, including ad-lib feeding conditions in a laboratory setting, the FEO is coupled with the LEO, helping to drive the appropriate activity and feeding profile of a given animal (Mistlberger, 1994). However, under conditions of metabolic challenge, such as when food is temporally limited to a predictable time, the FEO and LEO are capable of uncoupling, with FEO-generated

activity emerging prior to mealtime and LEO-driven activity continuing to occur in the appropriate phase of the LD cycle (Mistlberger, 2011). If the animal is then placed in constant lighting conditions FAA remains entrained to the mealtime while SCN/LEO-driven activity free-runs (Boulos et al., 1980).

In an attempt to determine the neurobiological mechanisms underlying FAA, chronobiologists systematically ablated areas of the hypothalamus involved in ingestive behavior and metabolism, including dorsomedial (DMH), paraventricular (PVN), and lateral (LH) hypothalamus (Honma, Honma, Nagasaka, & Hiroshige, 1987; Landry, Yamakawa, & Mistlberger, 2007; Mistlberger & Rechtschaffen, 1984; Mistlberger & Rusak, 1988). Although removal of certain areas resulted in altered expression (e.g.; lower amplitude), in no case did ablation of any hypothalamic structure, or combination of structures, consistently eliminate food anticipation (Davidson, 2009; Mistlberger, 2011). Removal of various basal forebrain, hippocampal, limbic, thalamic, brainstem, and cortical structures have similarly failed to eliminate FAA (Mistlberger & Mumby, 1992; Mistlberger, 1994; Mendoza, Angeles-Castellanos, & Escobar, 2005). Furthermore, given that clock gene expression in most tissues and brain regions outside the SCN preferentially entrain to restricted feeding (Damiola, 2000; Verwey & Amir, 2009), it was originally thought that FAA would rely at least in part on these genes. Surprisingly, however, mice bearing null mutations in the clock genes *Clk*, *Bmal1*, *Cry1*/*Cry2*, and *Per1*/*Per2* are all still capable of anticipating a daily meal, despite altered expression in some cases (Challet, Mendoza, Dardente, & Pevet, 2009; Iijima et al., 2005; Pitts, Perone, & Silver, 2003; Storch & Weitz, 2009), suggesting alternative molecular pathways involved in its regulation. The inability to localize the FEO and its resilience to disruption of canonical clock genes suggest that the FEO is both broadly distributed at the anatomical level (Escobar, Cailotto, Angeles-Castellanos, Delgado, & Buijs, 2009) and redundant at the genetic level (Challet et al., 2009). In sum, in the field of chronobiology, activity expressed prior to a daily meal is viewed as a circadian rhythm generated by a novel food-entrainable circadian oscillator that is distinct from, yet interacts with, the light-entrainable oscillatory system of the SCN.

1.2. Comparative Cognition

Understanding how animals psychologically represent the temporal relation between events is an important topic in the study of comparative cognition. The discrimination of durations of arbitrary intervals from seconds to minutes is referred to as interval timing (Gibbon & Church, 1990). In the best-known example of short-interval timing, fixed-interval (FI) schedules of reinforcement, a reward is delivered to an animal contingent on the first operant behavior performed after a target interval following the onset of a signal or previous reward presentation. An anticipatory pattern soon develops in these procedures, with the response rate, averaged across many trials, increasing as a function of time and reaching a maximum at the target interval (Buhusi & Meck, 2005). If reinforcement is occasionally withheld, responding takes on an inverted U-shape, coming to a peak at the time that reinforcement normally occurs (Gibbon 1977; Gibbon, 1991). The time at which the maximum response rate is reached during these peak trials can be used as an estimate of when the animal expected reinforcement, thus providing a measure of the animal's accuracy and variability in its ability to time the interval (Buhusi & Mech, 2005).

Defining characteristics of the interval timing system include its ability to begin at any arbitrary time, fully reset upon presentation of a stimulus, and time a broad range of intervals (Buhusi & Meck, 2005). The hallmark of this system however, is what is referred to as the scalar property. This property describes the linear relationship between physical intervals and variability in the estimates of these intervals, such that variability increases in proportion to the length of the interval being timed (Gibbon, 1977). It has been described as though subjective time is measured with a rubber ruler, capable of stretching to any interval length, but with discriminatory ability stretching along with it (Crystal, 2001b). One result of this property is that when probability of responding is plotted as a function of relative time, the response curves are superimposable regardless of the length of the interval (Gibbon, 1977).

To account for these properties, a set of mechanisms involving a pacemaker and an accumulator, known as linear accumulator models were initially proposed (Buhusi & Meck, 2005; Gibbon, 1991). In these models, time perception is based on pulses

generated by a pacemaker which are temporarily stored in an accumulator over a given time interval. At the time of reward, the number of pulses stored in the accumulator is stored in reference memory. Later temporal judgments are based on comparing this memory with the number of pulses currently stored in the accumulator. Reward presentation resets the accumulator and counting pulses begins anew.

Close inspection of the data, however, has cast some doubt on linear accumulator models of short-interval timing. Small departures from linearity at specific intervals have led some researchers to propose that the mechanisms involved rely on a series of endogenous oscillators, each with its own characteristic period (Crystal, 1999; Crystal, 2001b; Church & Broadbent, 1990). Oscillator models of interval timing involve the selection and entrainment of a range of oscillators with periods spanning milliseconds to hours, and possibly beyond (Crystal, 2006b). Further support for this model comes from reports of continued periodic responding to short intervals following the termination of stimulus presentation, consistent with an oscillatory driven mechanism (Crystal & Baramidze, 2007; Kirkpatrick-Steger, Miller, Betti, & Wasserman, 1996; Machado & Cevik, 1998; Monteiro & Machado, 2009).

Although there is evidence that, like most cognitive processes, interval timing is modulated by the circadian system (Agostino, do Nascimento, Bussi, Eguía, & Golombek, 2011; Bussi, Levín, Golombek, & Agostino, 2014), it appears that the mechanisms involved operate independently of the light-entrainable circadian system (LEO) at both the anatomic (Lewis, Miall, Daan, & Kacelnik, 2003) and genetic levels (Cordes & Gallistel, 2008; Papachristos, Jacobs, & Elgersma, 2011).

1.3. Recent Findings

The fact that in FAA an animal is able to time an interval (24h) using an oscillatory system (FEO) that is independent of the traditional circadian system (LEO) has led some to suggest that the FEO may be one of a larger array of oscillators, which includes those used to measure shorter intervals (Church & Broadbent, 1990; Crystal, 2006b). The similarities in the behavioral profile of animals anticipating short-intervals and daily meals would seem to lend further support to this hypothesis. There is,

however, a large gap between intervals in the traditional interval timing range (seconds-to-minutes) and those in the circadian range (24h). Although the comparative cognition literature has paid relatively little attention to the timing of intervals in the hours range, it has been reported that rats can anticipate long, but non-circadian intervals (14-21h) when anticipation is measured by operant responding. Animals were found capable of anticipating meals scheduled at either 14 or 24h, although the rate of anticipatory responding differed between the two groups (Crystal, 2001a). The rate of responding in the 24h condition increased more rapidly and came to a higher peak than in the 14h condition, potentially indicating greater temporal sensitivity to intervals in the circadian range. Furthermore, it was reported that animals anticipating meals scheduled at 16 and 21h intervals continued to display periodic anticipatory activity even after feedings were discontinued, suggesting that this timing was accomplished via an oscillatory mechanism (Crystal, 2006a). It was additionally reported that rats anticipating a daily meal utilized an interval timing strategy to time the meal in relation to the lighting schedule, suggesting that animals make use of interval timing mechanisms in FAA (Crystal, 2001a).

If there are non-circadian oscillators capable of producing anticipation to these long, but non-circadian intervals the question arises as to why the chronobiological literature has consistently failed to observe this behavior. One suggested possibility is the fact that the behavioral state of an animal anticipating food changes over the course of the interval, such that immediately after food presentation, food directed activity is generally inhibited in both FI schedules of reinforcement and FAA (Balsam, Sanchez-Castillo, Taylor, Van Volkinburg, & Ward, 2009). In animals anticipating a daily meal, this post-prandial suppression extends to all activity and lasts for several hours after feeding has concluded. As mealtime approaches, animals enter a focal search mode that involves both feeding directed behaviors and anticipatory physiological responses, preparing the animal for food consumption (Mistlberger, 1994). Animals timing short intervals also exhibit changes in their behavioral patterns as the interval elapses. Following withdrawal from the feeding area, animals first enter an exploratory mode in the middle of the interval, which is followed by a focal search mode as food presentation approaches (Silva & Timberlake, 1998). It has been argued that the single measures of anticipation that are typically employed in chronobiology (usually general cage activity or

wheel running) may limit researchers ability to detect anticipatory behavior if the form of that behavior changes as the interval elapses (Balsam et al., 2009). This could lead to the conclusion that a system was limited in the range of intervals it was capable of timing when no such limitation exists. It has been suggested that requiring an operant behavior to produce food, as is done in FI schedules of reinforcement, may address this potential issue and provide greater sensitivity in detecting anticipatory behavior (Balsam et al., 2009; Crystal, 2006b; Mistlberger, 2009). Although operant responding has been used in chronobiological studies, with animals displaying robust anticipatory responses to a daily meal, due to the added time and expenses involved, operant measures are not common practice, limiting the literature in this regard (Bolles & Stokes, 1965; Boulos et al., 1980; Terman, Gibbon, Fairhurst, & Waring, 1984).

1.4. Current Study

The present study attempts to address this inconsistency between the two literatures by re-examining circadian limits to food anticipation using both operant and non-operant measures of anticipation. Two groups of rats were maintained on 24 and 18h feeding schedules in a 12:12 LD cycle (N=16) or in constant bright light (N=20) to eliminate free-running activity rhythms. General activity was measured using telemetry and motion sensors, respectively. A third group (N=7) was placed on the same feeding schedules while housed in LD 12:12, with both operant (lever pressing) and non-operant (motion sensor) measures of FAA used. To test whether animals time FAA in relation to the lighting schedule via interval timing mechanisms, thus exhibiting the scalar property, this group was placed back on a 24h restricted feeding schedule and mealtime was then phase-delayed once every 14 days to sample 7 different mealtimes across the LD cycle.

Chapter 2.

General Methods

2.1. Animals and Ethics Statement

All procedures were approved by the University Animal Care Committee at Simon Fraser University (permit #1065p-12) and conformed to the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals and Canada's Animals for Research Act. Adult male Sprague-Dawley rats (Charles River, QC, Canada) were housed individually in a humidity- and temperature-controlled (~22°) vivarium. Water was available *ad libitum* throughout all experiments. Animal health was checked once daily. Cages were cleaned and animals weighed once weekly during food access periods. The only exception to this was during food deprivation tests in constant dark (DD), when animals were left undisturbed in their cages for the duration of the manipulation (2-3 days).

2.2. Data Analysis

For visual inspection, general cage activity (measured by overhead infrared motion sensor or intra-abdominal telemetry transmitters), body temperature, and lever pressing activity were plotted in 10 min bins in the standard raster plot format using Circadia (Dr. T.A. Houpt, Florida State University), and as 24h waveforms averaging one or more days within and across animals, using Prism 5.0 (Graphpad Software Inc., La Jolla, CA, US). Food anticipatory activity (FAA) onsets were identified using the Clocklab algorithm (Actimetrics, IL, US). FAA was further quantified as a ratio of the sum of activity occurring during a fixed window prior to mealtime (the 2-hours preceding mealtime for the 18h feeding schedules or 2-hours 40-min preceding mealtime for the

24h feeding schedules) to the total amount of activity between mealtimes. Changes in body temperature over the FAA window were calculated by subtracting body temperatures immediately before mealtime from body temperatures at the beginning of the FAA window. To evaluate differences in quantitative measures of FAA between feeding conditions, separate repeated measures, multivariate analyses of variance (MANOVAs), with Greenhouse-Geisser corrections where appropriate, were used for each feeding schedule. Repeated measures ANOVAs and paired t-tests with Bonferroni corrections were used to assess significance between paired comparisons of repeated measures where a significant main effect was found. Group means are reported in the text and plotted with standard errors. P-values are reported with SPSS Bonferroni corrections. All statistics were analyzed using SPSS software.

