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Rats Time Long Intervals: Evidence from Several Cases

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Long-interval timing fills the gap between the traditional range of short-interval timing (i.e., seconds to minutes) and the limited range of circadian entrainment (i.e., approximately a day). A number of reports suggest that rats time long intervals. However, a recent report proposed that anticipation of long, but noncircadian, intervals is highly constrained. We tested the hypothesis that long-interval timing is highly constrained by examining a number of cases: 7-, 8-, 9-, 11-, 12-, and 13-hour intermeal intervals. We found evidence for long-interval timing in each case. Long-interval timing appears to be robust.

Most research on interval timing focuses on times in the range of seconds to minutes (Gibbon, 1977). Two domains straddle this range, namely millisecond timing and circadian (i.e., approximately a day) timing. It has been proposed that different mechanism subserve timing in millisecond, seconds-to-minutes, and circadian ranges (Buhusi & Meck, 2005). Although millisecond and seconds-to-minutes span the lower range of timing, there is a large gap between minutes and the circadian range; I refer to this gap as *long-interval timing* (Crystal, 2006, 2009). The absence of evidence for long-interval timing has led to the belief that animals cannot time long intervals (e.g., Bolles & Stokes, 1965; Boulos, Rosenwasser, & Terman, 1980). This belief is reinforced by the widely held view that circadian mechanisms operate within a limited range of entrainment (Aschoff, 1981; Takahashi, Turek, & Moore, 2001). Integration of short-interval and circadian traditions may provide insight into underlying timing mechanisms (Balsam, Sanchez-Castillo, Taylor, Van Volkinburg, & Ward, 2009; Crystal, 2009, 2012).

A number of lines of evidence suggest that rats can time long intervals (Crystal, 2001a, 2009, 2012). Most research on short-interval timing uses a small reward (e.g., a small piece of food for a hungry rat). By contrast, a key ingredient to produce evidence for long-interval timing comes from using meals, which is a widely used method in circadian research (Mistlberger, 1994). Small meals (approximately 8 g) readily support timing of 1.5 hr (Wilson & Crystal, 2012; Wilson, Pizzo, & Crystal, 2013). We used large meals (approximately 15-20 g) to examine timing of inter-meal intervals using several long intervals (e.g., 14, 16, 21 hr) that are below the limited range of circadian entrainment (Crystal, 2001a, 2006). In each case, temporal gradients suggested that the rats timed long intervals; for example, food-trough entries increased as a function of time prior to the meal. Although the temporal gradients were characterized by high variability (i.e., response rates were low and responses were spread broadly across the temporal range), they increased as a function of time prior to the meal in each case. In one series of experiments (Crystal, 2006), we tested the hypothesis that long-interval timing (in the range of 16-21 hr) was based on a self-sustaining endogenous oscillator. In these experiments, rats earned food by interrupting a photobeam in a food trough during meals. After approximately a month of experience with the intermeal intervals,

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the meals were discontinued to determine if periodic output continued after cessation of periodic input. When the meals were discontinued, visits continued to be periodic, with a period of approximately 21 hr. These data are consistent with the hypothesis that long-interval timing is based on a self-sustaining erogenous oscillator.

Recently, it has been reported that rats cannot anticipate meals at long, but noncircadian, intervals (Petersen, Patton, Parfyonov, & Mistlberger, 2014) using an 18-hr intermeal interval. Petersen et al. proposed that anticipation of long, but noncircadian, intervals is highly constrained. We tested the hypothesis that long-interval timing is highly constrained by examining a number of cases. If long-interval timing is highly constrained, then we would expect to find limited, if any, evidence for anticipation of long intervals (i.e., flat temporal gradients). By contrast, if long-interval timing is robust, we would expect to find evidence for long-interval timing across many cases (i.e., temporal gradients that increase as the meal approaches). Our cases include: 7-, 8-, 9-, 11-, 12-, and 13-hr intermeal intervals. The rats lived individually in behavioral test chambers for approximately a month and earned all of their food by breaking a photobeam during 1-hr meals. Food trough responses prior to the meal were examined during the last 10 intermeal intervals. To handle the expected high variability in temporal gradients, response rates were expressed as a proportion of the maximum rate. The width of the temporal gradients was measured at 50% of the maximum rate to characterize variability.

