INVESTIGATING THE ROLE OF THE FOOD-ENTRAINABLE OSCILLATOR AND THE EFFECT OF LIGHT MANIPULATIONS ON HIPPOCAMPAL-DEPENDENT AND HIPPOCAMPAL-INDEPENDENT TASKS

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Abstract

Circadian rhythms are responsible for physiological processes necessary for survival. The presence of certain external cues (e.g., light, food) are necessary to maintain rhythmicity in the suprachiasmatic nucleus. However, disruption of circadian rhythms occurs through irregular patterns in external cues (e.g., shift work). Circadian disruption impairs hippocampal-dependent memory. Two experiments were conducted that explored the influence of circadian disruption (i.e., light manipulations) on performance of hippocampal-dependent and -independent tasks. Additionally, food access was restricted to assess any benefit of consistent feeding schedules in performance on these tasks. Experiment 1 used a modified 30-day light manipulation and had rats on either single or multiple meal schedules. The results of Experiment 1 found an impairment in acquisition of the hippocampal-dependent Morris Water Maze task in the groups exposed to the light manipulation, but no effect of meal schedule. There were no differences between groups in performance of the hippocampal-independent tasks (i.e., Elevated-Plus Maze, and Stimulus-Response Task). Experiment 2 used a previously validated light manipulation, with the addition of rats placed on either a single of multiple meal regimen. The results of Experiment 2 found no differences between groups in retention of the Morris Water Maze task. Overall, the light manipulation in Experiment 1 resulted in an impairment in the acquisition phase of the Morris Water Maze task, but the impairment was not present in the probes or other phases of the task. Experiment 2 was incomplete due to COVID-19; It is expected that with the addition of a second cohort an effect of meal will be observed in retention of the Morris Water Maze Task.

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Table of Contents

List of Tables

List of Figures

Investigating the Role of the Food-Entrainable Oscillator and the Effect of Light Manipulations on Hippocampal Dependent and Hippocampal-Independent Tasks

Circadian rhythms modulate many physiological processes and behaviors, such as sleep-wake cycles, hormone secretion, body temperature, and organ activity (Arendt, 2010; Craig & McDonald, 2008; Damiola et al., 2000; Li et al., 2016; McDonald et al., 2013; Schulz & Steimer, 2009; Verwey & Amir, 2009; Zelinski et al., 2013). These rhythms are shared by almost all organisms and provide a highly adaptive mechanism to anticipate daily environmental events (Gritton et al., 2013; Silver & Kriegsfeld, 2014).

The suprachiasmatic nucleus (SCN), located in the anterior hypothalamus, is known as the master clock of the brain as it controls and maintains circadian rhythms throughout the brain and body (Castillo et al., 2004; Mistleberger, 2009; Silver et al., 2011; Verwey & Amir; 2009; Zelinski, Hong, et al., 2014). The SCN acts as an internal pacemaker which drives a daily rhythm of nearly 24 hr and external cues (i.e., *Zeitgebers*), such as light, meals, and social interaction, synchronize the clock with the environment (Arendt, 2010; Craig & McDonald, 2008; Gritton et al., 2013; Silver et al., 2011; Verwey & Amir, 2009; Zelinski, Deibel, et al., 2014; Zelinski, Hong, et al., 2014). The SCN is commonly known as the Light-Entrainable Oscillator (LEO), given that light is the most salient *zeitgeber* for the SCN (Arendt, 2010; Li et at., 2016; McDonald et al., 2013; Mistleberger, 2009; Mulder et al., 2014; Zelinski et al., 2013; Zelinski, Deibel, et al., 2014). Additionally, there are non-photic cues that entrain peripheral oscillators (i.e., circadian oscillators that exist outside of the SCN). Entrainment refers to the alignment of the circadian system with the presence of external cues (Craig & McDonald, 2008). These cues include, but are not necessarily limited to, food, exercise, social cues, and learning/memory (Arendt, 2010; Gritton et al., 2013; Schulz & Steimer, 2009).

The clock mechanisms consist of molecular feedback loops containing positive and negative elements (e.g., clock gene products in the SCN that cycle with a near 24-hr period in the absence of external signals) (Castillo et al., 2004; Damiola et al., 2000; Schulz & Steimer, 2009; Silver & Kriegsfeld, 2014; Zelinski, Hong, et al., 2014). The SCN is largely responsible for maintaining oscillations throughout the brain, including areas such as the olfactory bulb, hippocampus, cerebral cortex, and amygdala (Zelinski, Deibel, et al., 2014). These areas of the brain are referred to as slave oscillators, given that without input from the SCN, the majority of these brain regions fail to generate circadian outputs (Zelinski, Deibel, et al., 2014). Overall, the SCN is vital in developing and synchronizing oscillations throughout the entire brain and body.

Although the SCN drives rhythms throughout the brain and the body, there are several oscillators that have SCN-like properties and are thus known as semi-autonomous (Silver et al., 2011). In addition to light, food access is another important *zeitgeber*. Food anticipatory activity (FAA) is the increase in activity one to three hours prior to a restricted feeding that occurs the same time each day (Davidson et al., 2003; Escobar et al., 2007; Silver et al., 2011; Stephan et al., 1979; Verwey & Amir, 2009). The anticipation is a result of entrainment to meal time, whereby food acts as the *zeitgeber* that can influence both the SCN and peripheral oscillators (Mistleberger, 2009). The FAA rhythms appear to depend upon a food-entrainable oscillator (FEO) which is independent of the light-entrainable circadian oscillator system (Rosenwasser et al., 1984; Silver et al., 2011; Verwey & Amir 2009; White & Timberlake, 1995). Mice and Syrian Hamsters that sustained complete ablation of the SCN lacked circadian organization when given *ad libitum* access to food, but when given a meal that occurred at the same time every day, they exhibited behavioural and physiological rhythms that were entrained to the meal (Mistleberger, 2009; Rossenwasser et al., 1984). These SCN-independent food-anticipatory

rhythms exhibit formal properties that meet criteria for an entrained, circadian clock-controlled process (Castillo et al., 2004; Mistleberger, 2009; Rosenwasser et al., 1983; White & Timberlake, 1995). That is, the behaviour associated with food entrainment exists, even when the SCN is not entrained to the LD cycle (Davidson et al., 2003; Mistleberger, 2009). Additionally, SCN lesions do not abolish or even reduce feeding entrained behavioral rhythms (Davidson et al., 2003). The FEO is independent of the SCN and access to food acts as a potent *zeitgeber* to which mammals can entrain.

Previous research showed that rats placed on a variable meal schedule were not able to exhibit FAA but exhibited a daily activation pattern in phase with their previous meal (Escobar et al., 2007). The variable feeding strategy acts as a 24-hour basis resetting mechanism for metabolism and general behaviour, meaning the rats attempt to entrain to their new mealtime every day (Escobar et al., 2007). In contrast, FAA tends to persist when animals are placed on a restricted feeding schedule (Davidson et al., 2013; Rosenwasser et al., 1983; Schulz & Steimer, 2009). When animals are placed on a restricted feeding schedule, the consistency of meal time acts as a potent *zeitgeber* that desynchronizes daily metabolic and clock gene oscillations in peripheral tissues from the SCN (Escobar et al., 2007; Stephen et al., 1979; Verwey & Amir, 2009; Zelinski et al., 2013). Furthermore, previous studies have shown that when rats are placed on a restricted meal regimen (i.e., one meal a day), they perform better on Time-Place Learning (TPL) tasks than rats on *ad libitum* food schedules (Lukoyanov et al., 2002) or rats in which mealtime varies each day (Wall et al., 2019). Rats on the restricted meal regimen got a higher percentage of first correct presses on a T-maze TPL task than rats on the variable meal schedule (Wall et al., 2019). In addition, rats on a restricted meal regimen were able to discriminate between platform locations at two different time points, whereas the rats on *ad libitum* did not

perform above chance (Lukoyanov et al., 2002). Furthermore, Mistlberger et al. (1996) showed that rats' activity entrained to a daily TPL task and SCN lesions did not impair task performance. Therefore, access to the FEO is considered a primary mechanism in successful TPL performance in rats (Lukoyanov et al., 2002; Mistlberger et al., 1996; Wall et al., 2019). Overall, manipulation of food access has an effect on both the activity and memory of lab animals.

Due to the influence of external cues on circadian rhythms, manipulation of the lighting (LEO) and food (FEO) schedules can induce circadian disruption. It is important to understand the implications of circadian rhythm disruption given that certain populations are consistently exposed to shifts in their work/sleep schedules (e.g., nurses, shift workers). Long-term shift work has been correlated with obesity, cancer, poor cardiovascular health, hypertension, immune dysfunction, gastrointestinal disorders, disrupted hormonal balance, and infertility (Arendt, 2010; Knutsson, 2003; McDonald et al., 2013). The health problems and increased risk for major disease in long-term shift workers are ascribed largely to working out of phase with the internal biological clock (Arendt, 2010; Knutsson, 2003). There are several mediators in all the aforementioned side effects of shift work (e.g., genetic susceptibility), but it is clear that shift work is one of the factors (Knutsson, 2003). Additionally, dysfunctional circadian clocks are characteristic of several disease states including dementia, mood/anxiety disorders, and Alzheimer's disease (Craig & McDonald, 2008; Schulz & Steimer, 2009; Zelinski et al., 2013; Zelinski, Hong et al., 2014). The fact that non-pharmacological (e.g., light therapy, sleep deprivation, rhythm therapy) and pharmacological (e.g., lithium, antidepressants, adomelatine) therapies of affective disorders influence circadian rhythms, indicates that biological clocks play a role in the pathophysiology of these disorders (Schulz & Steimer, 2009). It is therefore vital to learn more about what effects various forms of circadian disruption can have on an individual.