Chapter 3.

Experiment 1.
FAA on circadian and non-circadian feeding schedules in light:dark and constant light

3.1. Methods

3.1.1. Animals

Rats (N=20) were housed individually in standard plastic cages with corncob bedding. Activity was continuously monitored using infrared motion sensors located above the cage and stored at 1-min intervals using the Clocklab data acquisition system (Actimetrics).

3.1.2. Procedure

Rats were kept in a 12 hour light: 12 hour dark (12:12 LD) cycle and food was restricted to a daily 3h meal beginning 6h after lights-on (Zeitgeber Time 6, where ZT12 is lights off by convention) for 23 days, after which the rats were food deprived and left undisturbed in their chambers for 2 days in continuous dim (~1 lux) red light.

Constant bright light disrupts the circadian rhythms of nocturnal rodents, with long-term exposure producing arrhythmicity. Thus, animals were next tested in constant light to eliminate time of day signals, generated either externally (light cues) or by the SCN, that could be used to time FAA. Rats were exposed to constant bright light (LL, ~300 lux) with *ad libitum* food availability for 19 days to eliminate free-running rhythms. Food access was then restricted to a 3h daily meal provided at the same time each day for 15 days. For reasons unrelated to this study, on two separate occasions (the 6th and

12th days of this restricted feeding schedule) a single meal was skipped and feedings resumed the following day at the normally scheduled mealtime. These skipped meal days and the day immediately following each were excluded from all analyses.

Following a further 22 days in LL, with *ad libitum* access to food, rats were food deprived for 43h and then provided with 2h meals beginning at 18h intervals for 21 cycles. At the end of the 21 cycles, the animals were food deprived and left undisturbed in their chambers for 3 days (4 cycles of 18h).

3.2. Results

Visual inspection of raster plots (Fig. 3.1) and average waveforms (Fig. 3.2) confirmed that all rats exhibited anticipatory activity to a daily meal scheduled at 24h intervals when housed in either LD or LL. However, there was no evidence of anticipation to a meal scheduled at 18h intervals in LL.

Quantitative FAA measurements (Fig. 3.3) support the conclusions drawn from visual inspection of raster plots. FAA ratios during restricted feeding (RF) were significantly increased in both 24h feeding schedules as compared to their respective ad-lib baselines (LD 24h: 0.19±0.02 vs. 0.03±0.01; LL 24h: 0.22±0.01 vs. 0.13±0.01; both $p<0.001$). Furthermore, when placed in constant dark and food deprived (FD) following the 24h restricted feeding schedule, FAA ratios remained significantly higher than ad-lib baseline (0.20±0.02 vs. 0.03±0.01, $p<0.001$), but did not differ from restricted feeding ($p>0.999$). In the 18h schedule there was no main effect of feeding condition on FAA ratios (ad-lib: 0.11±0.01, RF: 0.09±0.01, FD: 0.11±0.01; $F(2,34)=2.8000$, $p=0.075$).

The total number of FAA counts was significantly increased during restricted feeding in both 24h feeding schedules as compared to their ad-lib baselines (LD 24h: 59.4±6.4 vs. 16.0±2.6; LL 24h: 95.0±9.4 vs. 28.3±2.7; both $p<0.001$), but not in the 18h feeding schedule (ad-lib: 22.6±3.3, RF: 14.7±1.5; $p=0.210$). Similarly, the peak level of activity reached during the FAA window (peak FAA) was significantly increased during restricted feeding in both 24h schedules as compared to their respective ad-lib baselines (LD 24h: 7.6±0.8 vs. 2.7±0.4; LL 24h: 10.4±0.9 vs. 4.5±0.4; both $p<0.001$), whereas in

the 18h schedule there was no difference between restricted feeding and ad-lib (2.5±0.2 vs. 4.0±0.5; p=0.097).

During the total food deprivation tests after the 24h feeding schedule in LD, FAA persisted with no attenuation in total amount (LD 24h: 66.9±8.5 vs. 59.4±6.4, p=0.273), and a significant increase in peak level (7.6±0.8 vs. 10.1±0.9, p=0.007), compared to restricted feeding days. During the total food deprivation test after the 18h feeding condition, there was a significant increase in the peak level of activity to occur over the FAA window relative to restricted feeding days (2.5±0.2 vs. 5.0±0.3; p<0.001), but not ad-lib baseline (p>0.999). There was no change in total activity over that same time period when on total food deprivation (14.7±1.5 vs. 15.3±1.6; p>0.999) relative to restricted feeding days.

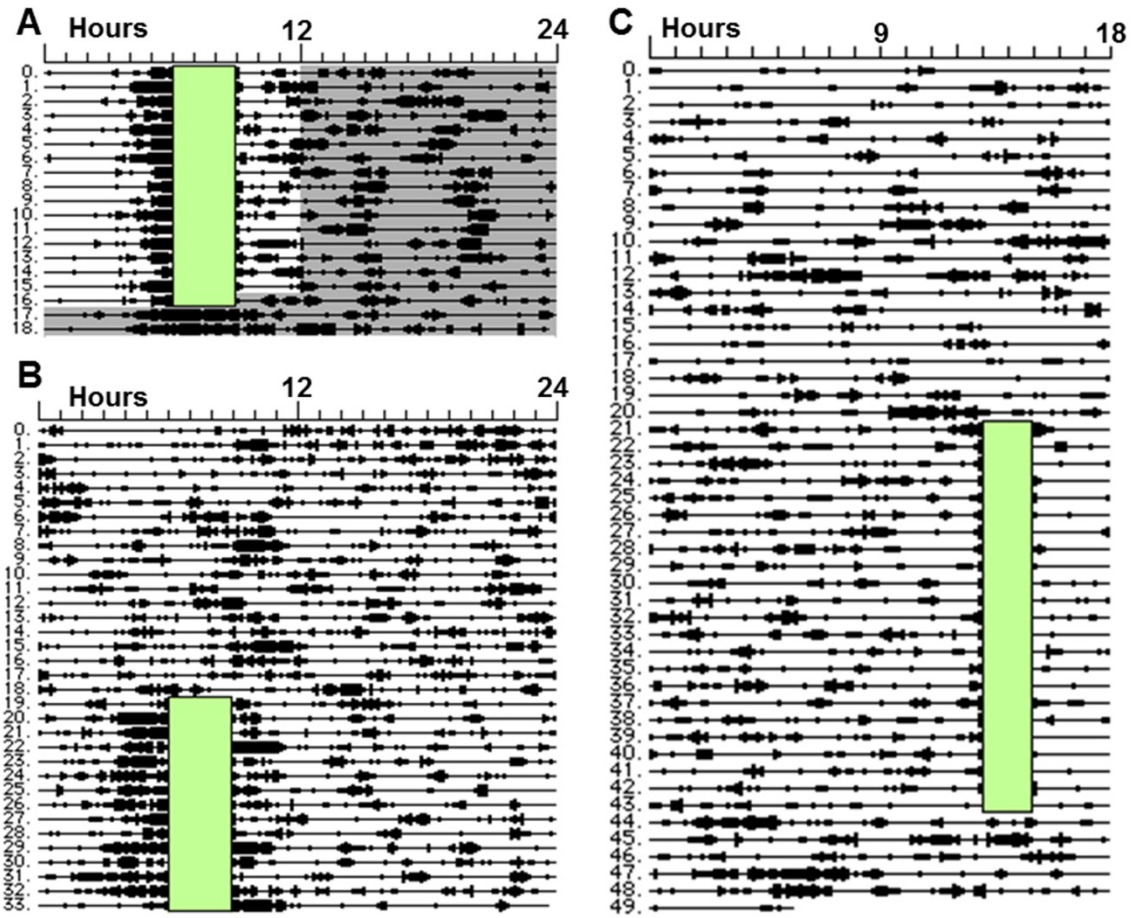

Figure 3.1. Representative actograms for Experiment 1
A. 24h feeding schedule in LD 12:12. Each line represents 24h, with time of day plotted left to right in 10 min bins. Meals are indicated by green boxes. Lights out is indicated by gray shading. Time bins during which activity was registered are denoted by heavy bars. **B.** 24h feeding schedule in constant light (LL). **C.** 18h feeding schedule in LL. Each line represents 18h, with time plotted left to right in 10 min bins.

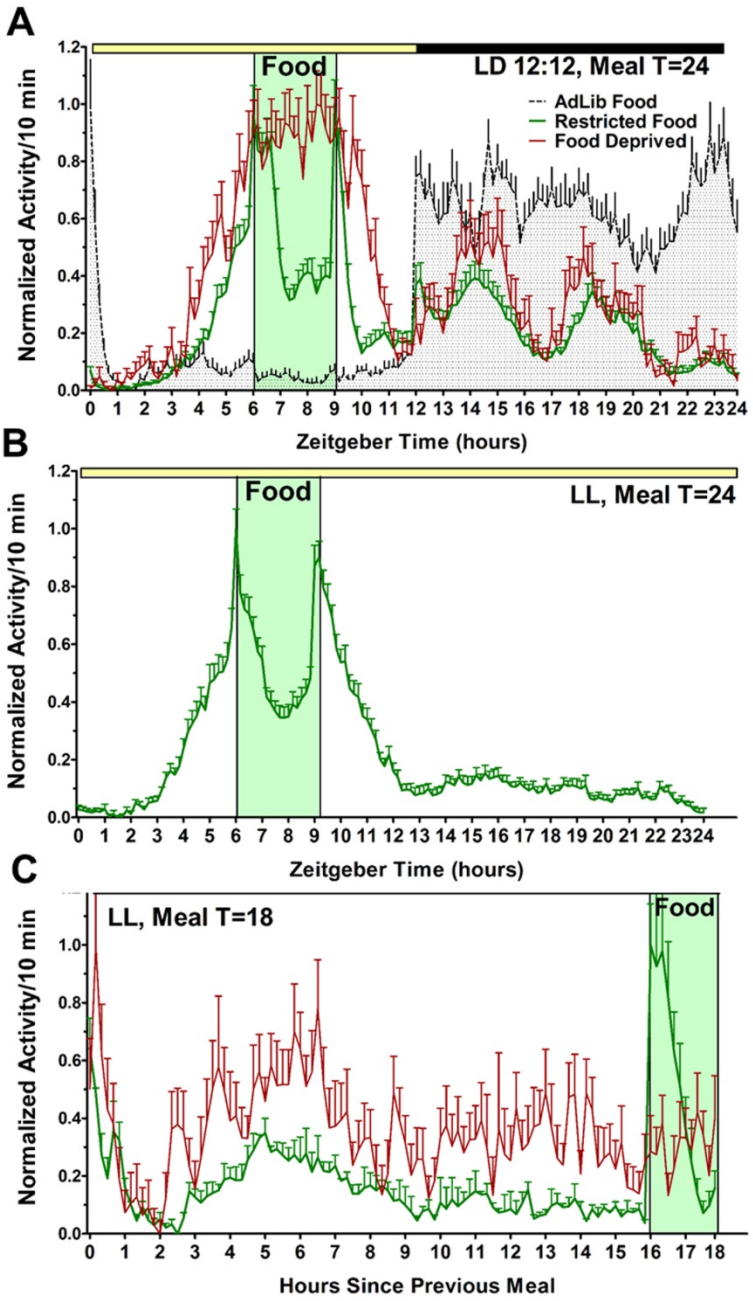

Figure 3.2. Waveforms of activity measured by motion sensor in Experiment 1
Normalized group means ±SEM, N=20 rats. Time is plotted in 10-min bins from lights-on (A), equivalent lights on time (B), or end of previous mealtime (C). Food availability is denoted by green shading. **A.** Days 5-15 of ad-lib food access (stippled black), days 12-23 of restricted feeding (green line), and 2 days of total food deprivation in DD (red line) in a LD 12:12 photoperiod (photoperiod indicated by horizontal black and yellow bars above graph) with meals scheduled at 24-h intervals. **B.** Days 1-15 (excluding days as mentioned in text) of restricted feeding (green line) in constant light (LL). **C.** Cycles 12-23 of restricted feeding (green line) in LL and 4 cycles of total food deprivation in DD (red line) with meals scheduled at 18-h intervals.