Method

Subjects

Male Sprague-Dawley rats (*Rattus norvegicus*; Charles River) were tested in groups of seven or eight (45 overall) at approximately 10 weeks of age at the start of the experiment. Before the experiment, rats were individually housed in a colony (for approximately 2.5 weeks) on a 12-12 light-dark cycle (lights turned off and on at 07:00 and 19:00, respectively). Dim red light was present in the colony and testing rooms at all times. The rats were on a restricted diet of approximately 15-20 g per day. During the experiment, the diet consisted of 45-mg pellets (PJAI-0045, Research Diets, Inc., New Brunswick, NJ). Water was continuously available. All procedures were approved by the institutional animal care and use committee at the University of Georgia and followed the National Research Council *Guide for the Care and Use of Laboratory Animals*.

Apparatus

Eight identical operant chambers (30 x 28 x 23 cm Width x Height x Depth; Med Associates ENV-007, Georgia, VT) were individually placed in ventilated sound attenuation cubicles (ENV-016M, 66 x 56 x 36 cm W x H x D). One wall had a recessed food trough (ENV-200R2M, 5 x 5 cm) horizontally centered (63 cm above the floor) between two retracted levers (ENV-112CMX). A photobeam (ENV-254) placed 1 cm inside the food trough (1.5 cm from the trough bottom) detected head entries. A 45-mg pellet dispenser (ENV-203-45IRX) was positioned outside the chamber and attached to the trough. Failures to dispense a pellet were monitored by a photobeam located on the feeder. If the feeder failed to dispense a pellet, up to four additional attempts were made. The number of pellet failures was monitored each day to identify feeders that required maintenance. The opposite wall had a water bottle placed outside the chamber with the sipper tube inserted behind a 1 x 1.5 cm (W x H) opening. A photobeam lickometer (ENV-251L) detected individual licks. A nose-poke opening was placed on left and right sides of the sipping tube. The remaining walls had four photobeams equally spaced to each other and 4 cm above the floor. The

chamber's floor consisted of 19 stainless steel rods (4 mm diameter, 15.5 mm spacing), with a stainless steel waste tray below the floor. Additional equipment included lights (ENV-215M and ENV-227M), speaker (ENV-225SM), and clicker (ENV-135M). In a nearby room, a Celeron computer (850 MHz) running Med-PC (Version 4.0) controlled experimental events and recorded the time at which each event occurred with 10 ms accuracy. Data were saved every two hr.

Procedure

Initial pretraining consisted of two 30-min, daily sessions in which a food pellet was delivered every 60 s; a click occurred 0.5 s before the delivery of a food pellet. The experiment began at 08:15, two or four days after the completion of pretraining. The experiment was conducted in constant darkness, with each rat remaining in the chamber (ENV-007) continuously throughout the study. Meals consisted of 1-hr access to food pellets; food was not available at any other time. The interval between meals (offset to offset) was 7, 8, 9, 11, 12, or 13 hr in independent groups (n = 7 or 8 per group). Because the meal was accessible for one hr, the amount of time available to evaluate anticipatory activity was 6, 7, 8, 10, 11, and 12 hr for the groups listed above (namely, 7, 8, 9, 11, 12, and 13 intermeal interval groups, respectively). During a meal, delivery of a pellet was contingent on breaking the photobeam located in the food trough using a variable-interval (VI) schedule. Food-trough activity was measured in the chambers as the dependent measure. A response was defined to occur at the time that the photobeam was first interrupted, and the interruption was required to terminate before the occurrence of another response. The VI was initially 30 s but was adjusted once per day after the meal to maintain daily consumption at approximately 15-20 g for each rat. Breaking the photobeam before or after a meal had no scheduled consequence. Each group was tested for approximately 33-35 days, during which each animal was continuously maintained in the chamber.

The experimenter accessed the operant chambers at quasirandomly determined times. Approximately once per day (at a randomly determined time between 08:00 and 17:00 but not 3 hr before or during a meal), the sound attenuation cubicle door was opened to check food and water levels (if a level was low, the level was increased). Approximately once per week (on a randomly determined day), the soiled waste tray was replaced with a clean tray.