In artificial environments, such as a lab setting, external cues can be manipulated to disrupt clock function and result in SCN arrhythmicity (Meijer & Reitveld, 1989). Ruby et al. (2008) showed that arrhythmic hamsters were unable to perform a novel-object recognition task, supporting the role of the circadian system in learning and memory that goes beyond that of simply providing temporal organization to memory function. The hippocampus often exhibits dysfunction following exposure to numerous forms of environmental disruption (e.g., light manipulation, and change in meal time) (Craig & McDonald, 2008; Devan et al., 2001; Ruby et al., 2008; Zelinski et al., 2013; Zelinski, Hong, et al., 2014). A common non-invasive method to disrupt circadian rhythms is altering the light/dark schedule as the SCN relies heavily on retinal input (McDonald et al., 2013). A common form of light manipulation is photoperiod shifting, which is a procedure that actively changes an animal's light:dark (LD) cycle, causing a healthy animal to modify its behaviour in an attempt to entrain to the novel schedule (Craig & McDonald, 2008; Zelinski et al., 2013; Zelinski, Hong, et al., 2014). Photoperiod shifts create alterations in the synchronization and integrity of the SCN, similar to what is observed during various disease states (Zelinski, Hong, et al., 2014).

A plethora of research has shown that circadian disruption can result in various forms of cognitive impairment (Craig & McDonald, 2008; McDonald et al., 2013; Zelinski et al., 2013; Zelinski, Hong, et al., 2014). Previous experiments have shown that chronic light manipulations resulted in impaired context learning and memory processes thought to be mediated by a neural circuit centered on the hippocampus (McDonald et al., 2013). Additionally, rats that were exposed to a light manipulation for six days and then trained on the Morris Water Maze (MWM) showed normal acquisition of the task, but impaired long-term retention when compared to control animals (Devan et al., 2001). Rats that were exposed to a chronic circadian disruption

(i.e., 64 days), that contained a combination of light manipulations and partial re-entrainment periods (i.e., the light schedule stayed the same time for consecutive days), were severely impaired on both acquisition and retention of the MWM task (Craig & McDonald, 2008). Acute phase shifted animals, that were exposed to one cycle of the chronic circadian disruption, were not impaired when trained subsequently on the MWM (Craig & McDonald, 2008). Craig and McDonald (2008) suggested that the longer an individual suffers from circadian disruption, the greater the likelihood that signs of dementia or cognitive impairments will develop. Relatively brief periods of circadian disruption (e.g., 6 days of photoperiod shifting) can prevent retrieval of associations acquired prior to manipulation and/or accelerate the decay rate of these memories in these subjects (Zelinski, Hong, et al., 2014). Overall, the amount and timing of lighting manipulations play a significant role in whether an impairment is observed in acquisition and retrieval of a hippocampal-dependent task.

The present study assessed memory impairments after lighting manipulations and examined whether entraining to a meal would have a protective role. The first goal of the study was to determine the nature of the cognitive impairments that arise from light manipulations and whether the duration of exposure to light manipulations was correlated with the severity of cognitive impairment. This was done by comparing performance on both hippocampal-dependent and -independent tasks. The second goal of this study was to determine the role that the FEO has in both acquisition and retention of a spatial memory task. FEO entrainment may play a role in protecting memory in cases where exposure to light manipulations or other forms of circadian disruption cannot be avoided.

Experiment 1: Thirty Day Light Manipulation

Craig and McDonald (2008) showed that chronic exposure to a light manipulation (i.e., 64-day paradigm) resulted in impairment on hippocampal-dependent tasks. However, acute exposure (i.e., 12-day paradigm) did not produce the same cognitive deficits. Deficits in performance of the hippocampal-independent task (i.e., tone fear conditioning) were not observed in either the acute or chronic light manipulation groups. Experiment 1 examined if a modified 30-day light manipulation paradigm would produce the same cognitive deficits as seen in the chronic light manipulation paradigm used in Craig and McDonald (2008). The full light manipulation schedule is presented in Table 1. The goal was to provide more knowledge on the dosage of light manipulation that results in cognitive impairment.

Additionally, the Craig and McDonald (2008) experiment examined the influence of FEO entrainment on hippocampal-dependent task performance. Entraining to a single meal schedule rescued spatial working memory in arrhythmic Siberian hamsters, as observed by improved performance on a spontaneous alternation task (Ruby et al., 2017). Conversely, the memory impairments persisted for three weeks when the animals were fed *ad libitum*. Furthermore, rats exposed to consistent meal times (i.e., FEO entrainment) perform better on a TPL task than rats that do not have FEO entrainment (i.e., FEO disruption) (Wall et al., 2019)**.** Additionally, Lukoyanov (2002) found that rats who were fed *ad libitum* did not show evidence of learning a daily TPL task in the MWM, while rats on a restricted feeding schedule (i.e., one meal a day) learned the task. Widman et al. (2004) argued that rats on restricted feeding may experience higher response costs compared to an *ad libitum* group on a TPL water maze task. That is, because the restricted feeding group consumed significantly fewer calories, they were more motivated than the *ad libitum* group to not make mistakes. Therefore, when rats fed one meal per

day outperform rats fed *ad libitum*, it may be due to either FEO entrainment or motivation to learn the task. Therefore, *ad libitum* feeding is an inadequate control group for FEO entrainment studies. Instead, one should control for the amount of food consumed, especially in learning and memory tasks where motivation is important.

The present study examined whether FEO entrainment protected memory and ameliorated performance on the hippocampal-dependent task compared to rats without FEO entrainment. Rats were given 28 days of food entrainment, then the light manipulation (LM) group was exposed to the light manipulation schedule for 30 days. On day 30 of the LM rats were tested on the elevated plus maze (EPM). Previously, rats exposed to constant light for eight weeks resulted in depressive and anxiety-like behaviours (Tapia-Osorio, 2013). Therefore, EPM was completed in the present study to ensure that any possible differences observed in behavioural testing was due to the light manipulation and not differences in anxiety levels. After the light manipulation was complete, the hippocampal-independent task (i.e., Stimulus-Response Task) and the hippocampal-dependent task (i.e., MWM task) were completed successively. The hippocampal-independent task was completed to ensure that any impairments we observed in learning were solely in hippocampal-dependent learning.

It was hypothesized that rats exposed to the 30-day light manipulation paradigm would exhibit impairments in the acquisition of the MWM task. It was also hypothesized that the acquisition of the SR task would not be impaired by exposure to the light manipulation. Furthermore, rats fed once a day (i.e., FEO entrainment) would perform better than rats fed multiple times a day (i.e., FEO disruption) on the MWM. Specifically, rats exposed to the light manipulation and multiple meal regimen would perform worse than rats exposed to the light manipulation and single meal regimen. Additionally, rats on the single meal regimen and control

light schedule would have superior performance than rats on the multiple meal regimen and control light schedule.

Method

Subjects

Thirty-two male Long-Evans rats were purchased from Charles River Laboratories (QC, Canada). The rats weighed an average of 160g upon arrival. Rats were singly housed in individually ventilated cages (IVCs) (35.8 x 30.9 x 18.4 cm) in a temperature-controlled room. Each cage contained corncob bedding (Netco, New York, NY), Crink-l'Nest (The Anderson, Maumee, Ohio), a Nylabone (Nylabone Products, Neptune, NJ), a wooden block and a piece of PVC pipe. Rats were handled daily and received enrichment sessions approximately five times per week starting four days after arrival to the colony room. The purpose of enrichment was to provide a stimulating environment for the rats' given that they were single housed due to the restricted food regimen. Each session took place during various times in the light phase. During enrichment sessions, rats were placed into one of two large Plexiglass boxes (Box A: 59.4 x 59.4 x 59.4 cm; Box B: 53.6 x 43.4 x 42.9) that contained a combination of a plastic house, a running wheel, wooden toys, and a plastic statue. Each session lasted approximately 20 minutes and rats were enriched with another rat (rat pairs were maintained throughout the entirety of the study to minimize conflict between rats) to allow for social interaction. Fruit Loops (Wal-Mart Canada, Corporation) were placed inside the enrichment boxes during each enrichment session to encourage exploration.

The rats were given *ad libitum* access to a standard rat food (PMI Nutrition International, St. Louis, MO) for the first three days after arrival, after which their food was restricted such that the rats gained approximately 10g per week. Their food was adjusted weekly to ensure this

weight gain was maintained. Rats were given four weeks on their restricted feeding regime prior to the onset of training, to ensure that they had entrained to the mealtimes, and thus granting time for the restricted feeding group to have FEO entrainment before training commenced. This feeding regime was maintained for the entirety of this study. Rats were given *ad libitum* access to water.