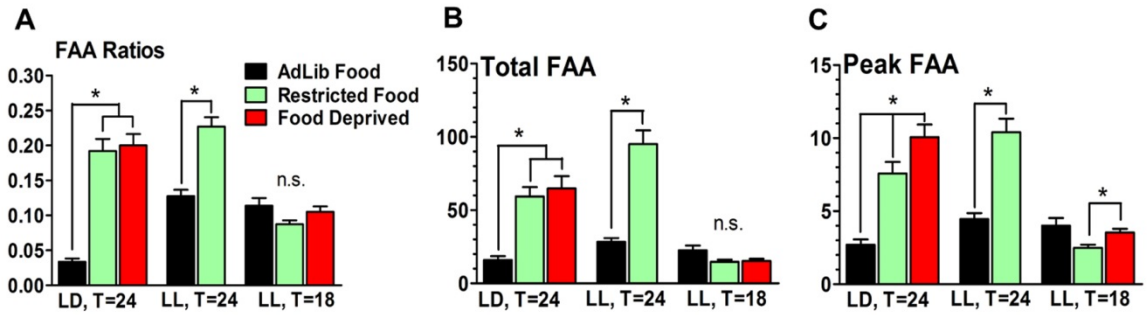

Figure 3.3. Quantitative measures of FAA in Experiment 1

Group means ±SEM, N=20 rats. **A.** FAA ratios for each feeding condition (black: ad-lib baseline, green: restricted feeding, red: food deprived in DD). FAA ratios were calculated by dividing the activity counts registered during either the 2-h (18-h feeding schedule) or 2-h 40-m (24-h feeding schedules) prior to mealtime by the total activity occurring outside of mealtime. **B.** Total activity counts occurring during the FAA window (as defined in A) for each condition. **C.** Peak level of activity reached during the FAA window. Significant differences at a Bonferroni-corrected $p<0.05$ are indicated by an asterisk.

Chapter 4.

Experiment 2.
FAA on circadian and non-circadian feeding schedules measured using telemetry

4.1. Methods

4.1.1. Animals

Rats (N=16) were individually housed in a 12:12 LD cycle in standard plastic cages with corncob bedding. Activity and core body temperature were monitored with intra-abdominal telemetry transmitters (ER-4000, Mini-Mitter Inc., Sunriver, OR, US) and stored at 1-min intervals using the Actiview data acquisition system (Mini-Mitter, Inc.).

4.1.2. Procedure

All rats were allowed to acclimate for at least 14 days with *ad libitum* food and water access. Animals were then food deprived for 43 hours and subsequently restricted to a 2h meal at 18h intervals for 3 weeks (24 cycles). The rats were then food deprived and left undisturbed in their chambers for 3 cyclesin continuous dim (~1 lux) red light.

Following food deprivation, the rats were re-entrained to a 12:12 LD cycle with *ad libitum* access to food for 12 days. The rats were then food deprived 64h and subsequently fed for 2h each day (ZT3-5) for 28 days.

4.2. Results

Visual inspection of activity and temperature raster plots (Fig. 4.1) and average waveforms (Fig. 4.2) revealed that all animals exhibited robust anticipatory activity/rise in body temperature to meals scheduled at 24h intervals, but not to meals scheduled at 18h intervals. This qualitative analysis was confirmed by measures of total and peak FAA counts (Fig. 4.3). Total FAA counts were significantly increased in the 24h feeding schedule as compared to ad-lib (142.8±6.0 vs. 48.0±3.4, $p<0.001$), but not in the 18h feeding schedule (83.1±2.4 vs. 80.2±2.3, $p=0.250$). Peak FAA was also significantly higher during the 24h schedule as compared to ad-lib baseline (14.0±0.5 vs. 8.3±0.8; $p<0.001$), but not in the 18h feeding schedule (9.75±0.29 vs. 9.71±0.36; $p>0.999$).

Quantification of FAA using anticipation ratios yielded results discordant with the visual and other quantitative measures. As expected, FAA ratios during the 24h feeding schedule were significantly increased compared to baseline (0.15±0.01 vs. 0.05±0.00, $p<.001$). Unexpectedly, FAA ratios during the 18h feeding schedules were also significantly increased compared to the ad-lib baseline (0.13±0.01 vs. 0.11±0.00, $p<0.001$), although the magnitude of the difference was much lower than was observed on the 24h feeding schedule (differences between ad-lib and restricted feeding FAA ratios for 24h and 18h schedules: 0.10±0.01 vs. 0.02±0.01, $p<0.001$). A significant anticipation ratio in the 18h feeding condition, despite the absence of a significant increase in FAA as measured by total counts or peak counts, appears to be an artifact of a post-prandial reduction in activity averaging about 3h across all mealtimes. On the 18h feeding schedule, about half of the post-prandial pauses occur during the lights-out period, when activity levels are normally high during ad-lib food access. This lowers the denominator in the ratio calculation for the 18h restricted feeding, artificially inflating the FAA ratio. A similar artifact does not occur in the 24h scheduled feeding condition because all meals occurred in the middle of the light period, when activity levels during baseline are asymptotically low.

Changes in body temperatures prior to mealtime confirmed anticipation to meals in the 24h schedule, but not the 18h schedule (Fig. 4.3). In the 24h schedule, rats in both the restricted feeding and food deprivation conditions displayed a statistically

significant increase in body temperature over the course of the FAA window as compared to a slight decrease observed during ad-lib baseline at this time of day (RF: +1.03±0.07º, FD: +0.93±0.11º, ad-lib: -0.27±0.04º; both $p<0.001$). In the 18h feeding schedule the change in body temperature prior to mealtime was not significantly different from ad-lib (+0.05±0.02º vs. +0.04±0.04º; $p>0.999$). During the 3 days of total food deprivation after the 18h schedule the rats showed a decreasing trend in body temperature over the FAA window that was significant compared to both the restricted feeding and the ad-lib food access days (FD: -0.16±0.07º; RF vs. FD: $p=0.004$; ad-lib vs. FD: $p=0.007$).

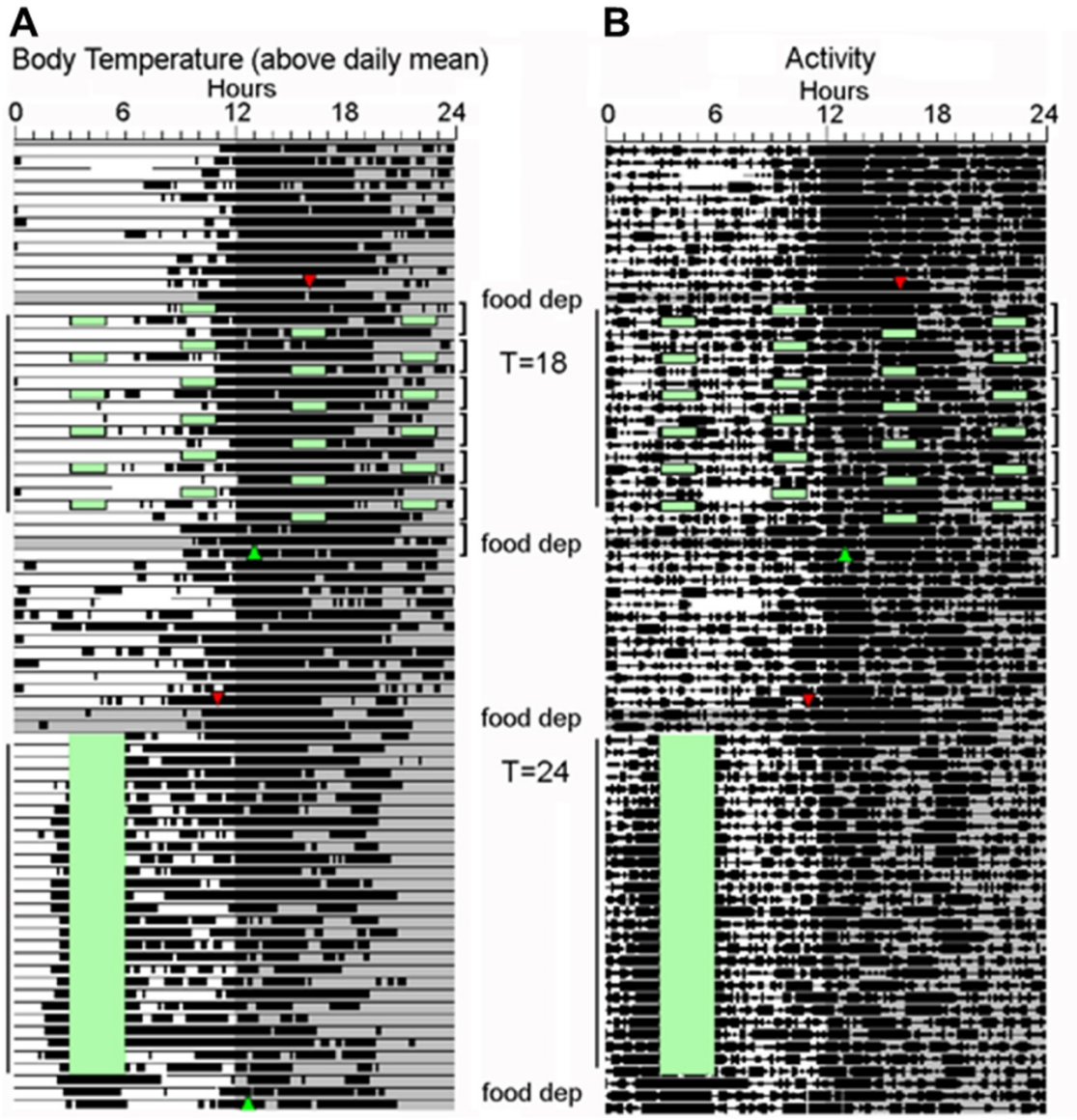

Figure 4.1. Representative actograms for Experiment 2
Each line represents 24h, with time of day plotted left to right in 10 min bins. Meals are indicated by green boxes. Lights out is indicated by gray shading. **A.** Time bins during which body temperature higher than the daily mean was registered are denoted by heavy bars. **B.** Time bins during which activity was registered are denoted by heavy bars.

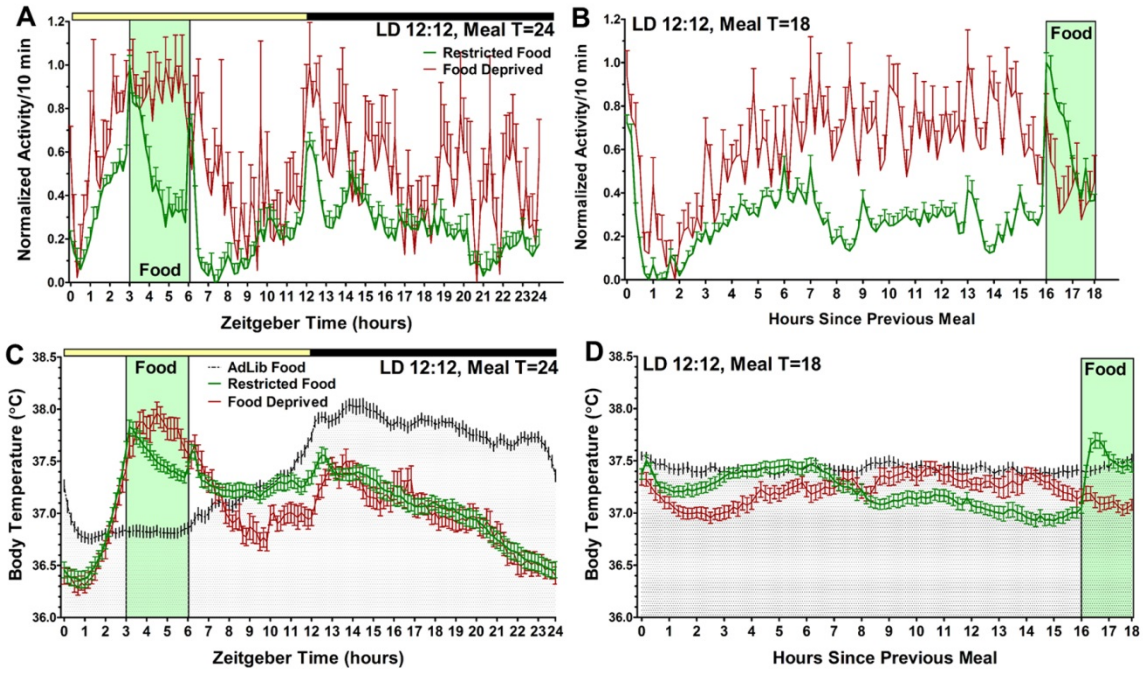

Figure 4.2. Waveforms of activity and temperature measured by telemetry in Experiment 2

Group means (normalized in A and B) ±SEM, N=16 rats. Time is plotted in 10-min bins from lights-on (A and C) or end of previous mealtime (B and D). Food availability is denoted by green shading. **A** and **C**. Days 1-10 of ad-lib food access (stippled black), days 17-28 of restricted feeding (green lines), and 2 days of total food deprivation in DD (red lines). **B** and **D.** Cycles 14-25 of restricted feeding (green line) and 3 cycles of total food deprivation in DD (red line).