Data Analysis

The response measure was the time of occurrence of photobeam breaks in the food trough. Temporal gradients were examined in 1-hr bins. The dependent measure for each rat was the rate expressed as a proportion of the maximum rate prior to the meal, which was then averaged across rats within each group. Mean response rates as a function of time were calculated using the 10 terminal intermeal intervals for each rat. Maximum rates are provided in Table 1. To assess the width of temporal gradients, a time series was obtained for each rat using the mean of the 10 terminal intermeal intervals by (a) calculating response frequency in 15-min bins, (b) subjecting response frequency to a 5-point running mean, and (c) finding the time of transition across 50% of the maximum rate by linear interpolation.

Results and Discussion

Figure 1 shows the proportion of maximum response rate plotted as a function of time prior to the meal. It is noteworthy that each group appears to time the interval: response rate increases to a maximum prior to the meal in each case. None of the intervals appears to be outside the range of long intervals that rats can time. The groups appear to have different variability properties. In particular, the 8-hr group appears to have less variability than the 7-hr and 9-hr groups (Figure 1A). Similarly, the 12-hr group appears to have less variability than the 11-hr and 12-hr groups (Figure 1B). We used a 6 x 5 two-way mixed analysis of variance to examine the effect of groups and time; there are 5 levels of time because we omitted times prior to the meal

that were not available in all groups and we omitted the terminal time immediately prior to the meal (because it is highly constrained to be approximately 1); there are 6 intermeal interval groups. As expected, there was a significant effect of time, $F(4, 156) = 146.75$, $p < 0.001$. There was also an effect of group, $F(5, 39) = 344.43$, $p < 0.001$, and a significant interaction, $F(20, 156) = 2.96$, $p < 0.001$. The significant interaction suggests that the rate of increase in responses as a function of time differed across groups (i.e., variability of temporal gradients differed across groups).

To quantify the variability of temporal gradients, the width of each rat's gradient was measured by identifying the time of transition across 50% of the maximum response rate. The width (measured as time prior to meal onset) is plotted as a function of intermeal intervals in Figure 2. The data represented in Figure 2 were subjected to a one-way analysis of variance, which documented a significant effect of intermeal interval on width, $F(5, 39) = 5.20$, $p < 0.001$. The width was smaller (i.e., sharper temporal gradient) for the 8-hr and 12-hr groups relative to nearby long intervals. This observation was supported by a post-hoc comparisons using the LSD statistic; the width was smaller for the 8-hr group relative to the data from the 7-hr and 9-hr groups ($p < 0.05$ and $p < 0.01$, respectively), and the width was smaller for the 12-hr group relative to the data from the 11-hr and 13-hr groups ($p < 0.01$ and $p < 0.05$, respectively). The widths for the 8-hr and 12-hr groups did not differ significantly ($p = 0.23$).

Figures 3 and 4 plot activity records on successive intermeal intervals for an individual rat from each group. Each actogram plots activity (photobeam breaks) in successive cycles as a function of time. Figure 3 plots activity records on successive intermeal interval cycles (i.e., time since the last meal in hours), beginning immediately after the first meal in the experiment; each line along the y-axis corresponds to a single intermeal interval, with the start of the experiment depicted at the bottom of each panel. The photobeam interruptions were examined in 1-min bins. If at least one photobeam interruption occurred in the bin, then a vertical line was placed on the activity record. The green and red lines indicate the start and end of each meal, respectively. Because each panel of data in Figure 3 is plotted as a function of intermeal intervals, all of the meals are depicted on the right side of the panel. Notice that anticipatory photobeam breaks are frequent prior to the meal and relatively sparse at earlier timepoints. Of course, photobeam breaks are also frequent during the meals, when the animals are obtaining food. Although the panels in Figure 3 are descriptive, they correspond well with the quantitative treatment of data described above (and shown in Figures 1 and 2).