Rats were first divided into two groups based on meal restriction. There was a single meal (1M) group that received its daily allotment of food at 4:30 p.m. everyday. The second group was the multiple meal (MM) group, which received one to three meals per day at random times during the light phase, at least 90 minutes apart. In addition, the rats were divided into two subgroups based on exposure to the light manipulation. The control (C) group were maintained on a 12:12 hour light-dark (LD) cycle (lights on at 7:00 a.m.) for the duration of the study, whereas the LM group had the 12:12 hour LD schedule for 30 days prior the being switched to the lighting schedule as outline in Table 1. Therefore, there were four groups in total (i.e., C-1M, C-MM, LM-1M, LM-MM), with eight rats in each group.

All methods and procedures used in the present study were carried out in accordance with the Canadian Council of Animal Care Guidelines and were approved by the Memorial University Institutional Committee on Animal Care.

Apparatus

Elevated-Plus Maze. Two identical wooden elevated plus mazes were used, both consisting of two open arms (15 cm x 122 cm) and two enclosed arms (15 cm x 122 cm x 50 cm) with an open roof. Both mazes were elevated 75 cm off the floor and were painted grey. There was a curtain placed between both mazes to prevent the rats from being distracted during the task. The elevated-plus maze room (617 cm x 358 cm) contained salient cues such as several

counter tops, a computer, chairs, doors, and two researchers. The lights remained on during testing. These cues remained constant throughout the entirety of testing.

Stimulus-Response Task. A wooden plus maze that was painted white and elevated 76.7 cm off the floor was used for the SR task. Each arm was 60.2 cm long and 15.5 cm wide. There was a circular indentation carved into the end of each arm where Fruit Loops were placed for reinforcement during the task. A wire mesh was used to cover the correct arm. Pantyhose were filled with Fruit Loops and attached underneath the end of each arm to reduce any confounding olfactory cues.

The SR room (594.7 x 49.7 cm) contained several cues that remained constant during testing. There was a curtain, computer, desk, two doors, posters, and the researcher that was present during testing. There was a counter in which the IVCs were placed in the same order every time they were tested on this task. The room was well lit, and a radio was turned on during each training session.

Morris Water-Maze Task. The water maze was a plastic pool that was 175.4 cm in diameter and 59.8 cm deep. An adjustable platform that was 11 cm in diameter was placed in a stationary position in the water maze. The pool was filled so that the water level was 2 cm over the adjustable platform and approximately 250 ml of non-toxic white Craft Smart Paint (MSPCI, Irving, Texas) was added to the water to make it opaque. The temperature of the water was held constant at approximately 21°C.

The training room (583.5 x 363.4cm) contained visual cues that remained constant throughout the training procedure. A door, windows, posters, two researchers, a cabinet, and a desk were all present in the room. The rats were switched from their IVCs into clear single conventional cages (45 x 25 x 21 cm) with metal lids. The cages were lined with paper towel to

absorb any excess water the rats had accumulated while in the water maze. For video recording to be possible, the overhead lighting was turned off and lamps were used to illuminate the room during testing. A radio was also turned on during testing.

Procedure

Rats were given four weeks to entrain to their meal regimen. The 1M rats were fed at 4:30 pm daily, while the MM rats were fed 1-3 times daily. The MM rats were fed at 4:30 pm along with the 1M rats, but the other mealtimes occurred randomly. After food entrainment was completed, the LM rats were transferred into another room where the light manipulation occurred for 30 days. SR habituation was completed on Days 28, 29, and 30 of the light manipulation. Additionally, on Day 30 of the light manipulation rats were tested on the elevated plus maze (EPM). The EPM is a commonly used measure to test for anxiety (Blundell et al., 2010; Pellow & File, 1986). Following EPM, both the SR and MWM were completed with all rats on a 12:12 LD schedule. See Figure 1 for an overview of the experiment timeline.

Light manipulation. The rats underwent the light manipulation following the 30 days of entrainment to their food regimen. The lights were shifted back three hours each day for seven days and then remained on a 10:00 am-10:00 pm LD cycle for 6 days (i.e., partial re-entrainment period). Two full cycles of the light manipulation and partial re-entrainment were completed followed by four days of the light manipulation, leaving them on a 10:00 am-10:00 pm LD schedule. Refer to Table 1 for the complete schedule.

Elevated-Plus Maze. Sixteen rats were moved from the colony room into a holding room outside of the testing room. Rats were counterbalanced so that the rats ran simultaneously on each maze were from different groups (e.g., C-1M, LM-MM) to control for any confounding variables (e.g., order effects, cart placement). The rats were placed on two carts that were left

outside the testing room for 30 minutes to allow habituation to the new location. Each researcher took one rat from each cart into the testing room and placed the cage on a table. Rats were placed on the maze in unison and were given five minutes to explore the maze. Each trial was recorded with Ethovision and the time spent in the closed and open arms were scored manually. Time spent in each arm was counted when the rat entered in arm (i.e., entire body of the rat minus the tail). Therefore, time spent in the center of the maze was not used in the analysis. Noise was kept at a minimum to avoid any confounds due to external stressors. Each of the mazes were cleaned after each pair of rats completed the trial. After all the rats completed one trial, they were returned to the colony room. The same procedure was used for the 16 rats in the second cohort.

Stimulus-Response Task. An equal number of rats from each group were taken from the colony room in groups of five, five, and six. Each day consisted of eight trials, such that all rats were run in chronological order eight times, resulting in an inter-trial interval of approximately 5 minutes. The radio was turned on to eliminate any confounding noise that may have distracted the rats during testing. Rats received 3 days of habituation which consisted of placing each rat on the SR maze for 5 minutes, while crushed Fruit Loops were spread out over the entirety of the maze to encourage exploration. This allowed the rats to get familiar with the maze, minimizing the amount of freezing and falling off the maze. Following habituation, the rats completed a minimum of 8 days of behavioural training, resulting in a minimum of 64 trials. During testing, the Fruit Loop was placed in the indentation at the end of the correct arm to act as a reinforcer and the wire mesh was wrapped around the arm leading up to the Fruit Loop (which acted as a cue for the reinforced arm). The location of the reinforced arm was randomized for each rat, so successive trials did not have the same arm reinforced. The rat was placed on a randomized start arm facing the researcher and away from the center of the maze. Once the rat was placed on the

maze the researcher started the timer. Each rat was given a maximum of 2 minutes to select an arm. During the first 20 trials, if the rat did not make a choice within 2 minutes, they were placed on the correct arm and given the Fruit Loop at the end of the correct arm. Additionally, every arm selection (i.e., defined as the body of the rat minus his tail entering an arm) was recorded for the first 20 trials, along with the latency to reach the Fruit Loop at the correct arm. After 20 trials, if the rats made a wrong selection or did not make an arm choice, they were taken off the maze immediately and therefore did not receive reinforcement on that trial. Once the first five rats completed the task they were moved to another room where they completed the water maze task. Training was complete once criterion was met (i.e., 18/20 consecutive trials correct). If rats did not reach criterion after 120 trials, training was stopped. The procedure was the same for the other two groups.

Morris Water Maze Task. Rats were transferred to the water maze room following completion of the SR task. The water maze task consisted of three phases: rapid acquisition, massed training, and competition (Craig & McDonald, 2008). Rapid acquisition lasted four days and the rats completed eight trials a day with the hidden platform stationed in Location 1. On Day 5, a no-platform probe was completed and was followed by 16 trials of massed training with the hidden platform stationed in Location 2 (i.e., diagonal to the original location). On Day 6, a no-platform probe was completed and the competition phase followed which consisted of eight trials with the platform in the original location. Finally, Day 7 consisted of the final no-platform probe. The entirety of the task was completed in seven days. There were four starting points that were randomized for each rat and the hidden platform remained stationary in the water throughout testing. The water was agitated between trials to control for any confounding olfactory cues. One researcher carried each cage to the appropriate start position in a counter

clockwise direction and placed it on the chair next to the pool. Rats were placed in the water facing the wall of the pool and each rat was given a maximum of one minute to reach the platform. If the rat reached the platform within one minute, it was left there for 10 s before being removed from the pool. If the rat did not reach the platform within one minute, the researcher directed the rat to the platform and left it there for 10s. The researcher remained stationed behind the starting position for the duration of each trial, as well as the 10 s each rat was left on the platform. Another researcher stationed away from the maze was responsible for the video recording and recording the latency of each trial. Once rats completed their eight trials, they were placed back in their IVCs and returned to their colony room.

There were three no-platform probe trials throughout the MWM task. Each trial consisted of removing the platform from the maze and placing the rat in either of the arms furthest away from the previous platform location. The release arms were counterbalanced based on meal group. Each rat was given a full 60 seconds in the maze and each trial was recorded using Ethovision.

