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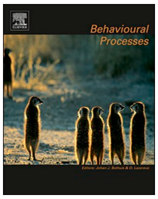

# Consistent meal times improve performance on a daily time-place learning task

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ABSTRACT

The ability of an animal to learn the spatiotemporal variability of stimuli is known as time-place learning (TPL). The present study investigated the role of the food-entrainable oscillator (FEO) in TPL. Rats were trained in an operant conditioning chamber which contained two levers that distributed a food reward, such that one lever provided food rewards in morning sessions, while the other lever provided food rewards in afternoon sessions. We expected that having access to the FEO would provide rats with more accurate depictions of time of day, leading to better performance. Rats received either one meal per day (1M group), which permitted FEO access, or many meals per day (MM group), which prevented FEO access. As predicted, 1M rats had a significantly higher percentage of correct first presses than MM rats. Once rats successfully learned the task, probe tests were conducted to determine the timing strategy used. Of the 10 rats that successfully learned the time-place discrimination, six used a circadian timing strategy. Future research should determine whether the advantage in learning seen in the rats having access to the FEO is specific to the daily TPL task used in this study, or to learning and memory tasks more generally.

## 1. Introduction

Acquiring spatiotemporal information of biologically significant events in the environment is vital for an animal's daily survival. It has been suggested that animals have developed complex cognitive systems to be able to identify how biologically significant events vary spatially and temporally (Carr et al., 1999). Learning the spatiotemporal availability of these stimuli allows organisms to better interact with their environment (Carr et al., 1999; Thorpe et al., 2007). Biologically significant stimuli may include food availability or avoidance of predators (Mulder et al., 2014; Thorpe and Wilkie, 2002, 2007; Widman et al., 2004). The ability of an animal to learn the spatiotemporal variability of stimuli is known as time-place learning (TPL) (Carr & Wilkie, 1997; Tello-Ramos et al., 2015; Thorpe et al., 2003; Thorpe and Wilkie, 2002). In daily time-place-learning (TPL) tasks, a rewarding or aversive stimulus is available in one location in the morning and in another location in the afternoon and the animal uses this information to modify its search patterns (Mulder et al., 2015; Thorpe and Wilkie, 2006).

Rats are able to learn a variety of different TPL paradigms that can vary in the nature of the biologically significant event, or the response required to approach or avoid the goal location (Aragona et al., 2002; Carr & Wilkie, 1997; Cole et al., 2016; Deibel et al., 2014, 2017; Deibel and Thorpe, 2012; Lukoyanov et al., 2002; Mistlberger et al., 1996; Pizzo and Crystal, 2002, 2004; 2006; Thorpe et al., 2012a,b; Widman et al., 2000; 2004). TPL is an adaptive characteristic displayed by a variety of species such as humans (Thorpe et al., 2012a,b), rats (Deibel and Thorpe, 2012; Thorpe and Wilkie, 2002), honeybees (Wahl, 1932; cited in Reebs, 1993), marine iguanas (Wikelski and Hau, 1995), and fish (Reebs, 1996), as well as many avian species such as warblers (Biebach et al., 1989), pigeons (Saksida and Wilkie, 1994), and hummingbirds (Tello-Ramos et al., 2015).

While it has been clearly demonstrated that a variety of species can successfully solve TPL tasks, less research has been focused on uncovering the mechanisms or systems mediating TPL performance. Cole et al. (2016) demonstrated that TPL performance recovers after an initial performance decrement when rats are given hippocampal lesions after task acquisition. In terms of timing, there are multiple candidates for the consulted clock. The two oscillators that have been examined in terms of TPL are the light-entrainable oscillator (LEO) and the food-entrainable oscillator (FEO).

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The first investigations into the possible oscillators used in daily TPL focused on the suprachiasmatic nucleus (SCN), as it is considered to be the master circadian pacemaker in the brain (Angeles-Castellanos et al., 2010; Davidson et al., 2009; Mendoza et al., 2005; Mulder et al., 2014). The SCN is located in the anterior hypothalamus (Zelinski et al., 2014) and is considered a LEO as it is entrained primarily by the LD cycle using photic cues received from the retina and hypothalamic nuclei via the retino-hypothalamic pathway (Mendoza et al., 2005; Mistlberger et al., 1996; Zelinski et al., 2013). Surprisingly, Mistlberger et al. (1996) found that rats with lesions to the SCN were still able to learn a daily TPL task in which they were required to press on one lever in morning sessions and another lever in afternoon sessions. Similarly, Boulos and Logothetis (1990) also found that rats with lesions of the SCN were not impaired on a daily TPL task. Both Mistlberger et al. (1996) and Boulos and Logothetis (1990) argued that because their rats were fed one or two meals per day at constant times of day they were able to use a FEO as the consulted clock. This is supported by the fact that the SCN-lesioned rats in the Boulos and Logothetis (1990) were not impaired in anticipating the feeding times. Prior to a regularly scheduled feeding time, rats will increase in activity (Pendergast et al., 2012; Pendergast and Yamazaki, 2014). This food anticipatory activity is thought to represent operation of the FEO (Pendergast et al., 2012; Pendergast and Yamazaki, 2014).