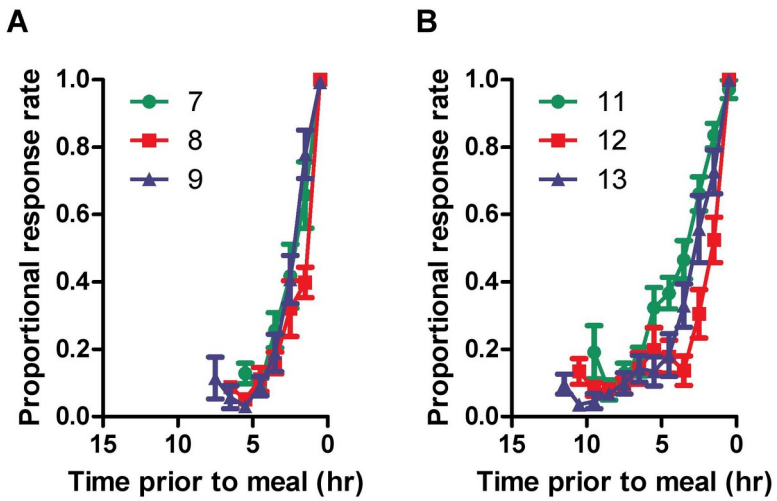

Figure 1. Response rate increases as a function of time prior to the meal using the 10 terminal intermeal intervals. The y-axis plots response rate expressed as a proportion of the maximum rate prior to the meal. The x-axis is plotted in reserves direction; note that the meal begins at time 0. (A). Intermeal intervals are 7, 8, and 9 hr. (B). Intermeal intervals are 11, 12, and 13 hr. Error bars are ± 1 *SEM*.

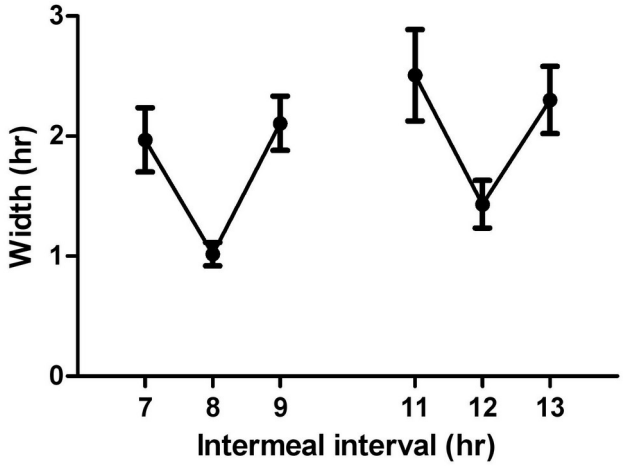

Figure 2. The width of temporal gradients is plotted as a function of intermeal intervals. Each width was calculated from an individual rat's mean response rate function, using the 10 terminal intermeal intervals. The y-axis plots width (expressed as time prior to the meal [hr] measured at 50% of the maximum rate prior to the meal). Error bars are ± 1 *SEM*.