Results

Elevated Plus Maze

To assess whether there were differences in anxiety levels across conditions, we compared time spent in open versus closed arms. The time spent in the open and closed arms of the EPM were analyzed and shown in Figure 2. A mixed model ANOVA with time spent in the open arms and closed arms as the repeated measures, and lighting, meals as between subjects factors was used to compare time spent in the open versus closed arms. There was a main effect of quadrant, $F(1,28) = 5.814$, $p = .023$, $\eta_p^2 = .172$, indicating that as a whole the rats spent more time in the open arms than the closed arms. There were no main effects of lighting, $F(1,28) =$

.224, $p = .640$, $\eta_p^2 = .008$, nor meals, $F(1,28) = .515$, $p = .479$, $\eta_p^2 = .018$, nor were there any significant interactions. These data suggest that the light manipulation did not act as a stressor for the rats' and any differences observed in behavioral testing were not due to difference in anxiety levels.

Stimulus-response task

All rats received a minimum of 64 trials, after which time they were removed once reaching criterion (i.e., 18/20 trials correct). The number of trials to criterion was examined to assess whether there were any differences in the acquisition of the SR task. Figure 3 shows the average number of trials to criterion for each group. As hypothesized, a univariate ANOVA determined there was no main effect of meals, F (1,28) = 1.367, $p = .252$, $\eta_p^2 = .047$, nor lighting, $F(1,28) = .147$, $p = .705$, $\eta_p^2 = .005$, nor was there a significant lighting × meals interaction, $F(1,28) = .069$, $p = .795$, $\eta_p^2 = .002$. These data suggest that exposure to the light manipulation and meal manipulation did not impair performance on the hippocampal-independent task.

Morris Water Maze Task

Acquisition. To analyze the acquisition of the MWM, the average latency to reach the platform across eight trials in each of the four days was calculated (see Figure 5). A mixed model ANOVA was then used with day as the repeated measures factor, and lighting, and meals as the between-subjects factors. There were main effects of day, $F(3,84) = 92.269$, $p < .001$, $\eta_p^2 = .767$, and lighting, $F(1,28) = 6.147$, $p = .019$, $\eta_p^2 = .180$, but no main effect of meals $F(1,28) = .001$, $p = .970$, $\eta_p^2 = .000$. The only significant interaction was the meals × day interaction, $F(1,28) = 3.375$, $p = .014$, $\eta_p^2 = .118$. Simple main effects analysis indicated that differences between the meal groups approached significance on Day 1, $p = .074$, with the MM groups having a shorter

latency than the 1M groups, but performance was not different on subsequent training days. As a whole these data suggest that the LM groups took significantly longer to find the platform during the entirety of acquisition training. Furthermore, these data suggest that performance between the meal groups differs depending on the day of acquisition training. This is, however, a subtle effect as performance only approaches significance on Day 1 of training.

Given the main effect of lighting, a pairwise comparison was conducted to further assess any differences between the LM-1M group and the LM-MM group. There was no significant difference between the LM-1M and LM-MM group, $p = .262$. This indicates that the single meal regimen did not afford an advantage to the LM group in acquisition of the MWM task.

To determine if the differences in latency were due to an impairment that negatively impacted the rats' swim speed, the velocity was analyzed throughout acquisition training to assess any differences between conditions. The average velocity across eight trials in each of the four days was calculated. A mixed model ANOVA was then used with day as the repeated measures factor, and lighting, meals as the between-subjects factors. There was a main effect of day, $F(3,75) = 7.549$, $p < .001$, $\eta_p^2 = .232$, but no main effects of meal, $F(1,25) = 3.806$, $p = .062$, $\eta_p^2 = .132$, or lighting, $F(1,25) = 1.639$, $p = .212$, $\eta_p^2 = .062$. There were no significant interactions. As a whole these data suggest that there were no differences in velocity between groups through acquisition training on the MWM task. This indicates that the differences in latency to the platform were not due to an impairment that negatively impacted the rats swim speed.

The distance travelled through acquisition training was analyzed to assess any differences between conditions. The average distance across eight trials in each of the four days was calculated (see Figure 5). A mixed model ANOVA was then used with day as the repeated

measures factor, and lighting, meals as the between subjects factors. There were main effects of day, $F(3,75) = 90.664$, $p < .001$, $\eta_p^2 = .784$, and lighting, $F(1,25) = 9.834$, $p = .004$, $\eta_p^2 = .282$, but no main effect of meals, $F(1,25) = .158$, $p = .695$, $\eta_p^2 = .006$. There were no significant interactions. These data as a whole suggest that the rats that endured the light manipulation traveled further throughout acquisition training than the control rats. This adds to the finding that the LM groups had longer latencies than the C groups throughout training. The LM groups spent more time finding the platform, therefore traveled further on average across trials.

Probe 1. To assess any difference in acquisition across groups, the first 30 seconds of the no-platform probe conducted on Day 5 of the MWM task were analyzed. Figure 6A shows the average time spent in the correct quadrant compared to the average time spent in the incorrect quadrants across all groups. A mixed model ANOVA with quadrant as the repeated measures factor, and lighting, meals as between-subjects factors was used to compare the time spent in the correct quadrant versus the average time spent in the remaining three quadrants. There was a main effect of quadrant, $F(1,28) =$, $p < .001$, $\eta_p^2 = .907$, indicating that as a whole the rats spent more time in the target quadrant than the other quadrants. There were no main effects of lighting, $F(1,28) = .762$, $p = .390$, $\eta_p^2 = .027$, nor meals, $F(1,28) = .269$, $p = .608$, $\eta_p^2 = .010$, nor were there any significant interactions. These data suggest that all groups of rats had a place memory for the location acquired during training and this memory did not differ among the groups.

The total distance traveled was analyzed in the first 30 seconds of the no platform probe to assess any difference between conditions (see Figure 6B). A univariate ANOVA determined there was no main effect of lighting, $F(1,28) = .422$, $p = .521$, $\eta_p^2 = .015$, nor meals, $F(1,28) = .001$, $p = .972$, $\eta_p^2 = .000$, nor a meal × lighting interaction, $F(1,28) = .813$, $p = .375$, $\eta_p^2 = .028$.

These data suggest that exploration of the maze was similar across all groups throughout the probe trial.

The velocity was analyzed in the first 30 seconds of the no platform probe to assess any difference between conditions (see Figure 6C). A univariate ANOVA determined there was no main effect of lighting, $F(1,28) = .438$, $p = .514$, $\eta_p^2 = .015$, nor meals, $F(1,28) = .002$, $p = .964$, $\eta_p^2 = .000$, nor a meal × lighting interaction, $F(1,28) = .797$, $p = .380$, $\eta_p^2 = .028$. These data suggest that velocity throughout the probe trial was similar across all groups.

Massed training. The latencies across the 16 trials were compared to assess any differences in acquisition of the platform in its new location. Figure 7 compares the latencies across groups during massed training. A repeated measures ANOVA determined there was no main effect of lighting, $F(1,28) = .152$, $p = .700$, $\eta_p^2 = .005$, nor meals $F(1,28) = .854$, $p = .363$, $\eta_p^2 = .030$, nor a meal × lighting interaction, $F(1,28) = .542$, $p = .468$, $\eta_p^2 = .019$. These data suggest that there were no significant differences across groups in the time spent finding the new platform location throughout massed training.

Probe 2. The first 30 seconds of the massed training probe were analyzed. Figure 8A shows the amount of time spent in the correct quadrant compared to the average time spent in the incorrect quadrant. A mixed model ANOVA with quadrant as the repeated measures factor, and lighting, and meals as between-subjects factors was used to compare the time spent in the correct quadrant versus the average time spent in the remaining three quadrants. There was a main effect of quadrant, $F(1,28) = 4.528$, $p = .042$, $\eta_p^2 = .139$, indicating that as a whole the rats spent more time in the target quadrant than the other quadrants. There were no main effects of lighting, $F(1,28) = .015$, $p = .903$, $\eta_p^2 = .001$, nor meals, $F(1,28) = .149$, $p = .703$, $\eta_p^2 = .005$, nor were there any significant interactions. These data suggest that all groups of rats had a place memory

for the location acquired during massed training and this memory did not differ among the groups.

Time spent in the previously correct quadrant was compared to time spent in the correct quadrant to assess if there were any groups that retained the first platform location over the novel location (see Figure 8B). A mixed model ANOVA with quadrant as the repeated measures factor, and lighting, meals as between-subjects factors was used to compare the time spent in the correct quadrant versus the time spent in the previously correct quadrant. There was no significant effect of quadrant, $F(1,28) = .194$, $p = .663$, $\eta_p^2 = .007$, indicating that the rats did not spend more time in the correct quadrant compared to the previously correct quadrant. There were no main effects of lighting, $F(1,28) = .006$, $p = .941$, $\eta_p^2 = .000$, nor meals, $F(1,28) = 1.143$, $p = .294$, $\eta_p^2 = .039$, nor were there any significant interactions. Given that the rats spent close to equal amounts of time in the correct and previously correct quadrants, these data suggest that the previous platform location may have partially impaired performance on the massed training probe trial.