To our knowledge, only one daily TPL study explicitly manipulated feeding, which would presumably affect access to the FEO. Lukoyanov et al. (2002) trained rats that the escape platform in a Morris water maze was in one location in morning sessions and in another location in afternoon sessions. Rats that were fed *ad libitum,* and therefore did not have access to the FEO, did not solve this task. However, rats that were food restricted and only fed once per day (at the same time each day) were able to solve the task. The authors argued that this was evidence that access to the FEO is required for animals to learn a daily TPL task. However, data from subsequent studies conducted by Widman et al. (2004) demonstrated that access to the FEO is not necessary to solve the water maze TPL task. They replicated the finding that rats fed *ad libitum* did not solve the task; however, a second group of rats that were also fed *ad libitum* but wore weighted vests were able to solve the task. They argued that the severe food restriction in the Lukoyanov et al. study resulted in an increase in response cost and that it was this high response cost (induced either by severe food restriction or weighted vests) that resulted in the rats being more motivated to find the platform. These results suggest that TPL is possible in the absence of predictable meal times, although it might be much more difficult.

While the FEO might not be necessary to solve daily TPL tasks, the LEO is also not critical, as SCN lesions and inversions of the light dark (LD) cycle do not have any effect on performance (Mistlberger et al., 1996). The present study was designed to determine whether having a predictable feeding schedule confers an advantage over an unpredictable feeding schedule when response cost was equated across the two conditions. One group of rats (1M) was fed one meal at the same time each day, which would make it possible for these rats to use the FEO. Another group of rats (MM) received many meals per day and at random times of day, which would presumably make it impossible to access the FEO. Our design is preferable to having an *ad libitum* group as the MM group was food restricted to the same amount as the 1M group, equating response cost across the two groups. Acquisition was compared between the two groups on a standard daily TPL task conducted in an operant conditioning chamber – one lever provided reinforcement in morning sessions, while another lever provided reinforcement in afternoon sessions. Once rats successfully learned the task (defined as meeting a criterion of 18/20 correct first presses) probe tests were conducted, allowing us to determine the timing strategy (circadian versus ordinal) used. Previous work has shown that rats may use a variety of strategies to solve daily TPL tasks, including circadian (i.e., learning when within a day the reward is available in a particular location – e.g., food is in Place 1 in morning sessions and in Place 2 in afternoon sessions), ordinal (i.e., learning the order in which locations provide food within a particular day – e.g., food is first in Place 1 and then in Place 2 each day), interval (i.e., learning that within a certain time since some external event food in Place 1 or Place 2 – e.g., 2 h after light onset food is in Place 1, but 6 h after light onset it is in Place 2), and alternation (i.e., learning a non-timing strategy in which the location rewarded is the location that was not rewarded on the last session) strategies (Carr et al., 1999; Carr & Wilkie, 1997; Deibel and Thorpe, 2012; Pizzo and Crystal, 2002; 2006).

We predicted that those rats receiving a single meal per day (1M Group) would perform better than those receiving multiple meals per day (MM Group). This extends from the rationale that 1M rats have access to the FEO and are, therefore, provided with the advantage of exhibiting FAA to aid in the determination of time of day (Angeles-Castellanos et al., 2010; Bolles and Moot, 1973; Mistlberger, 2011). We expected that having access to the FEO in addition to the LEO would provide an additional benefit in solving the temporal component of the daily TPL task. In this case, performance on the TPL task would be improved when rats had access to both the FEO and LEO. In other words, if the 1M Group performs better than the MM Group, it would suggest that access to the FEO in addition to the LEO provides more accurate time of day information. However, if both groups perform equally, it would suggest that the additional information provided by the FEO does not benefit acquisition. Furthermore, we sought to determine whether rats would be circadian or ordinal timers, since past research conducted in an operant conditioning chamber has shown that rats are exclusively ordinal timers (Carr and Wilkie, 1997, 1999).