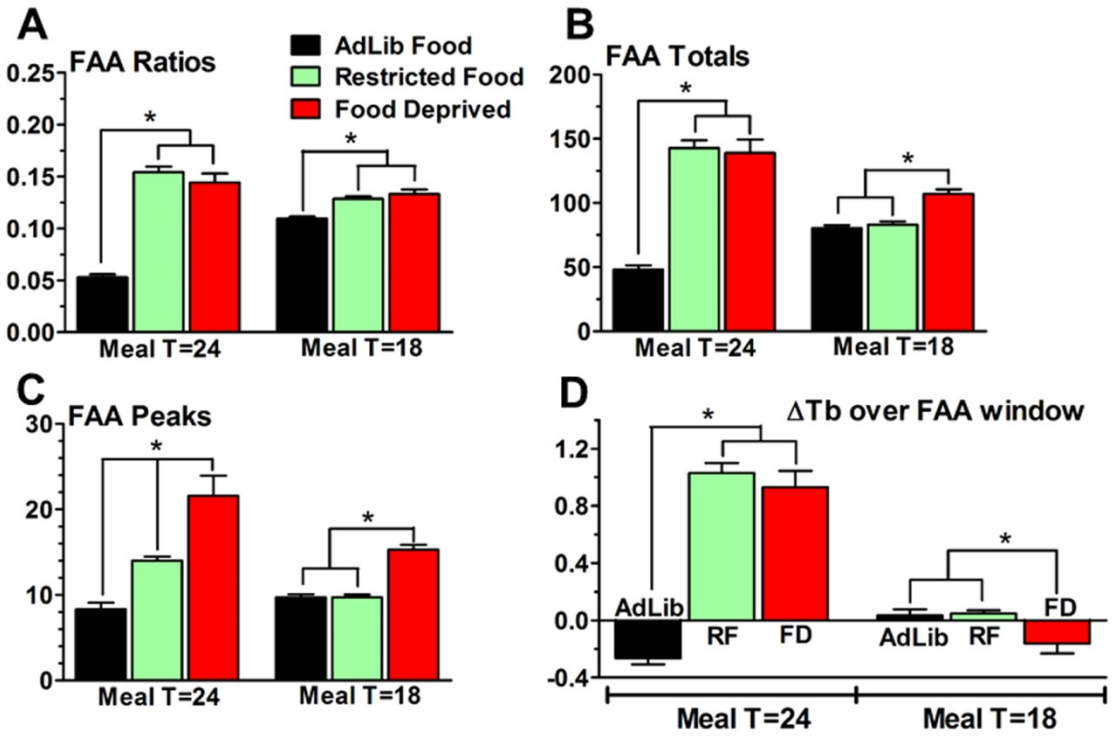

Figure 4.3. Quantitative measures of FAA in Experiment 2
Group means ±SEM, N=16 rats. **A.** FAA ratios for each feeding condition (black: ad-lib baseline, green: restricted feeding, red: food deprived in DD). **B.** Total activity to occur during the FAA window for each condition. **C.** Peak level of activity reached during the FAA window for each condition. **D.** Change in body temperature over the FAA window. Significant differences at a Bonferroni-corrected $p<0.05$ are indicated by an asterisk.

Chapter 5.

Experiment 3.
Operant mediated FAA on circadian and non-circadian feeding schedules

5.1. Methods

5.1.1. Animals

Rats (N=7, ~6 months age, 400-450 g) were housed individually in operant chambers (Med Associates, ENV-007, St. Albans, VT, US) in a 12:12 LD cycle. During the experiment, diet consisted of 20mg food pellets (Purina TestDiet Precision Pellets).

5.1.2. Apparatus

Seven identical operant chambers (Med Associates) were housed within individual ventilated sound-attenuation cubicles equipped with individual white lights (~60 lux) and an exhaust fan. The front panels of the operant chambers were outfitted with a retractable lever and a food trough. Pellets were provided via a 20mg pellet dispenser (Med Associates, ENV-203-20) positioned outside the chamber and attached to the food trough. Operant chambers were modified to allow for insertion of water bottles and plastic tops were replaced with wire mesh to allow for the recording of locomotor activity via overhead infrared motion sensors. Lever presses and reinforcements were summed and stored in 1-min intervals. Lever operation, reinforcement delivery, and data collection were controlled by a Pentium PC running Med-PC for Windows software (version 4.24; Med Associates). Activity counts, measured by overhead motion sensors, were summed and stored in 1-min intervals using the Clocklab data acquisition system (Actimetrics).

5.1.3. Procedure

Rats were first trained to press a lever for a food pellet reward (Purina TestDiet) using a continuous reinforcement schedule with food obtained only via lever pressing. All rats had acquired the task and were responding robustly after 36h of training. Following task acquisition, rats were left in the operant chambers with *ad libitum* access to food for 8 days. Food was obtained by lever pressing for 20mg food pellets following a fixed interval (FI) schedule of reinforcement. An interval of 1 second was used to discourage the stockpiling of pellets and to minimize the risk of pellet dispenser malfunction. Rats were then food deprived for 24h and subsequently restricted to a 2h mealtime occurring at 18h intervals. Outside of the mealtime, levers were left extended, with no rewards being administered, to monitor anticipatory lever pressing activity. The feeding schedule was maintained for 3 weeks (24 cycles) at the end of which the rats were food deprived and left undisturbed in their chambers for 3 days (4 cycles) in continuous dim (~1 lux) red light. The experimenter entered an adjacent anteroom ~5 min prior to each meal to initiate the feeding program, thus the 10 min prior to each meal has been excluded from all analyses.

Following food deprivation, the rats were once again housed in a 12:12 LD cycle with *ad libitum* access to food (via lever presses) and water for 14 days. Animals were then limited to 3h of food access daily from ZT6-ZT9. Following 28 days of food restriction, the rats were food deprived and left undisturbed in their chambers for 3 days in continuous dim (~1 lux) red light.

5.2. Results

Visual inspection of raster plots (Fig. 5.1) and average waveforms (Fig. 5.2) confirmed that all animals displayed general anticipatory locomotor activity as well as robust anticipatory lever pressing to a daily meal scheduled at 24h intervals, but not at 18h intervals.

FAA measurements (Fig. 5.3) confirmed the conclusions drawn from visual inspection of raster plots. FAA ratios for both lever responses and general cage activity

measured by motion sensors were significantly increased in the 24h feeding schedule as compared to the ad-lib baselines (lever: 0.75±0.02 vs. 0.02±0.01, motion: 0.24±0.02 vs. 0.03±0.01; both $p<0.001$) but were not increased in the 18h schedule (lever- ad-lib: 0.11±0.01, RF: 0.15±0.03, FD: 0.16±0.02, $p=0.128$; motion- ad-lib: 0.13±0.01, RF: 0.14±0.01, FD: 0.13±0.01, $p=0.649$). Furthermore, during food deprivation in constant dark following the 24h restricted feeding schedule, rats continued to show FAA ratios that were significantly higher than ad-lib baselines (lever: 0.53±0.03, motion: 0.24±0.02; both $p<0.001$). In the 18h schedule there were no significant differences between the ad-lib, restricted feeding, and food deprivation conditions (lever FD=0.16±0.02, $F(2,12)=2.447$, $p=0.128$; motion FD=0.13±0.01, $p=0.348$).

Both total lever pressing activity and general cage activity prior to mealtime were significantly increased during restricted feeding in the 24h feeding schedule as compared to ad-lib (lever: 102.2±5.6 vs. 25.6±11.8, $p=0.014$; motion: 79.6±13.3 vs. 18.3±2.9, $p=0.006$). In the 18h feeding schedule, total lever FAA in the restricted feeding condition was actually lower than during the ad-lib baseline (90.2±9.6 vs. 12.0±3.6, $p=0.002$). There were no differences in total general cage activity between feeding conditions ($F(2,12)=0.719$, $p=0.507$).

Although there was no significant difference in peak lever FAA for any of the feeding conditions in the 24h feeding schedule (ad-lib: 8.0±2.1, RF: 13.7±1.1, FD: 13.7±2.0; $F(1.02,5.12)=2.592$, $p=0.124$), peak general cage activity during both restricted feeding (9.2±1.5) and food deprivation (11.18±1.00) were significantly higher than ad-lib baseline (4.3±0.5, $p=0.046$). In the 18h feeding schedule peak lever FAA was significantly lower during both restricted feeding (3.1±1.1) and food deprivation (3.0±0.6) than ad-lib baseline (17.9±2.4, RF vs. ad-lib $p=0.009$, FD vs. ad-lib $p=0.004$). Locomotor activity was significantly lower during restricted feeding (7.6±0.5 vs. 11.3±1.2, $p=0.037$), but not food deprivation (8.0±0.7 vs. 11.3, $p=0.095$), as compared to ad-lib baseline in the 18h schedule.