Total distance moved was compared between groups to assess any differences between conditions (see Figure 8C). A univariate ANOVA determined there was no main effect of meals, $F(1,28) = .001$, $p = .980$, $\eta_p^2 = .000$, nor lighting, $F(1,28) = .077$, $p = .077$, $\eta_p^2 = .003$, nor a meals × lighting interaction, $F(1,28) = .059$, $p = .809$, $\eta_p^2 = .002$. These data suggest that exploration of the maze throughout the probe trial was similar across all groups.

Velocity was compared between groups to assess any differences between conditions (see Figure 8D). A univariate ANOVA determined there was no main effect of lighting, $F(1,28) = .184$, $p = .672$, $\eta_p^2 = .007$, nor meals, $F(1,28) = .064$, $p = .802$, $\eta_p^2 = .002$, nor a meals × lighting

interaction, $F(1,28) = 1.116$, $p = .300$, $\eta_p^2 = .038$. These data suggest that velocity through the probe trial was similar across all groups.

Competition training. Latency to the platform was compared across the eight competition trials to assess any group differences. Figure 9 shows the latencies to the platform across groups during competition training. A repeated measures ANOVA determined no main effect of meals, $F(1,28) = 3.911$, $p = .058$, $\eta_p^2 = .123$, nor lighting, $F(1,28) = .563$, $p = .459$, $\eta_p^2 = .020$, nor were any interactions significant. These data suggest that moving the platform back to the original location did not influence performance across groups.

Probe 3. The first 30 seconds of the competition training probe was analyzed. Figure 10A shows the time spent in the correct quadrant compared to the average time spent in the incorrect quadrant. A mixed model ANOVA with quadrant as the repeated measures factor, and lighting, meals as between-subjects factors was used to compare the time spent in the correct quadrant versus the average time spent in the remaining three quadrants. There was a main effect of quadrant, $F(1,28) = 28.067$, $p < .001$, $\eta_p^2 = .501$, indicating that as a whole the rats spent more time in the target quadrant than the other quadrants. There were no main effects of lighting, $F(1,28) = .993$, $p = .328$, $\eta_p^2 = .034$, nor meals, $F(1,28) = .617$, $p = .439$, $\eta_p^2 = .022$, nor were there any significant interactions. These data suggest that all groups of rats had a place memory for the originally acquired location and this memory did not differ among the groups.

Time spent in the previously correct quadrant was compared to time spent in the correct quadrant to assess if there were any groups that retained the first platform location over the novel location (see Figure 10B). A mixed model ANOVA with quadrant as the repeated measures factor, and lighting, meals as between-subjects factors was used to compare the time spent in the correct quadrant versus the time spent in the previously correct quadrant. There was a significant

effect of quadrant, $F(1,28) = 10.538$, $p = .003$, $\eta_p^2 = .273$, indicating that the rats spent more time in the correct quadrant than in the previously correct quadrant. There were no main effects of lighting, $F(1,28) = 2.834$, $p = .103$, $\eta_p^2 = .092$, nor meals, $F(1,28) = 1.315$, $p = .261$, $\eta_p^2 = .045$, nor were there any significant interactions. These data suggest that the previously correct quadrant did not impair performance on the competition training probe trial across groups.

Total distance traveled was compared to assess any group differences (see Figure 10C). A univariate ANOVA determined that there was no main effect of lighting, $F(1,28) = .001$, $p = .970$, $\eta_p^2 = .970$, nor meals, $F(1,28) = .105$, $p = .748$, $\eta_p^2 = .004$, nor a meals × lighting interaction, $F(1,28) = .001$, $p = .978$, $\eta_p^2 = .000$. These data suggest that exploration throughout the probe trial was similar across all groups.

Average velocity throughout the probe trial was compared to assess any group differences (see Figure 10D). A univariate ANOVA determined no main effect on lighting, $F(1,28) = .035$, $p = .853$, $\eta_p^2 = .001$, nor meals, $F(1,28) = .053$, $p = .820$, $\eta_p^2 = .002$, nor in the meals × lighting interaction, $F(1,28) = .370$, $p = 548$, $\eta_p^2 = .013$. These data suggest that velocity throughout the probe trial was similar across all groups.

Discussion

The purpose of Experiment 1 was to expand on the knowledge surrounding the dosage of light manipulation that results in cognitive impairment. Additionally, this experiment explored whether FEO entrainment could act as a compensatory mechanism for rats experiencing a light manipulation, as well as if FEO disruption resulted in cognitive impairment. We modified the chronic light manipulation schedule presented in Craig and McDonald (2008), to observe whether one month of the light manipulation resulted in a performance deficit on a hippocampal-dependent task (i.e., the MWM task). Furthermore, we used a multiple meal and single meal

paradigm to observe any possible ameliorating effect of FEO entrainment (i.e., 1M groups), as well as any cognitive deficit resulting from FEO disruption (i.e., MM groups). All variables were assessed by comparing differences in performance on both hippocampal-independent and -dependent tasks.

Hippocampal-Independent Tasks

The hypothesis that performance on a hippocampal-independent task would not be impaired by exposure to the light manipulation was supported. No group differences were found in the number of trials completed before meeting criterion. This shows that circadian disruption in the form of a light manipulation does not impair performance on hippocampal-independent tasks. This is consistent with the findings of Craig and McDonald (2008) using their hippocampal-independent task (i.e., tone fear conditioning task). However, Zelinski et al. (2013) showed that repeated exposure to a light manipulation impaired retention of their hippocampal-independent task (i.e., 8-arm radial maze). We did not assess retention of the SR task, and therefore cannot comment on whether we would have observed a cognitive deficit. Overall, the intermediate amount of light manipulation (i.e., 30 days) used in our paradigm did not result in impairment of performance on the SR task. Furthermore, all groups displayed similar behaviour on the EPM, which suggests that the anxiety levels across all groups were similar. Thus, any observed differences between the groups were not due to differences in anxiety.

Morris Water Maze Task

The LM group was significantly slower finding the platform location in the acquisition phase of the MWM task than the C group. Both LM groups exhibited an acquisition deficit throughout the four days of training. This shows that exposure to the light manipulation schedule, modeled after the Craig and McDonald (2008) chronic light manipulation, led to

impairment in acquisition of the MWM task for the LM groups. However, this deficit did not persist throughout the probes or the other phases of the MWM task. A similar effect was observed in a previous study assessing MWM performance in a rat model of Alzheimer's disease in that there was a deficit in the acquisition phase, but not the other phases of the MWM task (Deibel et al., 2016). Our results suggest that the area of the hippocampus responsible for performance of the MWM task was impaired by the light manipulation paradigm. However, the rats were able to recover throughout acquisition training, as indicated by the similar performance in all three no platform probes and other phases of the MWM. Overall, our hypothesis that acquisition of a hippocampal-dependent task would be impaired because of exposure to the light manipulation schedule was partially supported.

Additionally, there were no differences between meal groups across all phases of the MWM task. Therefore, our hypothesis that the rats fed once a day (i.e., FEO entrainment) would perform better than rats fed multiple times a day (i.e., FEO disruption) on the MWM task was not supported. These data suggest that FEO entrainment is not needed for performance in hippocampal-dependent tasks. However, as there were only slight deficits in MWM performance for the LM rats, it remains possible that entrainment to a meal can protect memory in paradigms that result in more severe impairments. Previously, it has been shown that a consistent daily feeding schedule rescued spatial memory in arrhythmic Siberian hamsters (Ruby et al., 2017). For an assessment of any ameliorating properties of FEO entrainment there would have to be a deficit caused by the light manipulation. Without the deficit, it is not possible for FEO entrainment to ameliorate performance.

One reason why these results may have occurred is because our modified version of Craig and McDonald's (2008) light manipulation was not disruptive enough. The data as a whole

indicate that in contrast to the 64-day paradigm used in Craig and McDonald (2008), approximately half of that exposure only has a slight impact on hippocampal functioning. While we did observe a deficit in acquisition of the MWM task, this deficit was not present in the other phases or probe trials. The LM rats recovered throughout training, which resulted in the global impairment not being observed in the present study. These results suggest that there may be a light manipulation schedule between 30 and 64 days that may result in the same deleterious effects in performance as seen by Craig and McDonald (2008). However, the present study did not replicate the deficit using our 30-day light manipulation schedule.

Additionally, Craig and McDonald (2008) did not manipulate meal access while exposing their rats to the chronic light manipulation. While there are behavioural effects observed when the FEO is entrained (i.e., FAA), the relationship between the LEO and FEO is still unclear. While effects of FEO (Lukoyanov et al., 2002; Wall et al., 2019) and LEO (Craig & McDonald, 2008; Zelinski et al., 2013; Zelinski, Hong, et al., 2014) entrainment on memory have been previously studied separately, there needs to be more research on the relationship between both oscillators and memory. When we broke down the main effect of lighting in acquisition training, the LM-1M and LM-MM groups did not significantly differ in performance. This indicates that FEO entrainment was not able to ameliorate performance on the MWM in rats exposed to the light manipulation schedule. Furthermore, there is a possibility that the MM groups were able to entrain to their meals, given that one of their meals were at 4:30 pm every day. If this were the case, there would be no differences across meal groups because all rats would have FEO access. However, it is still vital that the relationship between the FEO and LEO be explored further in future research. If the impairment from the light manipulation was more severe, we may have

been able to observe the FEO as a compensatory mechanism for the rats that experienced the light manipulation.