## 2. Method

All methods and procedures used in these investigations were carried out in accordance with the Canadian Council of Animal Care (CCAC) Guidelines.

### 2.1. Subjects

Eighteen male Long-Evans rats were obtained from Charles River, QC, Canada. The 18 rats were separated into two groups (1M and MM), each containing nine rats. All rats were approximately 120 days old at the start of training on the TPL task. Rats were weighed twice per week and food access was restricted. The amount of food provided to the animals was determined by allowing rats to gain approximately 10 g per week. They received standard rat diet (PMI Nutrition International, St. Louis, MO), as well as *ad libitum* water. The rats received 45 mg pellets (BioServ, Frenchtown, NJ) during training and test sessions as reinforcement.

Throughout the course of the experiment, rats were housed individually in transparent, plastic conventional cages (45 × 25 x 21 cm). Cages contained Corn Cob Bedding (Netco, New York, NY), Crinkl'Nest™ (The Anderson, Maumee, Ohio), cotton, a black pipe approximately 10 cm long, as well as a wooden block and a Nylabone (Nylabone Products, Neptune, NJ). Animals were kept in a colony room that was temperature and humidity controlled on a 12:12 h LD cycle, with lights on at 7:00 a.m.

### 2.2. Apparatus

During TPL sessions, rats were placed in a transparent Plexiglas operant conditioning box (47 × 47 x 32 cm). Two of the four walls of the operant conditioning box contained a lever (ENV-110 M, Med Associates, Inc., St. Albans, VT) with a food cup located beside it (ENV-200R1AM, Med Associates Inc., St. Albans, VT). Four pellet dispensers (ENV-203-45, Med Associates Inc., St. Albans, VT) were located on the top of the operant conditioning box. Additionally, there was a light (ENV-221M, Med Associates Inc., St. Albans, VT) located above the lever and food cup on each wall of the operant box. The floor of the operant conditioning box was lined with 2 cm of aspen woodchip bedding. The operant conditioning box was located in a small room (170 x 160 cm) that contained a cabinet (on which the operant box was placed), a radio, and a door. There was also a poster located on one of the room walls. The lights remained off in the room with the radio turned on to provide background noise during all sessions.

### 2.3. *Procedure*

Rats were randomly assigned to one of two groups. One group received a single meal per day (1M; n = 9) while the other received multiple meals per day (MM; n = 9). MM rats were fed 2 or 3 times daily (½ or ⅓ of their daily allotment of food per feeding). MM Group rats were fed two meals per day on Monday, Wednesday, and Friday, and three meals on Tuesday and Thursday. On weekends the MM Groups were fed once per day rather than multiple times. The MM rat feedings took place between 10:00 a.m. and 7:00 p.m. daily with at least 1.5 h between feedings. In contrast, 1M rats were fed their daily allotment of food at 6:00 p.m. The feeding schedules were continued throughout the entirety of the experiment.

#### 2.3.1. *Pre-training*

Rats were first shaped to lever press and then trained to a VR20 in the operant conditioning box.

#### 2.3.2. *Discrimination training*

The TPL task consisted of two trials per day, one in the morning and one in the afternoon, 5 days a week. The morning testing session began at 8:30 a.m., while the afternoon testing session began at 2:00 p.m. Each session lasted 300 s, with a 60-second time-out period at the beginning. This time-out period was given to allow rats time to explore the operant box without any choices during this time being counted as errors (Carr and Wilkie, 1997; 1999). During the time-out period, the lights in the operant box were off and lever presses did not deliver food rewards. Once the time-out period was over, the lights came on and the levers provided food rewards when pressed at the correct time of day. At the end of 300 s, the rat was removed from the chamber. Subjects were tested during the light phase of the cycle in the same operant box in which pre-training took place. For all rats, Levers 1 and 2 were available to press, which were on opposing walls of the operant box. In the morning, one lever provided a food reward based on a VR8 schedule, while the other lever was inactive. In the afternoon, whichever lever was inactive during the morning provided a food reward based on a VR8 schedule. The morning and afternoon locations of the food reward were counterbalanced across rats. Rats were tested individually and in the same order during each session. The rats that were not being tested were kept in their home cages on a cart in the laboratory. Once rats met criterion, skipped sessions took place to determine the timing strategy used.