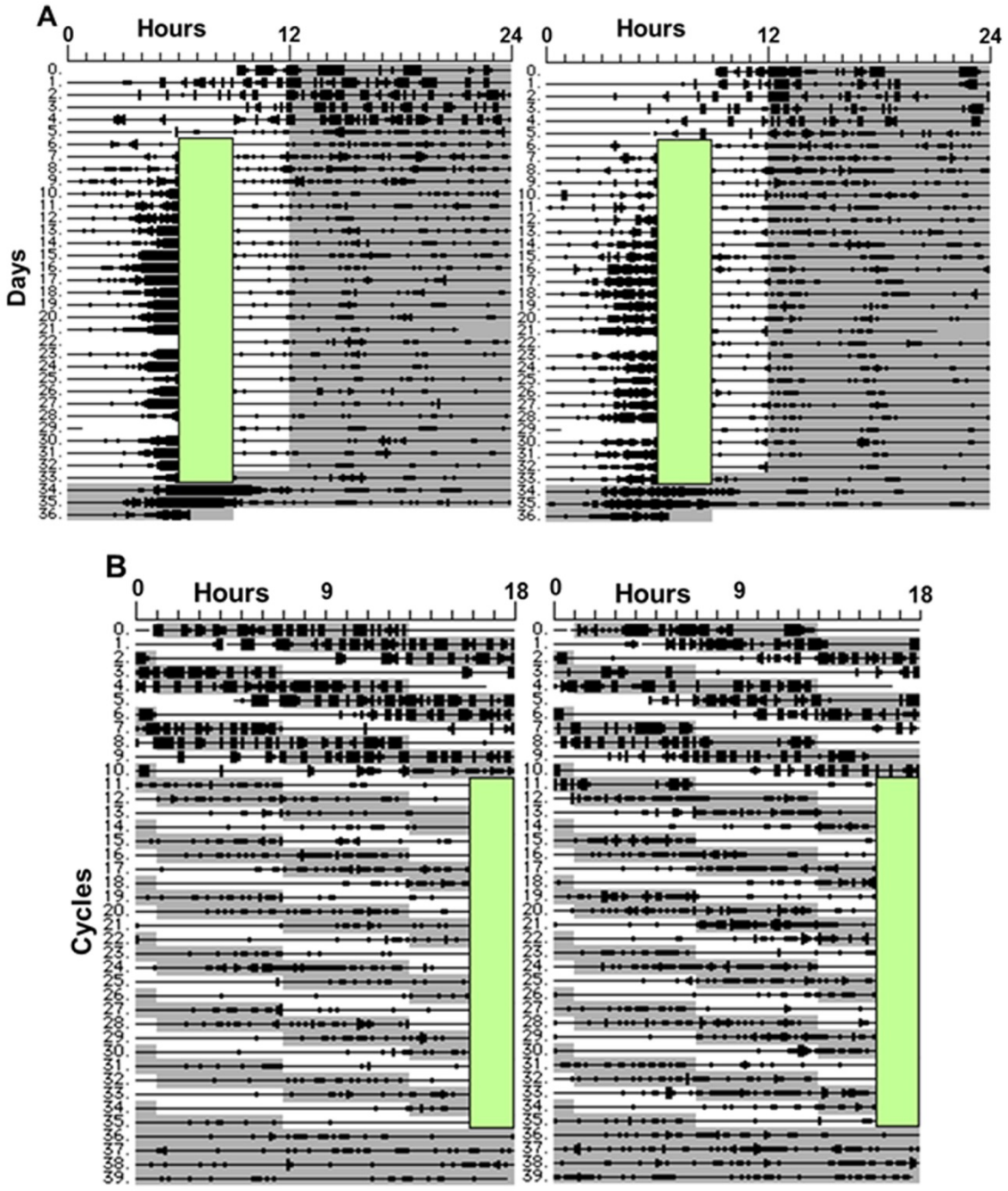

Figure 5.1. Lever pressing activity of two representative rats in Experiment 3
Time is plotted left to right in 10 min bins from lights on (A) or end of previous mealtime (B). Time bins during which lever presses were registered are denoted by heavy bars. Meals are indicated by green boxes. Lights out is indicated by gray shading. **A** and **B.** 24h feeding schedule. **C** and **D.** 18h feeding schedule. Two separate days of data collection in the 24h condition were lost due to equipment malfunction and are indicated by opaque white spaces in the plot.

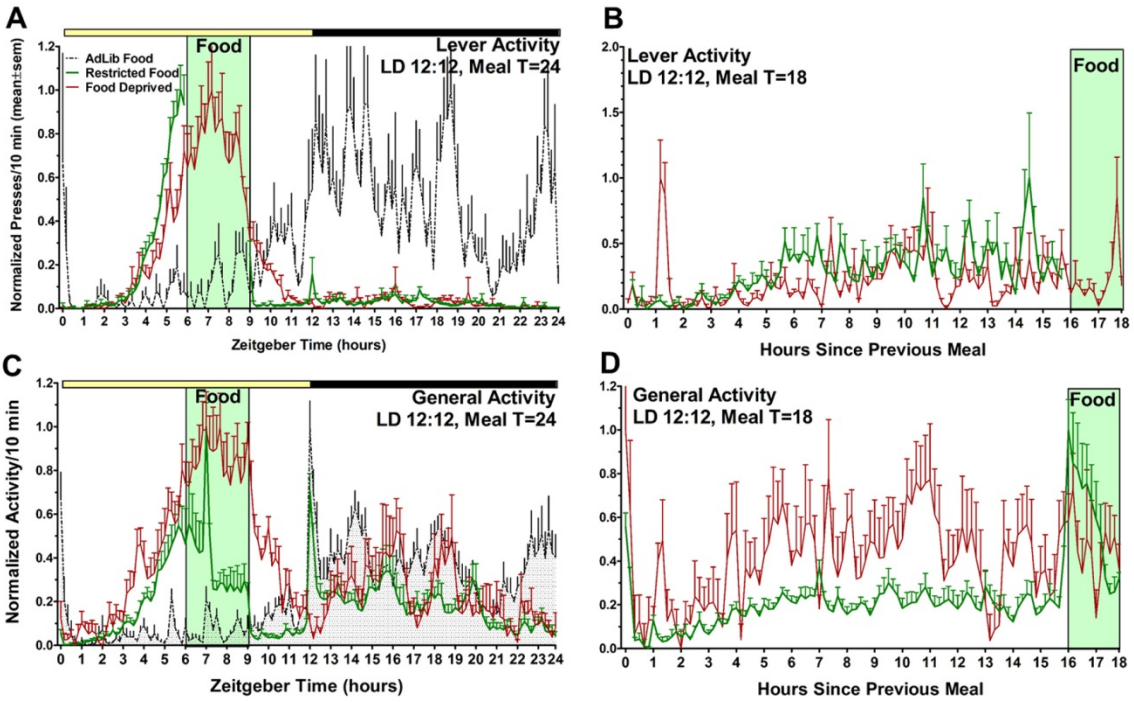

Figure 5.2. Waveforms of lever pressing activity and general cage activity in Experiment 3

Normalized group means ±SEM, N=7 rats. Time is plotted in 10-min bins from lights-on (A and C) or end of previous mealtime (B and D). Food availability is denoted by green shading. **A** and **C.** Days 1-5 of ad-lib food access (stippled black), days 17-28 of restricted feeding (green lines), and 3 days of total food deprivation in DD (red lines). **B** and **D.** Cycles 14-25 of restricted feeding (green line) and 4 cycles of total food deprivation in DD (red line).

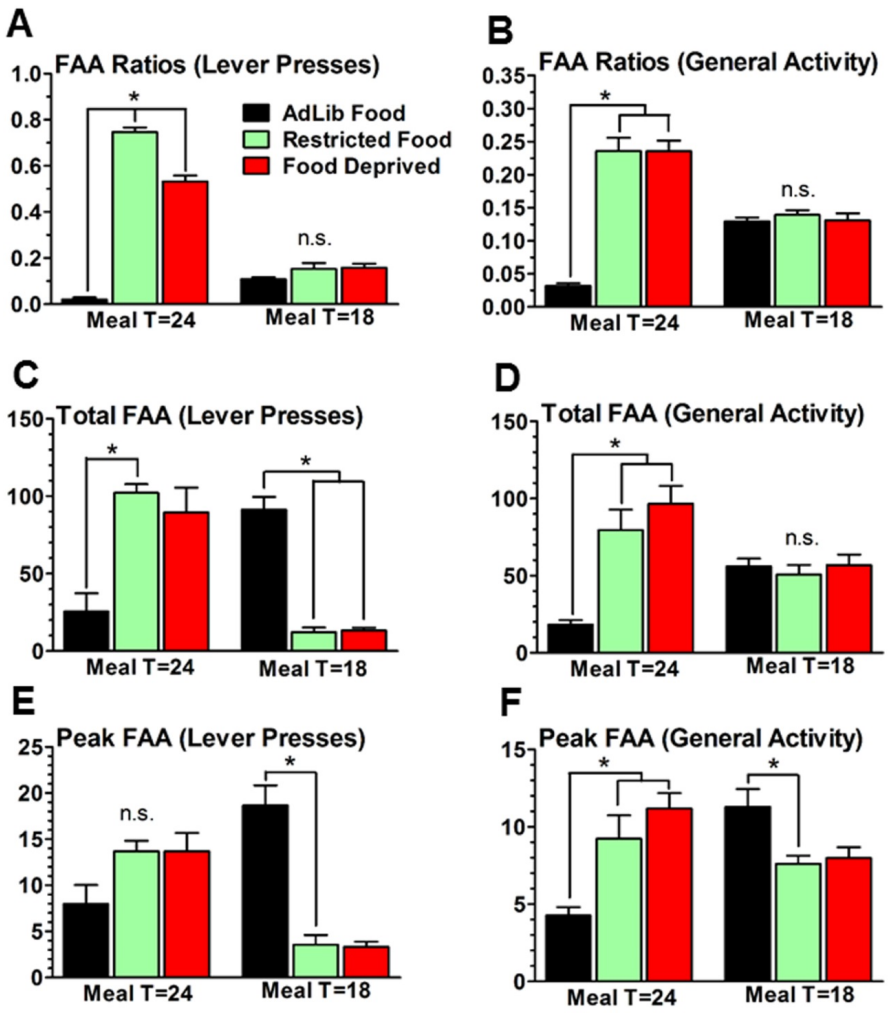

Figure 5.3. Quantitative measures of FAA in Experiment 3
Groups means ±SEM, N=7 rats. One animal was excluded from all non-normalized lever pressing analyses due to extreme outlier status. **A** and **B.** FAA ratios for each feeding condition (black: ad-lib baseline, green: restricted feeding, red: food deprived in DD). **C** and **D.** Total activity to occur over the FAA window for each condition. **E** and **F.** Peak level of activity reached during the FAA window for each condition. Significant differences at a Bonferroni-corrected $p<0.05$ are indicated by an asterisk.

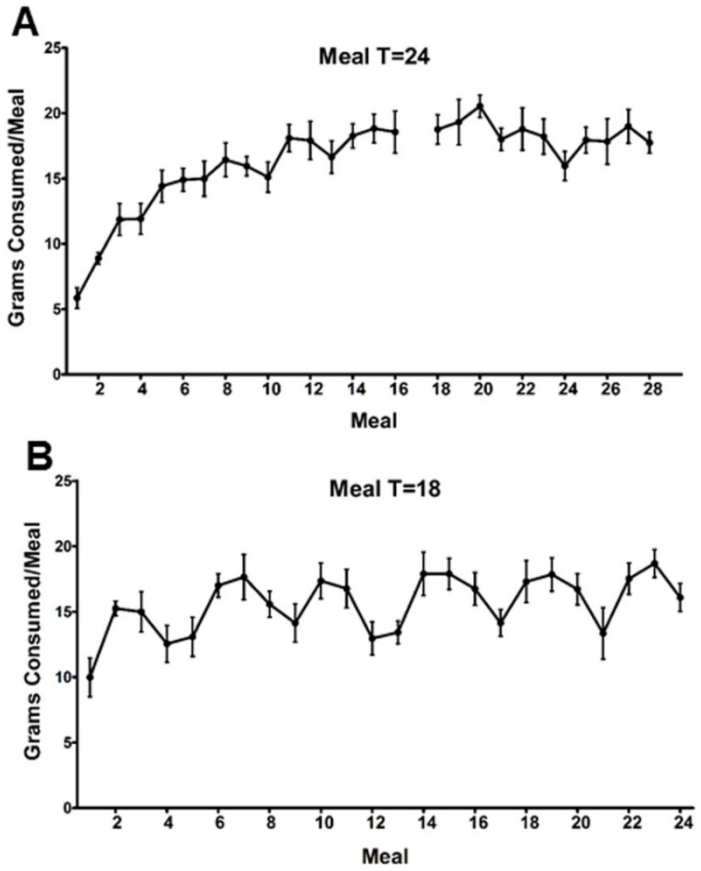

Figure 5.4. Grams consumed for each meal in Experiment 3
Group means ±SEM, N=7 rats. **A.** Grams of food consumed for each meal in the 24h condition. **B.** Grams of food consumed for each meal in the 18h condition.

Chapter 6.

Experiment 4.
Testing the scalar property:
Varying the interval from lights-on to mealtime

6.1. Methods

6.1.1. Animals and Apparatus

The same animals, operant chambers, and pellet dispensers were used as in Experiment 3.

6.1.2. Procedure

Following the food deprivation condition in Experiment 3, rats were again housed in a 12:12 LD cycle with *ad libitum* access to food (via lever presses) and water for 10 days. The rats were then food deprived for 24 hours and subsequently limited to 3h of food access daily from ZT9-ZT12 for 15 days. Mealtime was then advanced to ZT3 for 15 days, and then delayed to ZT11, ZT15, ZT19, ZT23, ZT3, and ZT6, at 15-day intervals.