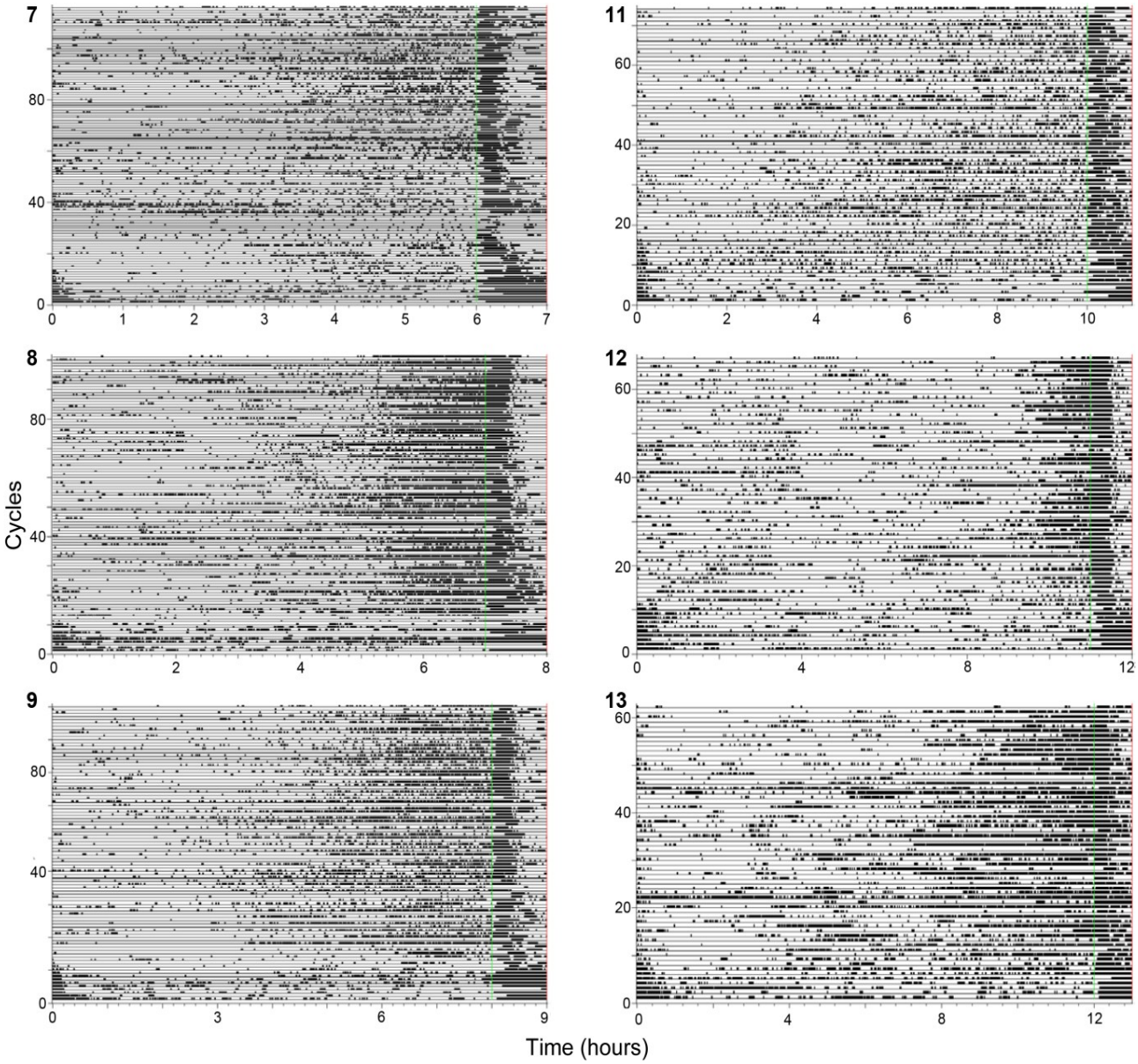

Figure 3. Activity records for an individual rat from each intermeal interval condition. The x-axis plots time into the intermeal interval, and each line along the y-axis corresponds to a single intermeal interval; the start of the experiment is depicted at the bottom of each panel, and terminal performance is depicted at the top of each panel. The data were examined in 1-min bins. If at least one response occurred in the bin, then a vertical line was placed on the activity record. Green and red lines demarcate the start and end of individual meals, respectively. In general, meals were preceded by a burst of anticipatory responses. Panels on the left side show data from intermeal intervals of 7 (top left), 8 (middle left), and 9 (bottom left) hr. Panels on the right side show data from intermeal intervals of 11 (top right), 12 (middle right), and 13 (bottom right) hr. Number at top left of each panel indicates the

The data shown in Figure 3 were replotted using a 24-hr time horizon on the x-axis, which is shown in Figure 4. In Figure 4, the 8-hr and 12-hr intermeal interval conditions produce meals (demarcated by green and red lines for onset and offset of meals) at constant times of day (3 and 2 times per day for the 8-hr and 12-hr conditions, respectively). By contrast, meals occur throughout the day in each of the

other intermeal interval conditions (i.e., the time of day of meals changed regularly across successive days).