#### 2.3.3. *Skipped session probes*

Skipped session probes were conducted to determine whether rats were using a circadian or ordinal timing strategy to solve the task. In these skip session probes, morning or afternoon sessions were omitted and performance on the following session was examined. If rats were using an ordinal timing strategy, then skipped morning sessions would result in rats incorrectly responding on the morning lever during the subsequent afternoon session. However, following skipped afternoon sessions, rats using ordinal timing would respond correctly on the morning lever in the subsequent morning session. In contrast, if rats were using a circadian strategy, they would always choose the correct lever following both skipped morning and afternoon sessions. Rats were given a minimum of two skipped morning sessions. If the results were inconsistent, they were given a third skipped morning session and whichever choice (correct or incorrect) was more popular was used in the judgment of timing strategy used. Likewise, rats were given a minimum of two skipped afternoon sessions and were given a third skipped session if the first two were not in agreement. Only one probe was given per week, and only if they had gotten at least 4/5 of the previous trials correct.

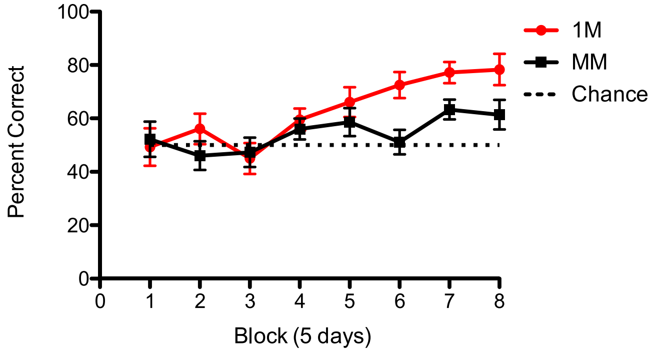

**Fig. 1.** Average percentage of first correct presses for Groups 1M and MM for the first 8 blocks (40 days, 80 experimental sessions) of training.

## 3. Results

One rat from Group 1M was excluded from all statistical analyses for uncharacteristically poor performance (rarely exceeding 11/20 successful trials). Every other rat, even those who never reached 18/20 correct trials, did frequently score between 15 and 16 correct trials out of 20. Therefore, a total of 17

For illustration purposes, trials were grouped into blocks of 5 days (10 sessions) to examine how performance changed across time, for each group. The average percent of correct first presses for Group 1M and Group MM is shown in Fig. 1, where percent correct was defined as the ratio of a rat's number of first correct presses after the 60-second time-out to total number of first presses. We chose to examine only the first eight blocks of acquisition (40 days) because, by Block 9, five of the eight rats in Group 1M had reached criterion and began probe sessions. At the beginning of training, rats were included in the statistical analyses. Success for each trial was defined by a rat's first press after the 60-second time out. Generalized Linear Mixed-Model analyses were conducted using the lme4 package v1.1–17 (Bates et al., 2015) in R v3.5.1. the percentage of correct presses was similar for both groups. As training continued, Group 1M displayed a higher percentage of correct first presses than Group MM. The pattern of data would also suggest that, over time, both groups of rats were able to discriminate which lever provided a food reward depending on time of day. To confirm this impression, a logistic Generalized Linear Mixed Model was conducted to predict the odds of success on each trial. Day, Group, and the interaction between Day and Group were entered as fixed effects and Rat was the random factor (with a random intercept). As can be seen from Table 1, there was a significant effect of Day, with Rat being 3.18 times more likely to make a correct first press at the end of the 40 days, which suggests rats in general were able to learn the task. There was also a significant difference in Group with rats in the 1M group more likely to be correct than rats in the MM group. However, there was also a significant Day by Group interaction. Looking at each Group independently, the MM group was 1.94 times more likely to choose correctly at the end of 40 days, which was still significantly different, CI [1.14, 3.27], $p = .014$. The 1M group, on the other hand, was 5.24 times more likely to choose correctly at the end of 40 days, CI [2.87, 9.55], $p < .0005$. In combination with the significant interaction, this indicates that the 1M rats' performance improved significantly more quickly than the MM rats.