6.2. Results

Visual inspection of raster plots (Figs. 6.1 and 6.2) and average waveforms (Figs. 6.5 and 6.6) confirmed that all animals displayed general anticipatory cage activity as well as robust anticipatory lever pressing to all 7 mealtimes. If rats use the interval between daily lighting changes and a daily meal to decide when to become active prior to mealtime (i.e., interval timing), then the duration of anticipation should scale with that interval. This can be quantified as the ratio between the mean duration of FAA and the

interval between lights-on (or off), which should be constant across mealtimes. Instead, for lever FAA, the ratios differed significantly across mealtimes, using the interval from lights-on ($F(6,36)=29.609$, $p<0.001$), lights off ($F(6,36)=53.16$, $p<0.001$) or the last lighting transition ($F(6,36)=22.064$, $p<0.001$) (refer to Fig. 6.3 and Table 6.1). Another interpretation of the scalar property would predict that if animals were utilizing interval timing strategies in meal anticipation then variability in FAA onset times should increase as mealtime moves later in a particular phase. The standard deviations of the durations of FAA however, did not differ across mealtimes ($F(6,36)=0.96$, $p=0.468$).

In the lever pressing data, parameters of FAA were remarkably constant across the 7 different mealtimes (Fig. 6.7). Although there were significant main effects of mealtime on FAA counts ($F(6,30)=2.607$, $p=0.037$), FAA ratios ($F(6,30)=8.190$, $p<0.001$), and FAA duration ($F(6,30)=3.078$, $p=0.015$), no pairwise comparisons survived Bonferroni corrections. Furthermore, there were no significant effects of mealtime on FAA peak level ($F(6,30)=0.992$, $p=0.493$). By contrast, in the motion sensor data, there were significant effects of mealtime on FAA ratios ($F(6,36)=21.098$, $p<0.001$) and total FAA counts ($F(6,36)=14.822$, $p<0.001$). FAA ratios tended to be greater to the nighttime meals compared to the daytime meals (refer to Figure 6.3) which is not surprising given that rats exhibit more general locomotor activity at night when fed in the day, than they exhibit in the day when fed at night. There were no differences in motion sensor parameters across nighttime meals. Across daytime meals, FAA ratio was lower in the ZT3 mealtime compared to the ZT6 mealtime.

When average measures of all daytime meals were compared to the average measures of all nighttime meals, no significant differences in any of the lever pressing FAA measures were found ($F(1,5)=1.818$, $p=0.235$). However, general locomotor FAA to meals scheduled for the daytime were characterized by significantly lower FAA ratios (0.21 ± 0.02 vs. 0.35 ± 0.02, $p<0.001$), total FAA (71.2 ± 12.2 vs. 135.0 ± 20.1, $p=0.003$), and peak FAA (9.2 ± 1.7 vs. 13.7 ± 2.2, $p=0.003$) than nighttime meals.

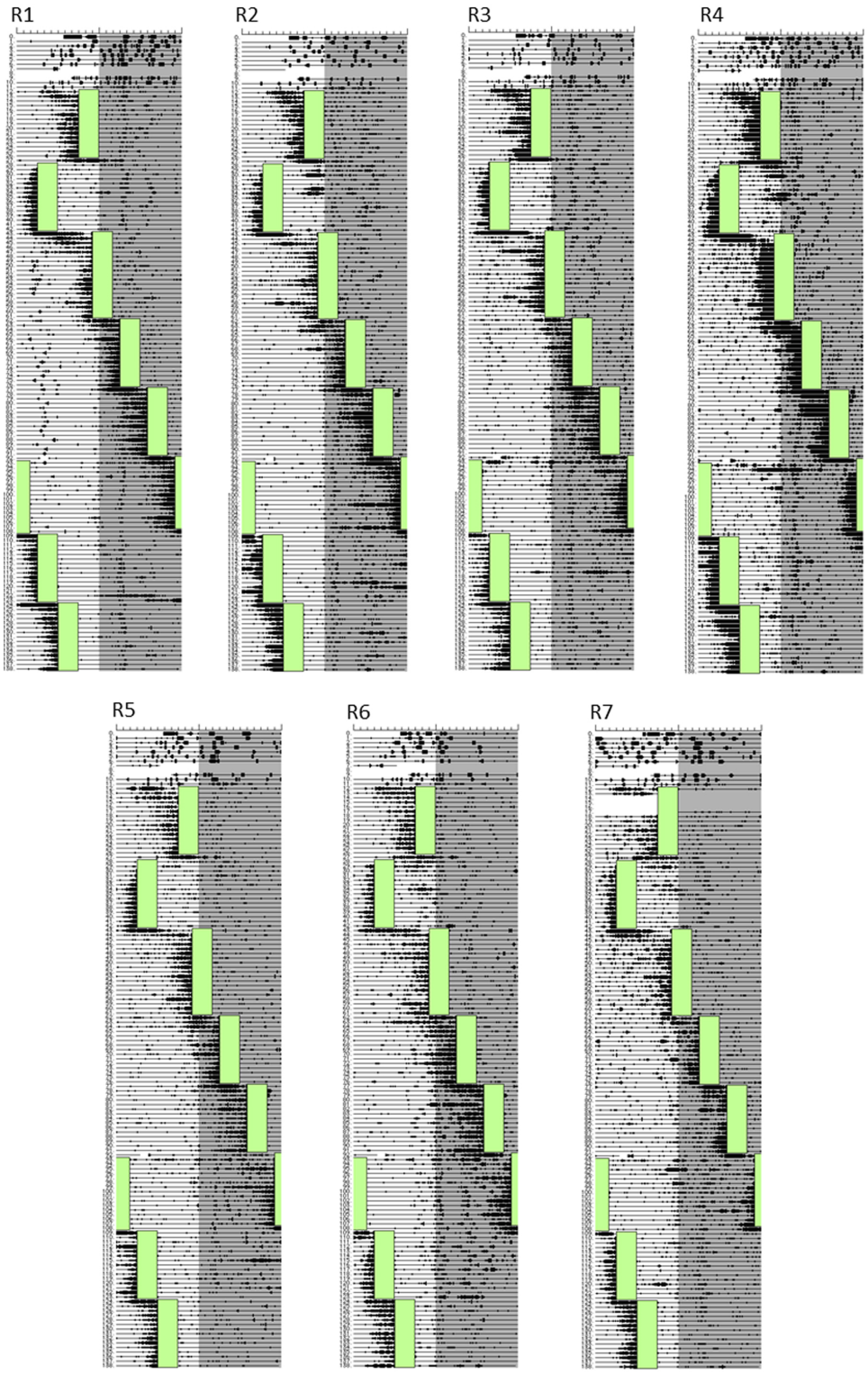

Figure 6.1. Lever pressing activity of all rats in Experiment 4
24h. feeding schedule. Each line represents 24h, with time of day plotted left to right in 10 min bins. Time bins during which lever presses were registered are denoted by heavy bars. Meals are indicated by green boxes.

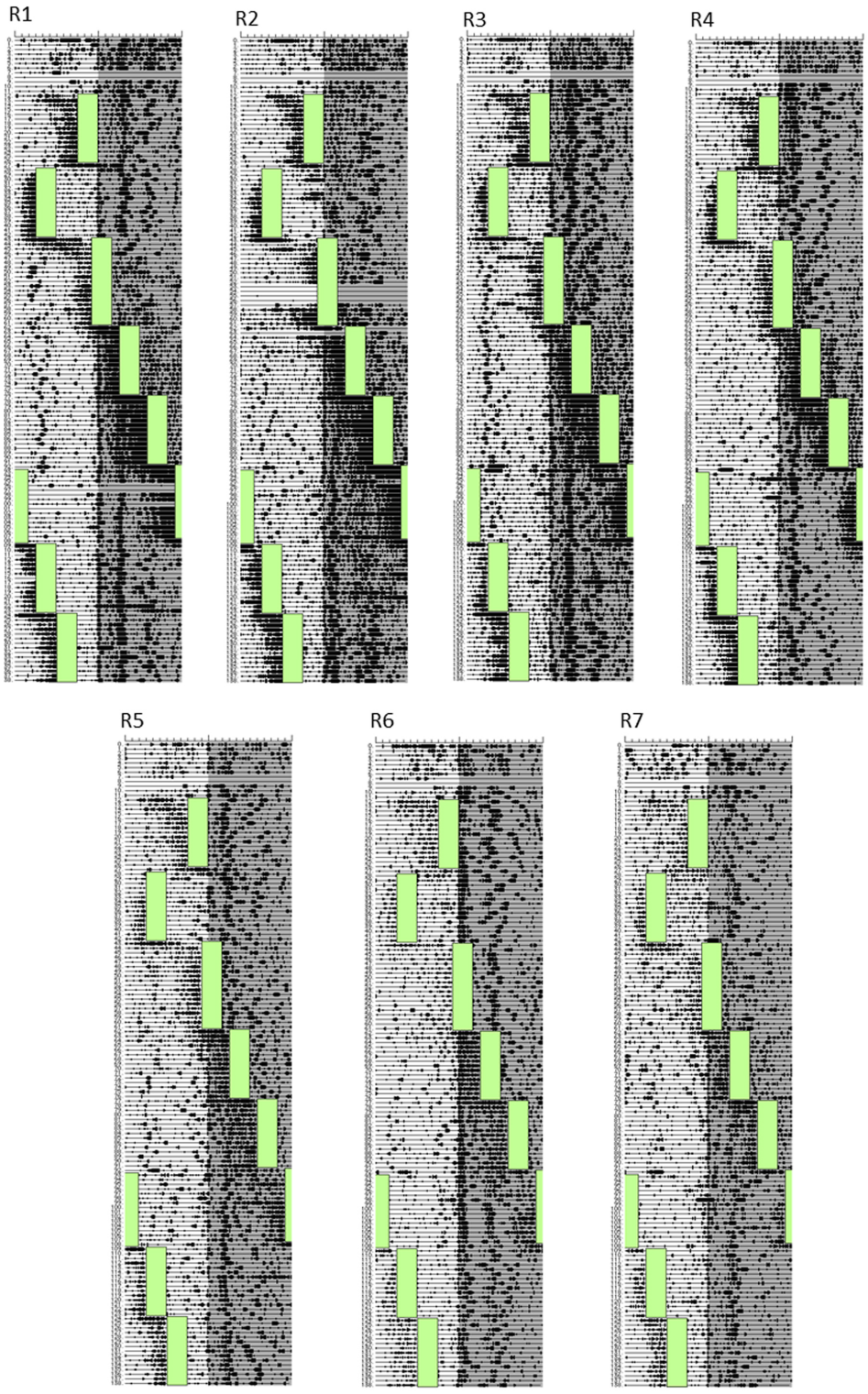

Figure 6.2. General locomotor activity of all rats in Experiment 4
24h. feeding schedule. Each line represents 24h, with time of day plotted left to right in 10 min bins. Time bins during which activity was registered are denoted by heavy bars. Meals are indicated by green boxes

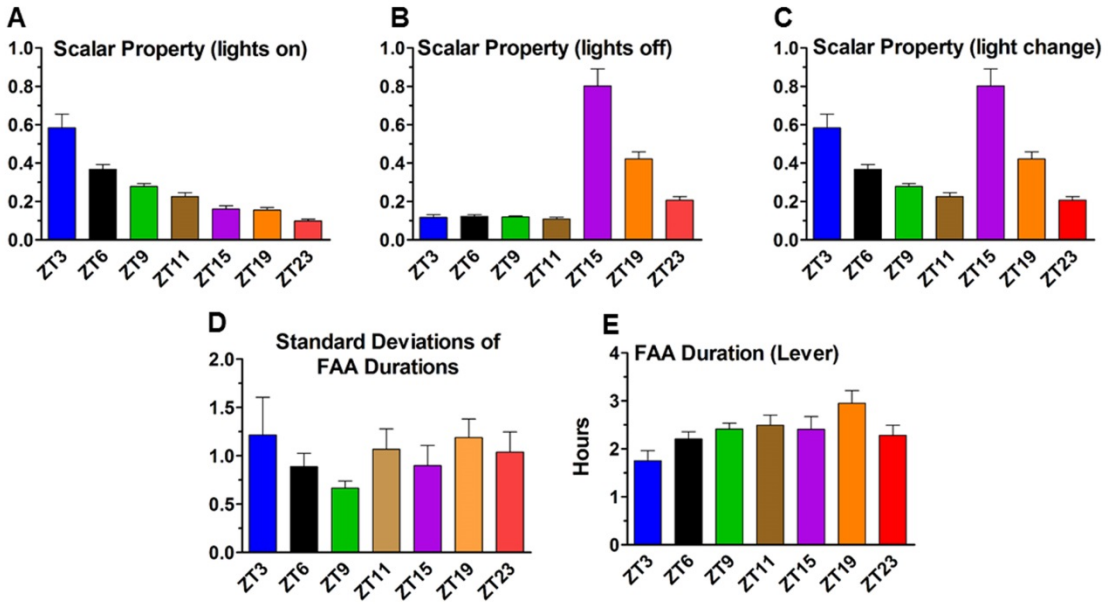

Figure 6.3. Scalar property tests
Group means ±SEM, N=7 rats. FAA duration divided by interval between mealtime and lights-on (**A**), lights-off (**B**), and most recent lighting transition (**C**). **D.** Standard deviations of FAA durations. **E.** FAA durations. For significant differences between meal times in A, B, and C refer to table 6.1. No significant differences were found in D and E.