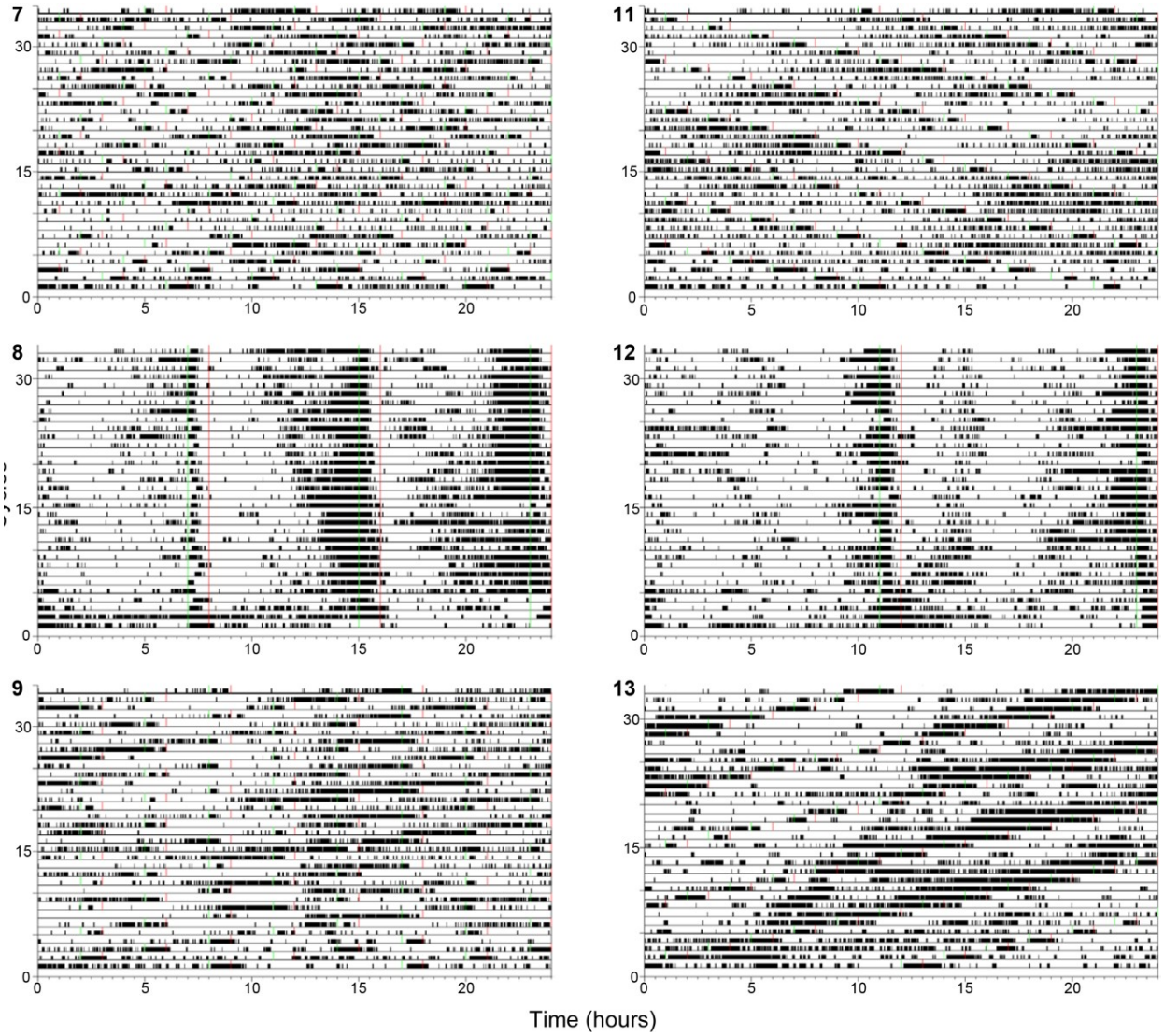

Figure 4. Activity records for an individual rat from each intermeal interval condition. The x-axis plots time in 24-hr segments. The data from the same rats shown in Figure 3 are reploted in Figure 4. Other features of the figure follow the format of Figure 3. The start of the experiment is depicted at the bottom of each panel, and terminal performance is depicted at the top of each panel. Number at top left of each panel indicates the intermeal interval (hrs).

Examination of activity as a function of time of day suggests that activity was primarily influenced by the time at which the meals occurred, rather than the time of day per se. It is difficult to detect patterns of drift as a function of time of day, which could occur when a free-running circadian oscillation impacts behavior. However, it is worth noting that the impact of a circadian oscillation cannot be ruled out for three reasons. First, intact rats are expected to have free-running rhythms unless the suprachiasmatic nucleus (SCN) is inactivated (e.g., Stephan, 2001). Although the

current work and none of the target articles (Crystal, 2001a, 2006; Petersen et al., 2014) used SCN-inactivated rats, studying long-interval timing in SCN-inactivated animals is an opportunity for future research. Second, it is difficult to evaluate the impact of a free-running rhythm in the current experiment because the rats were not maintained in the operant boxes prior to the commencement of intermeal-interval presentations. And third, the superior timing (reduction in widths in Figure 2) for the 8-hr and 12-hr intermeal intervals suggests that there is indeed a benefit for presenting meals at a consistent time of day. However, our objective here is to document that non-circadian, long intervals are indeed timed by rats, which does not preclude an impact of circadian systems. As noted above, SCN inactivation is one technique that may be used to disentangle circadian and non-circadian processes in future research.