A possible explanation for why Group 1M performed better than Group MM on the current task could be that rats from Group 1M were hungrier during their afternoon session (because they had not been fed

**Table 1**
Logistic Generalized Linear Mixed Models Examining the Effects on the Probability of Having a Correct First Press on Each Trial.

| Variable | Odds Ratio | 95% CI | $p$ |
|---|---|---|---|
| Model with Day and Group as Predictors | | | |
| Intercept | 1.44 | [1.27, 1.64] | < .0005* |
| Group | 0.70 | [0.54, 0.90] | .006* |
| Day | 3.18 | [2.14, 4.74] | < .0005* |
| Group * Day | 0.37 | [0.17, 0.82] | .014* |
| Model with Day, Group, and Time of Day as Predictors | | | |
| Intercept | 1.44 | [1.27], [1.63] | < .0005 |
| Group | 0.70 | [0.54], [0.91] | .006* |
| Day | 3.27 | [2.19], [4.90] | < .0005* |
| Time of Day | 0.74 | [0.59], [0.93] | .014* |
| Group * Day | 0.37 | [0.16], [0.82] | .015* |
| Group * Time of Day | 0.82 | [0.52], [1.29] | .377 |
| Day * Time of Day | 2.97 | [1.33], [6.65] | .008* |
| Group * Day * Time of Day | 0.61 | [0.12], [3.07] | .552 |

Note: Group and Time of Day were centered and coded such that a one-unit increase represented the difference between going from 1M to MM for Group and from Morning to Afternoon for Time of Day. Day was centered and scaled so that a one-unit increase represented the difference between the beginning and the end of the 40-day acquisition period. $^{*}p < .05$.

since 6:00 p.m. the evening before), compared to the MM Group. If this was so, we would expect better performance from the 1M rats in the afternoon session compared to the morning session. To test for this possibility, we repeated the same analysis as above but added Time of Day as another predictor. In this analysis, there were effects of Time of Day, but they did not support this alternate explanation. Looking at the lower model in Table 1, the significant effect of Time of Day suggests that rats were overall more correct in the morning sessions. Nevertheless, the significant Time of Day by Day interaction suggests that performance for afternoon trials improved more over time than morning trials did. This contradiction is possible because rats started off immediately having higher success on the morning sessions, with the afternoon sessions catching up over time. It is important to note, however, that there was no interaction between Group and Time of Day and no significant three-way interaction, which means these effects did not vary by Group. Although the effects of Time of Day are interesting, their pattern of favoring the morning, and not having any interaction with Group, rules out the possibility that the 1M rats' better performance was an artifact of them being hungrier than the MM rats in the afternoon session.

The trials to criterion data also indicated better performance for the 1M Group, with 7/8 1M rats (in an average of 45 days) achieving criterion and only 3/9 MM rats doing so in an average of 48 days (see Table 2). Fisher's Exact Test revealed that the number of rats reaching criterion was significantly different between the groups (rounded $p$ = 0.050).

Furthermore, a Mann-Whitney test was performed on the trials to criterion to compare which group reached criterion first. The non-parametric Mann-Whitney test was conducted because it allowed us to include the rats who did not reach criterion as the lowest rank scores. A parametric test would require these rats to either be assigned a value for when they would have theoretically reached criterion or not be included in the test, and either option would misrepresent the data. This test was significant, $U = 12.0$, $p = 0.021$, with rats in the 1M group reaching criterion before rats in the MM group.

Skip session probes were conducted to determine whether rats were using a circadian or ordinal timing strategy. Following a skip session probe, the subsequent session was analyzed to determine whether the rat's first press following the 60-second time-out was correct or incorrect (see Method for an explanation of circadian and ordinal timing strategies). Table 2 shows the results of these skip session probes, as well as the trials to criterion for each rat. Of the 10 rats that reached criterion, six used a circadian timing strategy.

**Table 2**
Criterion and Timing Strategies of Rats.

| Meal Group | Rat | 18/20 | Skip AM Probe | Skip PM Probe | Timing Strategy |
|---|---|---|---|---|---|
| 1M | 1 | – | – | – | – |
| 1M | 2 | Day 64 | CC | ICC | Circadian |
| 1M | 3 | Day 57 | CII | CC | Ordinal |
| 1M | 4 | Day 41 | CC | CC | Circadian |
| 1M | 5 | Day 42 | CCI | ICC | Circadian |
| 1M | 11 | Day 37 | ICC | ICC | Circadian |
| 1M | 12 | Day 34 | CC | ICI | Indeterminable |
| 1M | 14 | Day 42 | ICC | ICI | Indeterminable |
| MM | 6 | – | – | – | – |
| MM | 7 | – | – | – | – |
| MM | 8 | – | – | – | – |
| MM | 9 | Day 48 | CC | CC | Circadian |
| MM | 10 | – | – | – | – |
| MM | 15 | Day 50 | CII | CC | Ordinal |
| MM | 16 | – | – | – | – |
| MM | 17 | | | – | – |
| MM | 18 | Day 46 | CC | ICC | Circadian |