Overall, the results from Experiment 1 suggest that our modified light manipulation schedule was not disruptive enough to result in a global deficit across performance on the MWM task. Additionally, the potential compensating factor of FEO entrainment was unable to be observed here, given the small deficit present in performance on the MWM task.

Experiment 2: Do Consistent Meals Protect Memory?

While there was a deficit in the LM group in acquisition of the MWM task in Experiment 1, this deficit did not carry over into the other phases of the task. This indicated that our modified light manipulation paradigm resulted in partial impairment of acquisition of the MWM task. Additionally, Experiment 1 showed no significant differences in performance on the MWM between the 1M and MM groups. This indicates that FEO entrainment did not significantly improve performance on the hippocampal-dependent task as expected. However, Experiment 1 used a modified version of the light manipulation paradigm used in Craig and McDonald (2008). Our light manipulation schedule did not produce the hippocampal-dependent task performance deficit that was seen in the original experiment. As a result, it was impossible to determine any ameliorating effect of FEO entrainment. Therefore, Experiment 2 was developed to answer the questions surrounding the possible benefit of FEO entrainment.

Experiment 2 used a previously validated acute light manipulation schedule that was shown to impair retention of the MWM in rats (Zelinski, Hong, et al., 2014). To observe any ameliorating effect of FEO entrainment, a significant difference between the LM and control rats needed to be observed. If the LEO is disrupted, the question of whether FEO entrainment protects memory can be answered. As we aimed to replicate the cognitive deficit caused by the

light manipulation, we followed the method outlined in Zelinski, Hong, et al. (2014). As a result, we did not test the rats on the SR task or EPM in Experiment 2.

Rats were given three weeks of food entrainment inside running wheels to observe their activity while entraining to the meal regimen. After food entrainment, the MWM task was completed over 6 days, followed by the light manipulation for 6 days, and a re-entrainment period of 13 days. Finally, the no platform probe was completed 19 days post acquisition training.

It is hypothesized that rats exposed to the light manipulation paradigm will experience impairments in the retention of the MWM task. Furthermore, rats fed once a day (i.e., FEO entrainment) will perform better than rats fed multiple times a day (i.e., FEO disruption) on the MWM. Specifically, rats exposed to the light manipulation and multiple meal regimen will perform worse than rats exposed to the light manipulation and single meal regimen. Additionally, rats on the single meal regimen and control light schedule will have superior performance than rats on the multiple meal regimen and control light schedule.

Method

Subjects

Sixteen male Long Evans rats were purchased from Charles River Laboratories (QC, Canada). The rats weighed an average of 160 g upon arrival. The rats were placed on free feed of a standard rat food (PMI Nutrition International, St. Louis, MO) for the first seven days after arrival, after which their food was restricted such that the rats gained approximately 10 g per week. Their food was adjusted weekly to ensure this weight gain was maintained. Rats were given three weeks on their restricted feeding regime prior to the onset of training, to ensure that they had entrained to the meal times, and thus granting time for the 1M group to have FEO

entrainment before training commenced. Such feeding regime was maintained for the entirety of this study. Rats were given *ad libitum* access to water.

Rats were singly housed in individually ventilated cages (IVCs) (35.8 x 30.9 x 18.4 cm) in a temperature controlled room and were maintained on a 12:12 light-dark (LD) cycle (lights on at 7:00 a.m.). Each cage contained corncob bedding (Netco, New York, NY), Crink-l'Nest (The Anderson, Maumee, Ohio), a Nylabone (Nylabone Products, Neptune, NJ), a wooden block, and a piece of black PVC pipe. After seven days, rats were placed in running wheels to entrain to both the wheel and the meal regimen.

Rats were first divided into two groups based on meal restriction. There was a single meal (1M) group that received their daily allotment of food at 4:30 p.m. every day. The second group was the multiple meal (MM) group, which received one to three meals per day at random times during the light phase, at least 90 minutes apart. The MM rats were only fed once a week at 4:30 pm with the 1M rats, meaning the MM feeding regimen was more random in Experiment 2. The reason for this change was to ensure that the MM rats were not able to entrain to the meal times. In addition, the rats were divided into two subgroups based exposure to the light manipulation paradigm. The control group (C) received a regulated 12:12 LD schedule, and the light manipulation (LM) group were exposed to the light manipulation for six days. Therefore, there were four groups in total, with four rats in each group. [Note: The intention was to run a second cohort of rats so that there would be 8 rats in each group. However, due to COVID-19 restrictions I was unable to run the second cohort. Once restrictions on research are lifted, the second cohort of rats will be included.]

All methods and procedures used in the present study were carried out in accordance with the Canadian Council of Animal Care Guidelines and were approved by the Memorial University Institutional Committee on Animal Care.

Apparatus

The water maze that was described in Experiment 1 was used for this experiment.

Procedure

After one week of acclimation to the new environment and having *ad libitum* access to food and water, twelve rats were placed in individual running wheels. The remaining four rats had motion sensors placed inside their IVC's for the entirety of the experiment. The meal regimen started once the rats were placed in the running wheels. Activity was monitored for three weeks to allow enough time for entrainment to the meal regimen as well as having accurate activity recordings. Rats remained in the running wheels for the remainder of testing. Following the food entrainment period, the MWM task was completed with six days of eight trials a day. After MWM, the light manipulation paradigm took place with six shifts occurring over six days. The re-entrainment period was thirteen days, with the retention probe being completed on Day 19 post acquisition training. See Figure 11 for an overview of the experiment timeline.

Light Manipulation. The rats underwent the light manipulation following three weeks of entrainment to their food regimen. The light manipulation used in this experiment was a replication of the one previously used by Zelinski, Hong, et al. (2014). The lights were shifted ahead three hours each day for 6 days and ended on a lights on 1:00pm-1:00am schedule for the remainder of the experiment. Refer to Table 2 for the complete schedule. The feeding schedule remained consistent for both the 1M and MM groups throughout the light manipulation. This resulted in the rats occasionally being fed during their dark phase.

Morris Water Maze. Rats were removed from the running wheels and placed in IVCs and transferred to the training room upstairs. Given that Experiment 2 assessed retention of the MWM task, all rats were on a 12:12 LD schedule with lights off at 7:00 pm during the MWM task. Testing began 1.5 hours before lights off (i.e., 5:30 pm) and the eight rats in the control room were always tested first, followed immediately by the rats in the light manipulation room. Rats were tested in chronological order and were released from one of four pseudorandom positions around the maze. Each trial had a maximum time of one minute and each rat completed eight trials a day, with an intertrial interval of approximately 10 minutes. If the rat did not find the platform within one minute, they were guided to the platform and left there for ten seconds to observe their surroundings. When the first group of eight were done, they were returned to the colony room and the other eight were brought to the testing room. The rest of the water maze procedure was identical to what was described in Experiment 1.

Retention Probe. Nineteen days after completing acquisition of the water maze task, the retention probe was completed. The probe trial took place 1.5 hours before lights off time for each group. This meant that the probe for the control rats started at 5:30 pm and the probe for the light manipulation rats started at 11:30 pm. Each rat was released from the same position that was furthest away from the original platform location. The platform was removed from the maze and each rat completed a one-minute free swim that was recorded using Ethovision.

Results

Given the closure of the university due to COVID-19, the acquisition videos were unable to be analyzed using Ethovision. Additionally, the running wheel data was unable to be analyzed, and therefore actograms are not included in this write up. The probe data was analyzed using Ethovision, but the manually entered data was used for the acquisition analysis.

Morris Water Maze Task

To analyze the acquisition of the water maze task, the average latency to reach the platform across eight trials in each of the six days was calculated (see Figure 12). A mixed model ANOVA was then used with day as the repeated measures factor, and lighting, meals as the between subjects factors. There were main effects of day, $F(5,60) = 76.914$, $p < .001$, $\eta_p^2 = .865$. However, there was no main effect of lighting, $F(1,12) = .177$, $p = .682$, $\eta_p^2 = .015$, nor meals, $F(1,12) = 2.405$, $p = .147$, $\eta_p^2 = .167$, and no significant interactions. Because the light manipulation had not yet occurred, it is not surprising that there is no lighting effect. We had expected to see a Meal effect and this remains a possibility if the sample size had been larger.

Retention Probe

The first 30 seconds of the retention probe were analyzed. A mixed model ANOVA with quadrant as the repeated measures factor, and lighting, and meals as between subjects factors was used to compare the time spent in the correct quadrant versus the average time spent in the remaining three quadrants (see Figure 13A). There was a main effect of quadrant, $F(1,12) = 4.907$, $p = .047$, $\eta_p^2 = .290$, indicating that as a whole the rats spent more time in the target quadrant than the other quadrants. There were no main effects of lighting, $F(1,12) = .328$, $p = .578$, $\eta_p^2 = .027$, nor meals, $F(1,12) = .669$, $p = .429$, $\eta_p^2 = .053$, nor were there any significant interactions. These data suggest that all groups of rats had a place memory for the location acquired during training and the ability to retain this memory did not differ among groups.