If rats cannot time long intervals, then temporal gradients are expected to be approximately flat. None of the temporal gradients were flat. Critically, we found evidence for long interval timing in each case. These data suggest that long-interval timing is robust. These data suggest that rats can time intervals in the range of 7-13 hr. Elsewhere we have shown that rats can time other long intermeal intervals, namely 1.5, 14, 16, 21, and 48 hr (Crystal, 2001a, 2006; Pizzo & Crystal, 2007; Wilson & Crystal, 2012; Wilson et al., 2013). It is unlikely that there are any long intervals that rats cannot time. By contract, Petersen et al. (2014) proposed that anticipation of long, but noncircadian, intervals is highly constrained. There are a number differences in methodology that may contribute to the discrepancy (including number of days that the animals were left undisturbed in their cages prior to restricted feeding, the duration of food access [1 hr here vs. 2 or 3 hr], different dependent measures [food-trough inspections here vs. general activity and lever presses], exposure to different light cycles). Moreover, temporal gradients for long-interval timing are characterized by high variability and low maximum rates (Table 1), which makes it difficult to detect anticipation, especially given that response rates during the meal (which are not of primary interest) are much higher. Our approach is to normalize response rates relative the observed maximum rate prior to the meal for each rat, which reveals robust anticipation (Figure 1). Overall, it is difficult to conclude that timing long intervals is constrained when so many cases of long-interval timing have been documented (1.5, 7, 8, 9, 11, 12, 13, 14, 16, 21, and 48 hr).

Table 1

Maximum response rate (responses per minute) prior to the meal.

Intermeal Interval (hr)	*M*	*SE M*	*n*
7	1.8	0.2	7
8	3.9	0.5	8
9	3.6	0.7	8
11	2.2	0.4	8
12	4.9	0.7	8
13	6.1	1.1	7

Note. The response is a photobeam interruption in the food trough.

It is noteworthy that variability properties provide some clues about factors that influence long-interval timing. Temporal gradients were sharpest (i.e., smallest width) for 8-hr and 12-hr intermeal intervals. These groups received meals at a constant time of day, but with 3 or 2 meals per day. By contrast, the other groups (7-, 9-, 11-, and 13-hr intermeal intervals) received meals at times of day that changed gradually over successive days. Improved temporal gradients likely arise from the ability of the 8-hr and 12-hr groups to anticipate the constant time of day at which meals occurred, which was not possible for the other groups.

Variability properties in long-interval timing identify the location of endogenous oscillators. Previous work suggests that the width of temporal gradients for intermeal intervals near 24 hr is smaller than other intermeal intervals (Crystal, 2001a). The current data are consistent with this observation because 8-hr and 12-hr groups received meals at constant, although multiple, times each day. Short-interval timing (i.e., in the range of milliseconds, seconds, and minutes) are also characterized by local peaks in temporal sensitivity (Crystal, 1999, 2001b).

Local peaks in sensitivity to time have been interpreted as a feature of multiple endogenous oscillators (Crystal, 2012). Although the current data do not identify the type of timing mechanism, other work distinguishes between endogenous oscillators and pacemaker accumulators (Crystal, 2006; Crystal & Baramidze, 2007). Briefly, a hallmark feature of an endogenous oscillator is that it is self-sustaining after termination of periodic input. We have documented that long-interval timing (16-21 hr; Crystal, 2006) and short-interval timing (1-3 min; Crystal & Baramidze, 2007) are endogenous and self-sustaining. Identification of timing properties may ultimately lead to the development of a unified theory of timing that encompasses the discrimination of temporal intervals across several orders of magnitude – from milliseconds to days.

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