*Note.* A "C" indicates that the first press made on the session following the skip-session indicated in the table was correct. An "I" indicates that the first press made on the session following the skip-session indicated in the table was incorrect. Rats that pressed on the correct lever following a skip-session probe for both skip AM and skip PM probes demonstrated a circadian timing strategy. Rats that pressed incorrectly following a skip AM session, but not following a skip PM session were determined to have used an ordinal strategy. And, if rats pressed incorrectly following both skip AM and skip PM sessions they were determined to be alternating between levers on successive sessions. For two of the rats in Group 1M their pattern of responding did not conform to any of these patterns and were labeled as "indeterminable".

## 4. Discussion

The purpose of the present study was to determine whether rats that received one meal per day at the same time each day (and likely had access to the FEO) acquired a daily TPL task quicker than rats that received multiple meals per day at unpredictable times of day (and likely did not have access to the FEO). As expected, rats that had predictable meals (1M group) performed better than rats with multiple unpredictable meals (MM groups), as indicated by a greater number of correct trials and more rats reaching criterion.

Rats that are restricted to one meal per deal will display FAA a few hours before the predicted meal (Mistlberger, 1994, 2011; Stephan et al., 1979). Subsequently, FAA can comprise a large proportion of daily activity. For instance, past research has suggested that 54% of daily activity was in anticipation of a restricted meal that occurred during subjective day (Stephan, 1986). If one includes the two training sessions involved in the current study, our 1M Group could be considered to have been fed three times daily. However, the vast majority of daily calories were from the restricted feeding session at the end of the day, rather than the two training sessions. In a very similar TPL paradigm, Mistlberger et al. (1996) found that most of the daily activity occurred around and between the two daily testing sessions. However, their rats received food after both training sessions, and not just after the last session, as in the current study.

Due to the lack of monitoring activity, we can only speculate what our 1M and MM rats' activity rhythms would look like. We choose not to record wheel running activity for fear that it might affect learning and memory performance (e.g., Anderson et al., 2000). We predict that, despite the two daily training sessions, activity data would show 1M rats to be entrained to the meal at the end of the day. This is supported by data suggesting that even when given free access to food, rats will entrain to nutrient rich meals (Mistlberger and Rusak, 1987). As the bulk of the caloric intake for the 1M rats came from the feeding rather than the testing sessions, we postulate that the 1M rats were entrained to the 6:00 p.m. feeding session.

In contrast, it is very unlikely that the MM rats entrained to their meals. Furthermore, as all of the feeding occurred during the light phase of the cycle, it would be likely that there was more activity during the light phase than normal. Escobar et al. (2007) demonstrated this by observing that a meal that occurred at an unpredictable time during the light phase of the cycle increased activity during this phase from 22% to 37% of the daily amount. However, they did not see any FAA in this group, but instead saw FAA for the previous day's mealtime. As we also had an unpredictable number of meals, it is unlikely that the MM rats displayed FAA, but rather just increased activity during the light phase of the cycle.

An obvious question is why entrainment to a restricted mealtime might mediate time-place discriminations. It is hypothesized that training times in TPL are discriminated in relation to zeitgebers (Mulder et al., 2015; Thorpe and Wilkie, 2006). As mentioned earlier, the possibility that rats in TPL tasks discriminate using the light dark cycle was ruled out using SCN lesions and light-dark cycle inversions (Mistlberger et al., 1996). This opens up the possibility that TPL performance might rely on entrainment to a mealtime. In our past studies (Deibel et al., 2014, 2017; Deibel and Thorpe, 2012; Thorpe et al., 2012a,b), the feeding regime was identical to our current 1M Group. When the present data are compared with those mentioned above, entrainment to a mealtime appears to be the primary mechanism by which rats can learn TPL tasks.