To determine if each group retained the task we ran planned comparisons that compared time spent in the target quadrant to the average of the time spent in the other quadrants. There was a trend toward significance in the C-1M group, $p = .093$. The comparison was not significant in the C-MM group, $p = .361$, LM-1M group, $p = .247$, or the LM-MM group, $p = .671$. This

indicates that the C-1M group were the closest to retaining the task. Additionally, this indicates that the LM-MM group were the furthest from retaining the task. To analyze this further a Jonckheere-Terpstra test was conducted. This demonstrated that the median amount of time spent in the target (T_{JT} = 50.000, Z = .187, p = .852) and average of the other three (T_{JT} = 46.000, Z = -.187, p = .852) quadrants were not different among the groups.

Total distance traveled was compared between groups to assess if there were any differences between groups. The average distances can be seen in Figure 13B. A Univariate ANOVA determined that there was no main effect of lighting, $F(1,12) = 1.117$, $p = .311$, $\eta_p^2 = .085$, nor meals, $F(1,12) = .002$, $p = .956$, $\eta_p^2 = .000$, nor in the meals × lighting interaction, $F(1,12) = .035$, $p = .856$, $\eta_p^2 = .003$. These data suggest that exploration of the maze throughout the probe trial was similar across all groups.

Velocity was compared between groups to ensure there were no differences in swim speed between groups. The average velocities can be seen in Figure 13C. A Univariate ANOVA determined that there was no main effect of lighting, $F(1,12) = 1.125$, $p = .310$, $\eta_p^2 = .086$, nor meals, $F(1,12) = .002$, $p = .957$, $\eta_p^2 = .000$, nor the meals × lighting interaction, $F(1,12) = .038$, $p = .849$, $\eta_p^2 = .003$. These data suggest that velocity throughout the probe trial was similar across all groups.

Discussion

Experiment 2 investigated the possible compensating factor of FEO entrainment when LEO disruption has occurred. Additionally, using a previously validated light manipulation schedule from Zelinski, Hong, et al. (2014), the effect of LEO disruption on hippocampal-dependent task performance was furthered assessed. This was done by comparing performance on the MWM task across all four groups. The purpose was to ideally observe a performance

deficit caused by LEO disruption and a compensating factor of FEO entrainment on MWM performance.

Morris Water Maze Task

The hypothesis that exposure to the light manipulation schedule would have deleterious effects on MWM performance was not supported. There were no differences in acquisition of the MWM task, which was to be expected as the light manipulation occurred after learning the task. However, there was no difference in retention of the task across all groups. Zelinski, Hong, et al. (2014) found a cognitive impairment in their rats that were placed on an *ad libitum* food schedule and exposed to an acute light manipulation schedule. However, the major difference with the present experiment is the inclusion of both multiple meal and single meal schedules. Manipulating both LEO and FEO entrainment simultaneously could have influenced our results. While the majority of the rats' meals were given in their light phase, during the light manipulation there were meals given during the dark phase. The conflict of mealtime with the rats' LD cycle could have impacted their activity, which in turn could have shifted when they were primarily active. This possible shift in activity may have influenced their performance on the MWM task. Unfortunately, due to COVID-19 restrictions we have been unable to analyze the activity data to observe if this conflict in LD cycle and mealtime influenced the rats' activity levels. A previous experiment in our lab showed that rats on an *ad libitum* feeding schedule had impaired retention on the MWM task when exposed to the light manipulation schedule used in Experiment 2 (Higdon, 2020). There were no memory impairments in Experiment 2, but there were memory impairments when rats were fed *ad libitum* (Higdon, 2020; Zelinski et al., 2014) using the same light manipulation paradigm. This suggests that one meal may have ameliorated memory retention in the Experiment 2.

To determine whether FEO entrainment would have a compensating factor in rats exposed to a light manipulation schedule, we would need to find evidence of an impairment in the LM group. Because we did not find this, we could not address our original hypothesis. Unpublished data from our lab suggests that we are able to replicate the deleterious effect of LM in rats maintained on an *ad lib* diet (Higdon, 2020). It would be interesting to determine if rats fed multiple meals but during the dark phase exhibited impairments when exposed to the LM. If this were the case, we could include a group that was also given 1M during the dark portion of the LD cycle and determine whether in this instance the 1M was able to compensate. Furthermore, there were no significant differences between meal groups in acquisition training or in the retention probe trial. However, the pattern observed in the results of the pairwise comparison suggest that a meal effect may be present with the inclusion of a second cohort. The C-1M group were the closest to retaining the task, followed by groups LM-1M, C-MM group, and LM-MM. This is the order in which the groups were hypothesized to perform, given that FEO access (i.e., 1M groups) was hypothesized to ameliorate performance on the MWM task. Given that there was no significant impairment observed in the LM group, the possible compensating factor of FEO entrainment may not be possible to observe here. We have not completed the second cohort of testing for this experiment, so the possibility of seeing this effect once testing can resume is still possible.

Previously in our lab, we conducted an experiment with everything the same as Experiment 2 except the groups assessed were: control lighting and food available ad libitum (n = 8); lighting manipulation and food available ad libitum (n = 8) (Higdon, 2020). We found that the lighting manipulation rats were impaired in the probe trial. The effect size for the significant main effect of group was small/medium (partial $\eta^2 = .293$), with an observed power of .612. The

addition of feeding schedule as an independent variable in Experiment 2 could change the nature of the effects and therefore the observed power. Nonetheless, detection of a significant effect with similar groups, the same n and learning/lighting manipulations suggests that n's of eight are adequate to detect an effect of the size we appear to be observing. This further amplifies the importance of completing the second cohort of this experiment, as a main effect of meal may be present once all the rats are completed.

Overall, due to COVID-19 the results from Experiment 2 are inconclusive. The results from the first cohort suggest that the light manipulation did not result in deleterious effects on retention of the MWM task. In turn, there was no opportunity to observe whether there is a compensating factor of FEO entrainment.

General Conclusions

Further research on the relationship between circadian disruption and spatial memory in rats is important due to the possible implications in humans. It is known that the presence of circadian disruption is common in a variety of illnesses, both physical and mental (Craig & McDonald, 2008; Schulz & Steimer, 2009; Zelinski et al., 2013; Zelinski, Hong, et al., 2014). Previous research has shown that rats exposed to chronic circadian disruption showed an impairment in performance on a hippocampal-dependent task. (Craig & McDonald, 2008). Experiment 1 showed that 30 days of exposure to our light manipulation paradigm resulted in an impairment in acquisition of the MWM task. While the impairment did not persist through the other phases of the MWM, it is important to recognize that only a month of exposure resulted in a learning deficit in a hippocampal-dependent task. In cases such as shift work, humans are exposed to circadian disruption for far longer than one month, so it is important to uncover more

information on the influence of circadian disruption on hippocampal-dependent task performance.

Interestingly, the Experiment 2 did not replicate the performance deficit seen in Zelinski et al. (2014), which previously found a retention deficit in the MWM task after exposure to six days of light manipulation. However, it is important to reiterate that once the second cohort is able to be completed, these results may change. Experiment 2 examined the relationship between the FEO and LEO entrainment and its influence on hippocampal-dependent task performance. Where the location of the FEO is still unknown, it is hard to draw a conclusive reason as to why the effect of the light manipulation washed out when FEO entrainment was also involved. However, the present results suggest the possibility that FEO entrainment may have prevented a memory impairment in the 1M-LM rats. Overall, the relationship between the FEO and LEO is still unclear, which is why more research is important in this area. Both effects of LEO and FEO entrainment on hippocampal-dependent memory have been observed individually, but more research needs to be conducted on the relationship between all variables.

Both Experiments 1 and 2 were unable to observe whether FEO entrainment compensates for LEO disruption in terms of performance on the MWM task. Given that the light manipulation schedules in both experiments did not produce the deficits we hypothesized, we were unable to assess the potential protective effects of meal entrainment.

Future Research

Future research is necessary in the area of circadian rhythms and memory, specifically the relationship between the LEO and FEO. Once the second cohort of Experiment 2 is complete, there is hope that more information about this relationship will be observed. Replications of this research are key in determining if consistent mealtimes can ameliorate

memory performance in rats that are experiencing other forms of circadian disruption. Additionally, a comparison should be made between restricted meals, multiple meals, and *ad libitum* food access, to further unveil any compensating factors of FEO entrainment. Finally, future research should assess whether a light manipulation schedule between 30 and 64 days will produce the same deleterious effects as previously observed in Craig and McDonald (2008). It is important to assess the amount of circadian disruption that a rat can adjust to and whether or not there is a certain time point in which the global deficit of hippocampal-dependent task performance occurs.

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Day	Lights off
1	7:00 pm
2	4:00 pm
3	1:00 pm
4	10:00 am
5	7:00 am
6	4:00 am
7	1:00 am
8	10:00 pm
9	10:00 pm
10	10:00 pm
11	10:00 pm
12	10:00 pm
13	10:00 pm
14	7:00 pm
15	4:00 pm
16	1:00 pm
17	10:00 am
18	7:00 am
19	4:00 am
20	1:00 am
21	10:00 pm
22	10:00 pm
23	10:00 pm
24	10:00 pm
25	10:00 pm
26	10:00 pm
27	7:00 pm
28	4:00 pm
29	1:00 pm
30	10:00 am

Table 1. Light Manipulation Schedule for Experiment One.