Table 6.1. Pairwise comparisons of statistical tests of the scalar property

Comparison	Lights on	Lights off	Light change
ZT3 v. ZT6	ns	ns	ns
ZT3 v. ZT9	ns	ns	ns
ZT3 v. ZT11	0.048	ns	0.048
ZT3 v. ZT15	0.026	0.006	ns
ZT3 v. ZT19	0.027	0.009	ns
ZT3 v. ZT23	0.011	ns	ns
ZT6 v. ZT9	0.039	ns	0.039
ZT6 v. ZT11	0.022	ns	0.022
ZT6 v. ZT15	0.003	0.004	0.037
ZT6 v. ZT19	0.011	0.006	ns
ZT6 v. ZT23	0.002	ns	ns
ZT9 v. ZT11	ns	ns	ns
ZT9 v. ZT15	0.008	0.004	0.015
ZT9 v. ZT19	0.032	0.006	ns
ZT9 v. ZT23	0.001	ns	ns
ZT11 v. ZT15	ns	0.006	0.022
ZT11 v. ZT19	ns	0.008	ns
ZT11 v. ZT23	ns	ns	ns
ZT15 v. ZT19	ns	0.038	0.038
ZT15 v. ZT23	ns	0.006	0.006
ZT19 v. ZT23	0.014	0.003	0.003

SPSS Bonferroni-corrected p-values for all pairwise comparisons
FAA duration divided by interval between mealtime and lights-on, lights-off, or most recent lighting transition.
ns = not significant

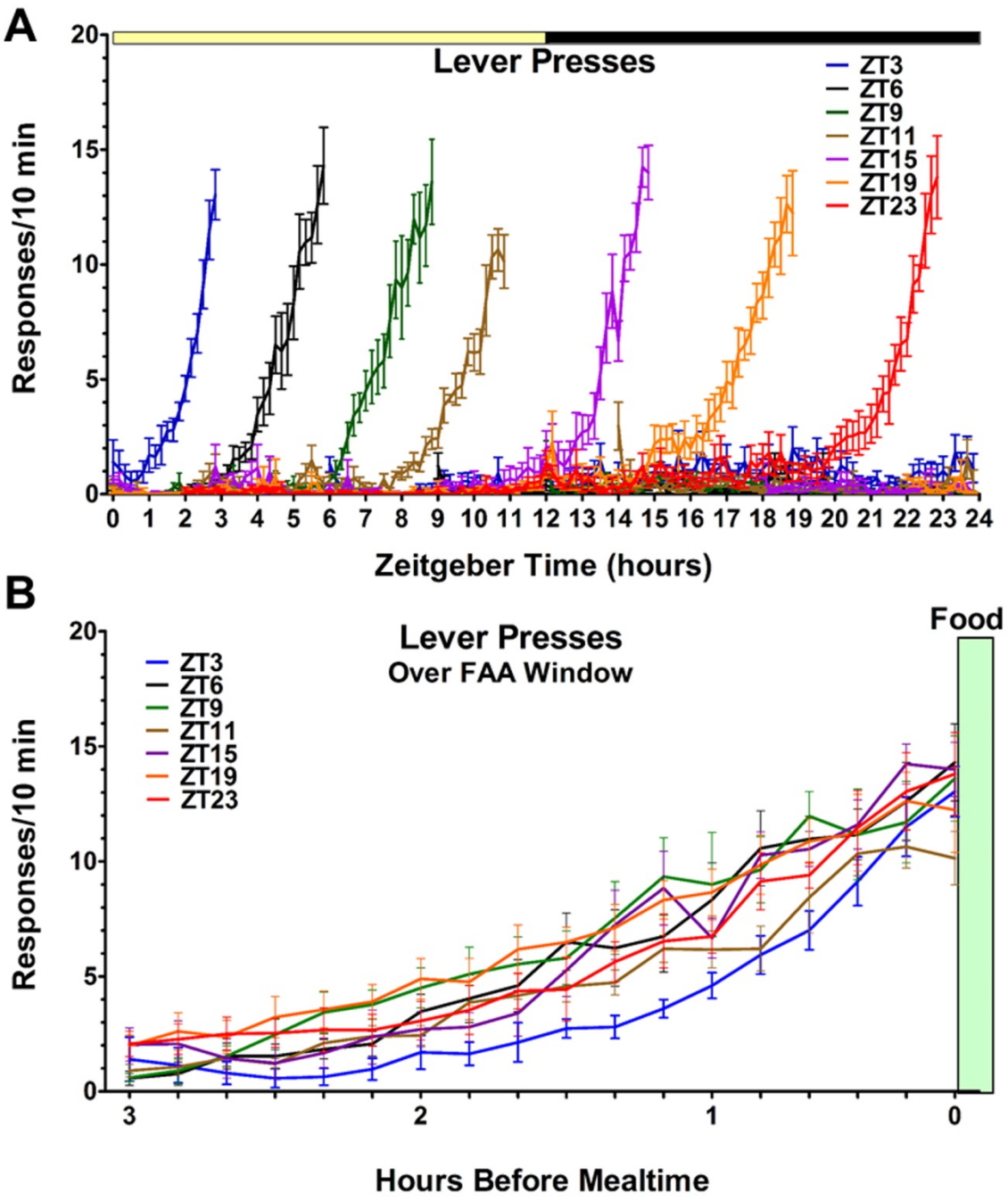

Figure 6.4. Waveforms for lever pressing activity in Experiment 4
Group means ±SEM, N=7 rats. One animal was excluded due to extreme outlier status. Time is plotted in 10-min bins. **A.** Mealtimes start where each waveform ends. Each mealtime averaged over restricted feeding days 10-14. **B.** Lever pressing activity for the 3 hours prior to mealtime in each condition averaged over restricted feeding days 10-14.

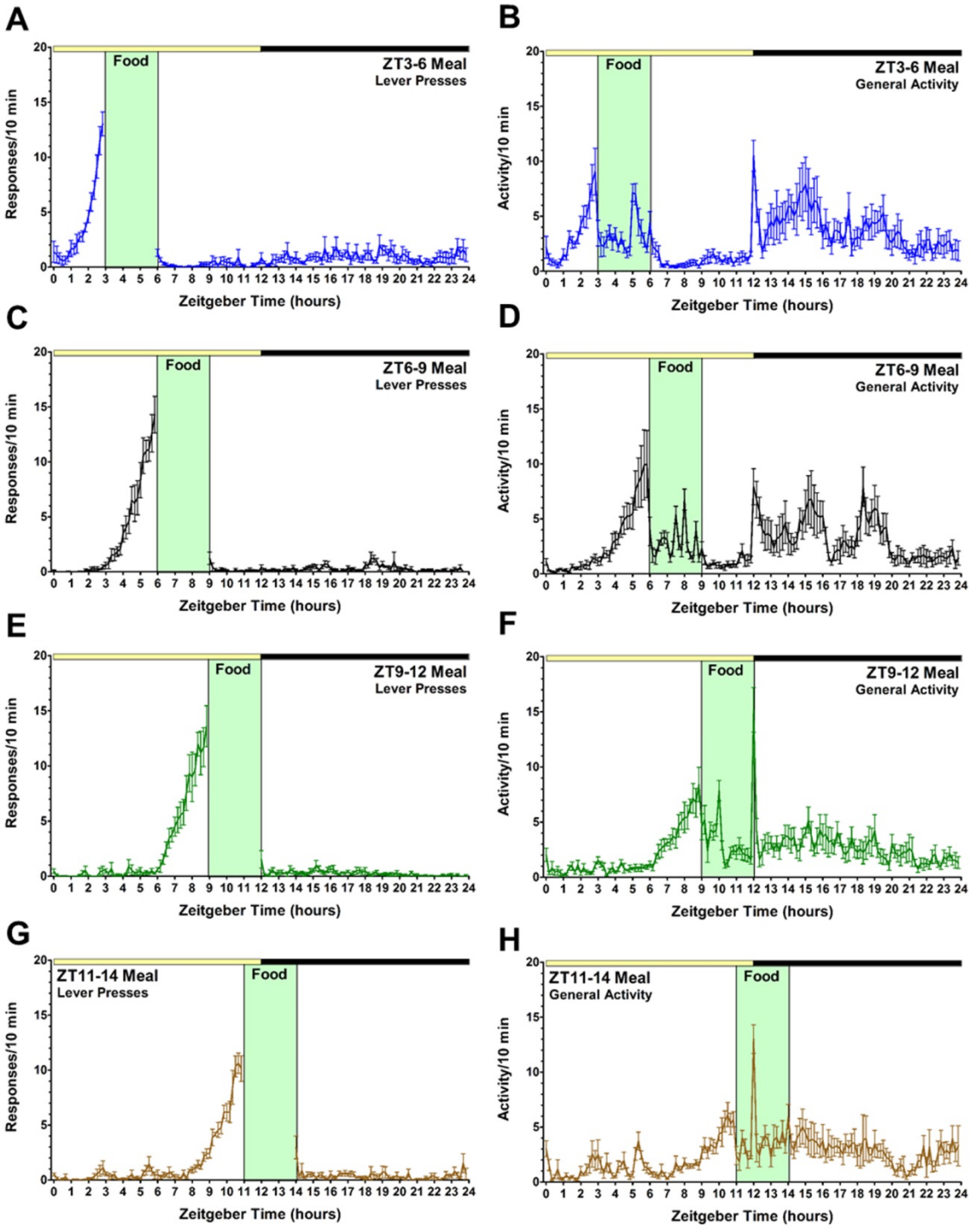

Figure 6.5. Waveforms of lever pressing activity and general cage activity for each daytime meal in Experiment 4

Group means ±SEM, N=7 rats. One animal was excluded due to extreme outlier status. Time is plotted in 10-min bins from lights-on. Food availability is denoted by green shading. Each mealtime averaged over restricted feeding days 10-14.