Another study supports our argument that entrainment to a meal is a primary mechanism in successful TPL. While the vast majority of TPL studies use food as the event, Lukoyanov et al. (2002) used an aversively motivated TPL task in the Morris water maze (Morris, 1984). When free fed rats trained to swim to one escape location in the morning and to another escape location in the afternoon failed to learn the task, they ingeniously tested another group of rats that were food restricted. These rats were fed once a day at one of two times in the morning separated by three hours, and were restricted to 60% of their *ad libitum* weight. Interestingly, the corticosterone rhythm in these animals was entrained to both the onset of darkness and the restricted meal in the morning. Furthermore, these food-restricted rats learned the task. The authors suggested that "it is possible to assume that the ability to acquire time-of-day discrimination is largely dependent upon the access to temporal information provided by the food-entrainable circadian oscillator" (p. 288, Lukoyanov et al., 2002).

Additionally, the fact that of the 10 rats that reached criterion, six used a circadian timing strategy further supports the role of the FEO and other circadian oscillators in TPL. It should be noted however that the rats in the present study could have been using an interval timing strategy in conjunction with a circadian timer. Pizzo and Crystal (2002) conducted probes tests in which rats that had learned a daily TPL task received constant lighting and no daily meal for 24 h prior to the probe. They wanted to determine if rats were using interval timing from either colony light onset or meal time. Rats continued to search the correct locations at the correct time of day although there was a decrement in performance (i.e., the proportion of correct responses decreased on probe trials). They argue that this suggests that rats were using both interval and circadian timing strategies. Unfortunately, we were unable to do these colony lighting probes as this requires additional testing space that we did not have available.

Therefore it is possible that rats were using an interval strategy in conjunction with a circadian strategy. However, it is unlikely that they were using an interval strategy on its own. Scalar timing predicts that temporal expectations are more variable when the timed duration is longer. If the rats were using an interval timer set to either meals or lights on, afternoon sessions after a skipped morning session might be harder than skipped morning sessions because the timed duration would be five and half hours longer. While we did find better performance during acquisition in morning sessions, this was marginal and performance during afternoon sessions improved greatly as the experiment progressed. In the probe trials this was not the case as qualitatively performance was better after skipped morning sessions (72%) versus skipped afternoon sessions (69%). If an interval timer, started from meal time was mediating performance, the MM group should not have acquired the task at all. In contrast, although it took longer than the 1M group, 33.3% of the MM rats acquired the task. Although for the above reasons we feel it is unlikely that the rats were using only an interval timing strategy, future studies should investigate the use of interval timing in this daily TPL paradigm.

Our finding of predominately circadian timers is in contrast to previous studies suggesting that rats strictly utilize an ordinal timing strategy in similar operant conditioning box versions of the daily TPL task (Carr and Wilkie, 1997; 1999). In fact, Carr and Wilkie (1999) were unsuccessful in demonstrating circadian timing strategies in a daily TPL task in which rats only received one session per day on some days, thereby making it that rats had to use a circadian strategy to solve the task. However, Carr and Wilkie (1999) provided rats with meals following their experimental sessions, which differed daily from morning-only, afternoon-only or both morning and afternoon sessions. The randomness of these experimental sessions, and the subsequent randomness of the following mealtimes would prevent FEO access. Even when rats within such an experiment were switched to a schedule of two typical TPL sessions daily, no time was permitted to allow rats to entrain to the subsequent regular mealtimes. Therefore, this suggests that without FEO access, utilization of a circadian strategy to solve daily TPL tasks may not be possible.

At the same time, the role of an FEO in TPL does not appear to be absolute. Three of the rats in the MM Group learned the task, even if the other six did not. Furthermore, Widman et al. (2004) did a follow-up study of Lukoyanov et al. (2002) and found that rats could learn the TPL water maze task if the response cost of swimming was increased by the use of weighted vests. These results suggest that TPL is possible in the absence of FEO access, although it might be much more difficult.

Alternatively, in the absence of consistent meal times such as *ad libitum* conditions, the FEO is thought to entrain to the SCN (Stephan, 1986). If a consistent meal is introduced the FEO is thought to uncouple from the SCN and entrain to the meal over the coming days. As it takes on average five days for food anticipatory rhythms to entrain to a four-hour delay in feeding time (Stephan, 1986), it is unlikely that the FEO in the MM group was able to entrain to the random meals. Thus, in the present study it is likely that the FEO was synchronized to the SCN. This opens up the possibility that the FEO could still be mediating performance in the MM group, but as its amplitude and/or stability would likely be attenuated, performance suffers.