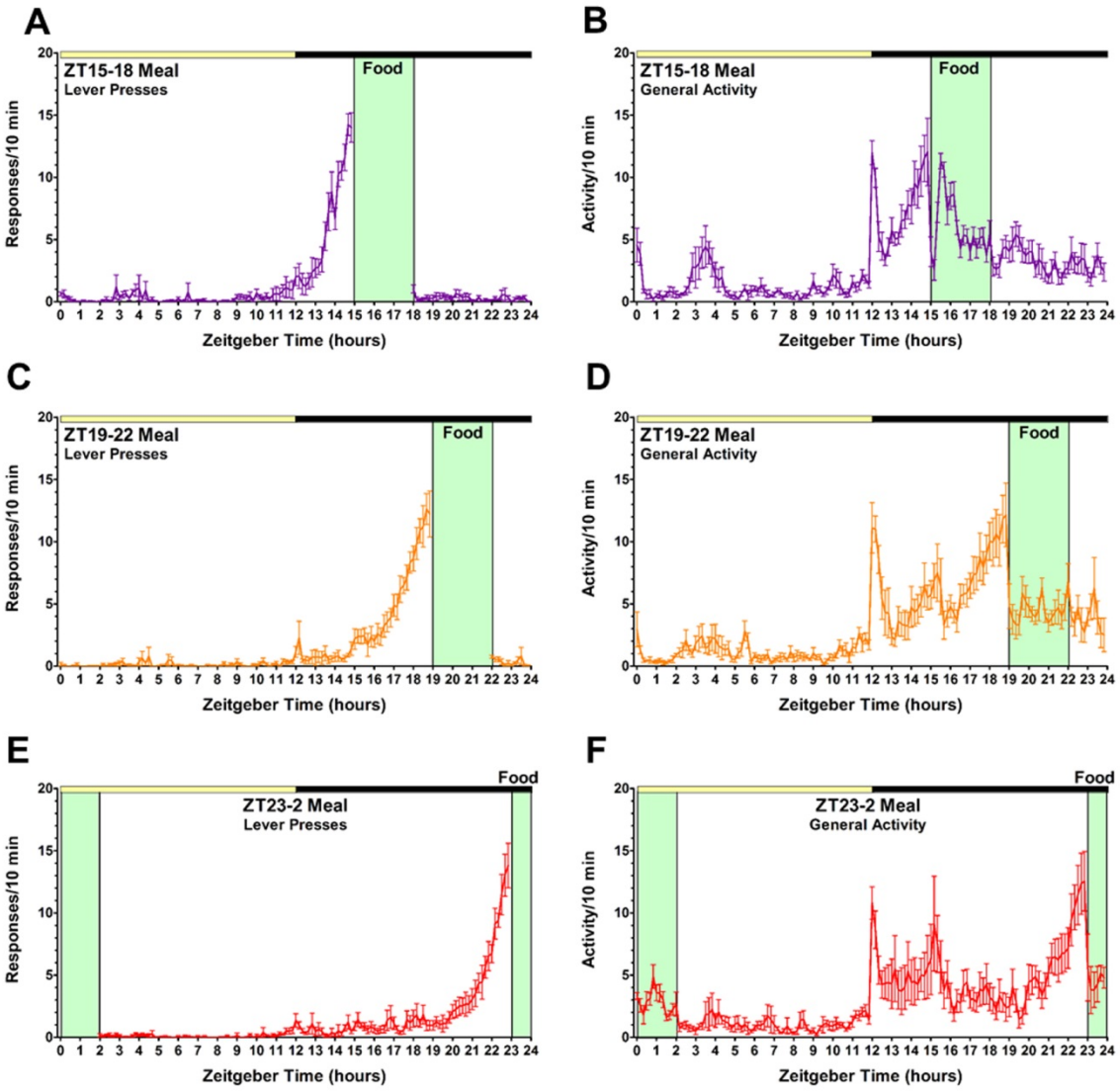

Figure 6.6. Waveforms of lever pressing activity and general cage activity for each nighttime meal in Experiment 4

Group means ±SEM, N=7 rats. Time is plotted in 10-min bins from lights-on. Food availability is denoted by green shading. Each mealtime averaged over restricted feeding days 10-14.

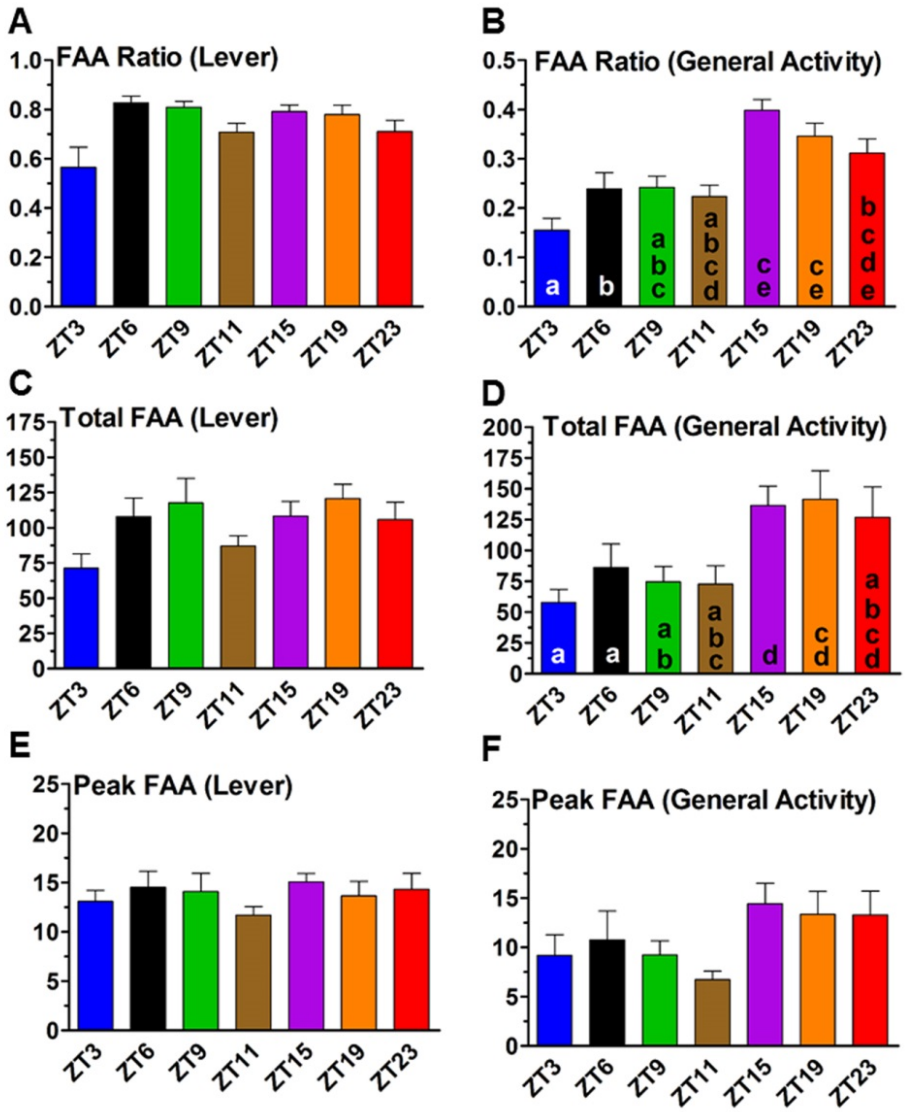

Figure 6.7. Quantitative measures of FAA as measured by lever presses or general activity for each mealtime in Experiment 4

Group means ±SEM, N=7 rats. One animal was excluded from all non-normalized lever pressing analyses due to extreme outlier status. **A** and **B.** FAA ratios for each mealtime. **C** and **D.** Total activity to occur over the FAA window for each mealtime. **E** and **F.** Peak level of activity reached during the FAA window for each condition. In graphs with lettering, means lacking common letters are significantly different ($p<0.05$, SPSS Bonferroni corrected). Graphs with no letters had no significant differences.

Chapter 7.

Discussion

Circadian limits to entrainment have been an important constraint in chronobiological theories of food anticipatory activity. Recent findings have challenged entrainment limits for FAA by showing anticipation to long, but non-circadian intervals (Crystal, 2001a; Crystal, 2006). Anticipation of mealtimes at long intervals that fall outside of the circadian range would provide evidence that the food entrainable oscillator is either non-circadian or is one among a larger array of oscillators used to time a broad range of intervals. The purpose of the current experiments was to test the hypotheses that rats can anticipate meals scheduled at long, but non-circadian intervals and that they make use of interval-timing strategies when anticipating daily meals. To do this we tested rats on 24h and 18h feeding schedules in a LD cycle or in constant light using a variety of measurements of anticipatory activity. To test whether animals time FAA in relation to the lighting schedule, a final group of rats were placed on a 24h restricted feeding schedule, which was then phase delayed once every 14 days to sample 7 different mealtimes across the LD cycle.

All animals anticipated meals scheduled at 24-, but not 18-, hour intervals regardless of lighting condition (LD or LL) or measure of anticipation (general locomotor activity or operant lever pressing) used. We found no evidence that rats show anticipatory behaviors to long intervals that fall outside of the circadian range.

Anticipation of meals at long, non-circadian intervals has been reported in studies using operant measures of anticipation (Crystal, 2001a; 2006). It has been suggested that anticipatory operant behaviors may emerge at an earlier point in the interval than wheel-running or general locomotor behavior, making operant behaviors a more sensitive measure of anticipatory activity in feeding schedules with meals provided at intervals of less than 24h (Crystal, 2001a; 2006; Balsam et al., 2009). Our results

provide no support for this hypothesis. Animals required to press a lever for food delivery showed no evidence of anticipation to 18h intervals (independent of non-specific increases in daily activity or post-prandial hypoactivity), whether anticipation was measured by lever presses or general locomotion. In contrast to this, animals displayed robust anticipatory activity, in both lever pressing and general locomotion, to 24h intervals.

Visual inspection of average waveforms revealed that when used concurrently, the two measures of anticipation were remarkably similar in both the time of activity onset and slope (see Fig. 5.2). Interestingly, once on restricted feeding, normal circadian variations in operant responding that were evident in the ad-lib condition fail to appear, regardless of the length of the inter-meal interval. In the 18h condition, operant responding fell to very low levels with no discernable rhythmic pattern, other than a distinctive pause in activity following the meal.

Our results also emphasize the importance of considering post-prandial suppression of activity in measures of FAA. In average waveforms, post-prandial suppression can create the illusion of anticipation as activity levels rise back to basal levels. Hence, the importance in using food deprivation tests of multiple cycles, ensuring that the rise in activity is truly rhythmic behavior and not due to an hourglass timing mechanism, such as a stomach emptying its contents. In quantitative measures of anticipation, such as activity ratios, especially in regards to non-circadian intervals, post-prandial suppression of activity can occur at times when normal baseline activity is high, thus inflating the ratio and potentially creating the appearance of anticipation.

Animals anticipating daily meals synchronize their daily physiological rhythms (such as body temperature) to food access (Mistlberger, 1994; refer to Figure 4.2c for example). This does not occur in animals anticipating short intervals. Additionally, there is evidence that behavioral anticipation and anticipatory rise in body temperature are dissociable phenomena (Recabarren, Valdés, Farías, Serón-Ferré, & Torrealba, 2005). Therefore, if it were possible for animals to anticipate long, but non-circadian intervals, it is not clear whether they would display a corresponding increase in body temperature. Thus, lack of physiological entrainment does not necessarily rule out anticipation to the

18h intervals, but lack of both behavioral and physiological anticipation is a strong argument against the hypothesis.

To test whether rats use the interval between changes in lighting conditions and a daily meal as a cue to time their behavior, rats were placed on a 24h restricted feeding schedule which was then phase delayed once every 14 days to sample 7 different mealtimes across the LD cycle. If rats utilize changes in lighting as a timing cue, then the duration of anticipatory activity should scale with that interval, resulting in longer bouts of FAA to mealtimes occurring later in a particular lighting phase, thus demonstrating the scalar property. We found no evidence of scalar proportionality between FAA durations and the intervals between lights-on, lights-off, or the nearest light-change and mealtimes. Another interpretation of the scalar property would predict that if animals were utilizing interval timing strategies in meal anticipation then variability in FAA onset times should increase as mealtime moves later in a particular phase. We also failed to observe differences in variability in FAA durations between the mealtimes. Taken together, our results indicate that our animals did not utilize interval timing mechanisms when anticipating a daily meal (see Figs. 6.3, 6.4, and Table 6.1).

Our results are limited by the fact that only one non-circadian interval was examined. 18h intervals were used to ensure that the temporal distribution of meals was equal between the light and dark phases without requiring extended durations of restricted feeding. An 18h interval also allows the examination of each of the four times of day that a meal could occur to determine whether anticipation occurred to any of these four mealtimes. Crystal (2006a) however, reported oscillations in operant responding with a period of 21h following anticipation to either a 16h or 21h interval, thus hypothesizing the existence of an oscillator with an intrinsic periodicity of 21h. It should be noted, however, that this oscillator was reported to be flexible in its periodicity, in that it was capable of timing both intervals. Thus, the 18h intervals employed in the current study should have been capable of engaging such an oscillator.

Taken together, our results reinforce prior evidence supporting the role of a circadian FEO in the generation of food anticipatory activity to a scheduled daily meal. These data do not rule out the possibility of non-circadian oscillators as a basis for timing

intervals below 18h. Our results do challenge the idea that animals are capable of timing 18h intervals between meals using oscillatory or non-oscillatory mechanisms. Furthermore, our results suggest that animals do not make use of interval timing processes when anticipating daily meals.

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