Essentially, accuracy of the circadian clock may be improved by access to two circadian oscillators (i.e., both the LEO and FEO). This, in turn, may be helpful in solving the temporal component of the daily TPL task. Rats could presumably use information from both oscillators to ensure that they have access to more accurate circadian information. One method to calculate circadian time of a food-related event might involve the phase angle difference from the FEO and the SCN. As touched on above, when the FEO is entrained to the SCN instead of meals, calculating circadian time of meals would be more difficult as the phase angle difference information would be minimal or inconsistent.

An important tenet to consider is that is that improved circadian rhythmicity from access to multiple oscillators improves performance on learning and memory tasks more generally. Recent research has suggested that circadian rhythm disruption can lead to impairments in memory. For example, Zelinski et al. (2013) disrupted rats' circadian rhythms by advancing their light schedule by three hours each day for six consecutive days. These acute episodes of photoperiod shifts disrupted circadian timing, negatively affecting spatial memory in a hippocampal-dependent task, as well as retrieval of previously acquired associations. If circadian disruption leads to impairments in learning and memory, it is possible that improvements in circadian rhythmicity will lead to improved learning and memory.

We propose that access to the FEO provides a way for temporal cues to be relayed to learning and memory structures such as the hippocampus. Corticosterone rhythms entrain to predictable restricted feeding (Ahlers et al., 1980; Honma et al., 1983), but are arrhythmic when the time of the restricted meal is variable (Verwey and Amir, 2012). Interestingly, along with blunted corticosterone rhythmicity, unpredictable meal time also caused a blunting of *Per2* rhythmicity in learning and memory structures such as the dorsal striatum and dentate gyrus region of the hippocampus, while SCN *Per2* rhythms were unaffected (Verwey and Amir, 2012). These data suggest that unpredictable mealtimes actually disrupt clock gene expression in learning and memory structures and the mechanisms appear to be corticosterone driven.

However, clock genes, TPL, FEO access and FAA all compile to create a complex story. *Cry1* and *Cry2* are needed for an aversively motivated TPL task in mice, but *Per1* and *Per2* are not (Mulder et al., 2015; Van der Zee et al., 2008). In terms of FAA, single deletions of all canonical clock genes did not abolish FAA, although FAA is typically affected in these clock gene mutants (for review see, Mistlberger, 2011). Interestingly, clock gene mutants are typically impaired in hippocampal dependent learning and memory tasks (for review see, Deibel et al., 2015). In summary, although normal clock gene function is not necessary for FAA, it is for learning and memory in many tasks. Our interpretation of the present finding is that unpredictable meal times are affecting clock gene expression in learning and memory structures and this is, subsequently, impairing TPL performance. It remains to be seen if learning and memory in the gold-standard hippocampal dependent tasks is affected by unpredictable meals.

Future research is needed to determine whether the advantage of FEO access is specific to tasks in which time of day information is crucial, or if the advantage would also extend to other tasks. It is important to understand how access to peripheral circadian oscillators such as the FEO, and subsequent potential improvements in circadian rhythmicity, can affect behavior and physiology in rodent models, as this can have implications for humans. Lack of stable circadian rhythmicity is common in individuals with diseases such as Alzheimer's disease and cancer, as well as in mood and anxiety disorders (Zelinski et al., 2014). Additionally, individuals who experience long-term shift work tend to exhibit a lack of stable circadian rhythmicity (Davidson et al., 2009; Zelinski et al., 2014). There are numerous health detriments associated with long-term shift work, such as heart disease, cancer, dementia, obesity, immune dysfunction, hypertension, and infertility (Davidson et al., 2009; McDonald et al., 2013). If providing access to the FEO can improve circadian rhythmicity and benefit learning and memory, it is possible that by regulating mealtimes, we can reduce some of the negative physiological and cognitive impacts in humans that have been associated with disrupted circadian rhythms.

In conclusion, the current data suggest that access to a predictable mealtime and subsequent FEO access is crucial for TPL and, to our knowledge, is one of the only demonstrations that unpredictable meals can impair learning and memory. Future research is needed to discern whether the effect of FEO access improves general circadian rhythmicity, improves performance on cognitive tasks that specifically utilize time of day information, or enhances general cognitive functioning. Furthermore, future research is also needed to determine whether meal regularity will help humans, specifically to improve learning and memory as in the current study. If so, there is the potential to utilize rodent models to improve cognitive impairments associated with circadian arrhythmia problems in humans. Not only would this better reflect the impact of arrhythmicity on learning and memory in human populations, it would also provide insight into the impacts of circadian arrhythmia on processing time of day information in both animals and humans.

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