Day	Lights off
0	7:00pm
1	4:00pm
2	1:00pm
3	10:00am
4	7:00am
5	4:00am
6	1:00am
6+	1:00am

Table 2. Light Manipulation Schedule for Experiment Two.

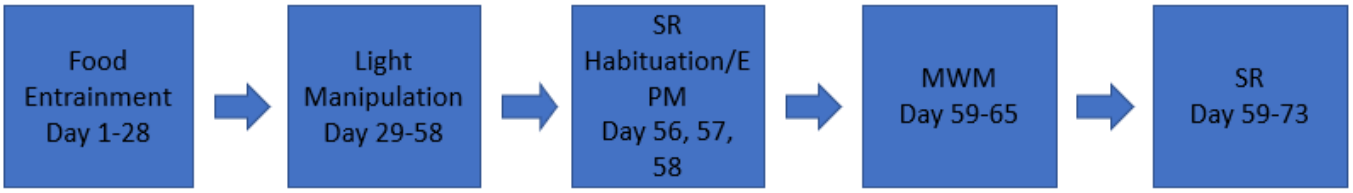

Figure 1. Timeline of Experiment One.

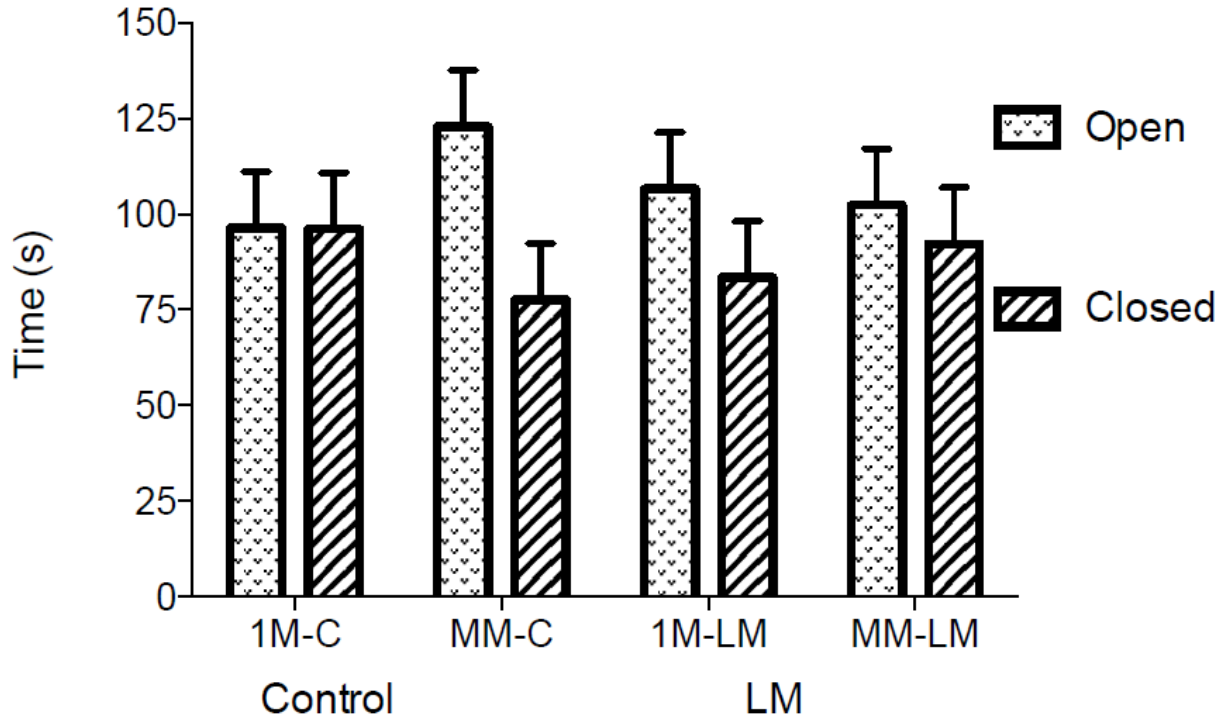

Figure 2. Average (±SEM) duration spent in the open and closed arms during the 5-minute testing period of the elevated plus maze task for each of the four groups for Experiment One.

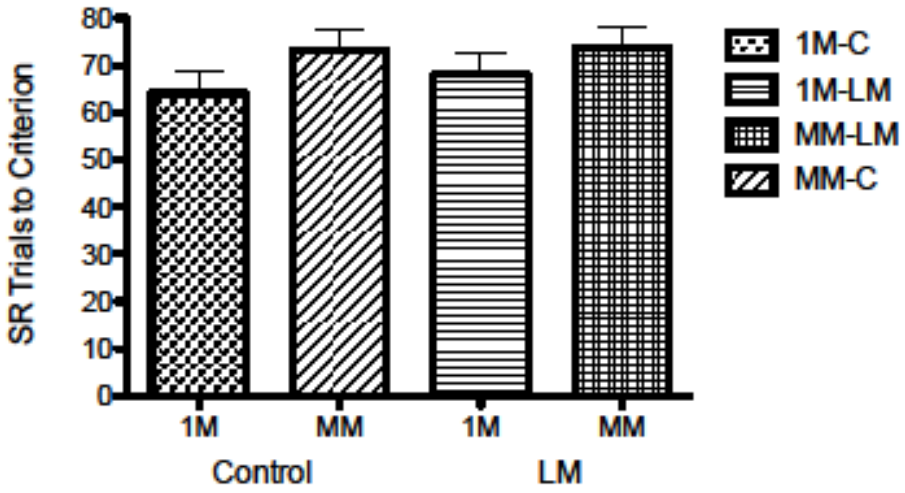

Figure 3. Average (±SEM) trials to criterion for each of the four groups on the SR task For Experiment One. Criterion was set as 18/20 trials correct.

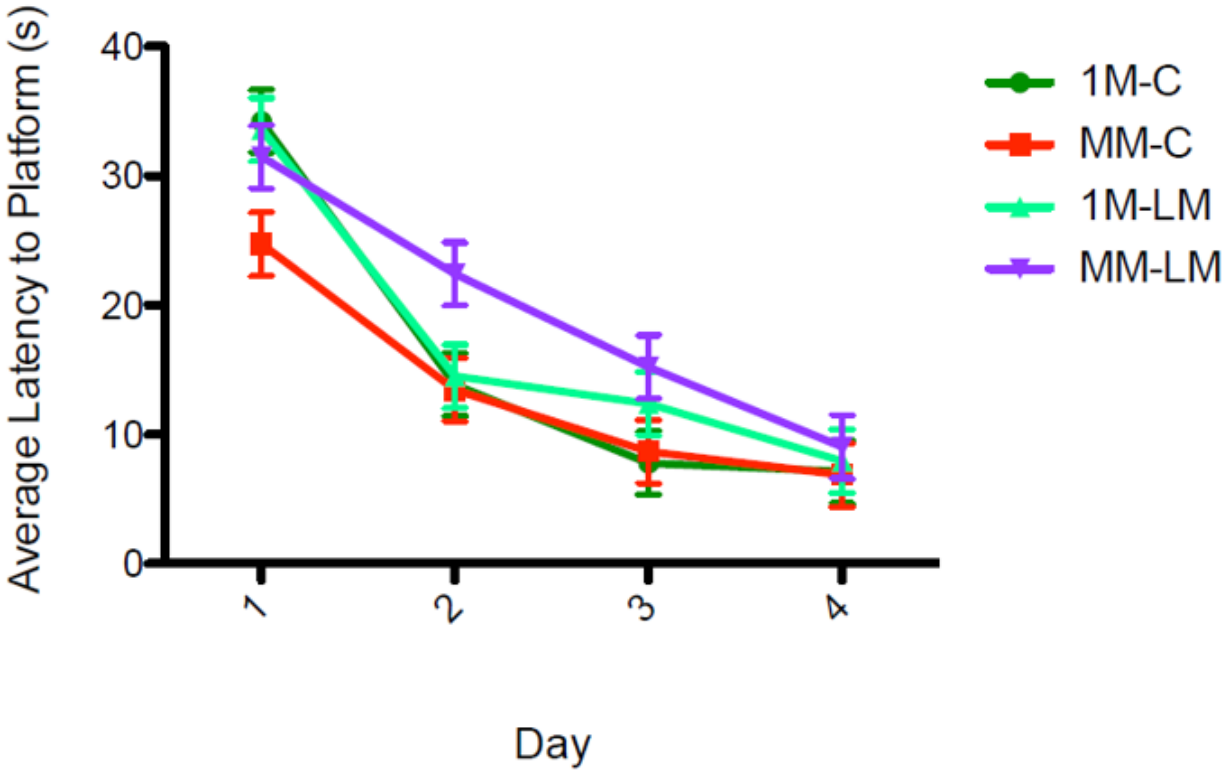

Figure 4. The average latencies (±SEM) on the Morris water maze task blocked into four days of eight trials per day for Experiment One.

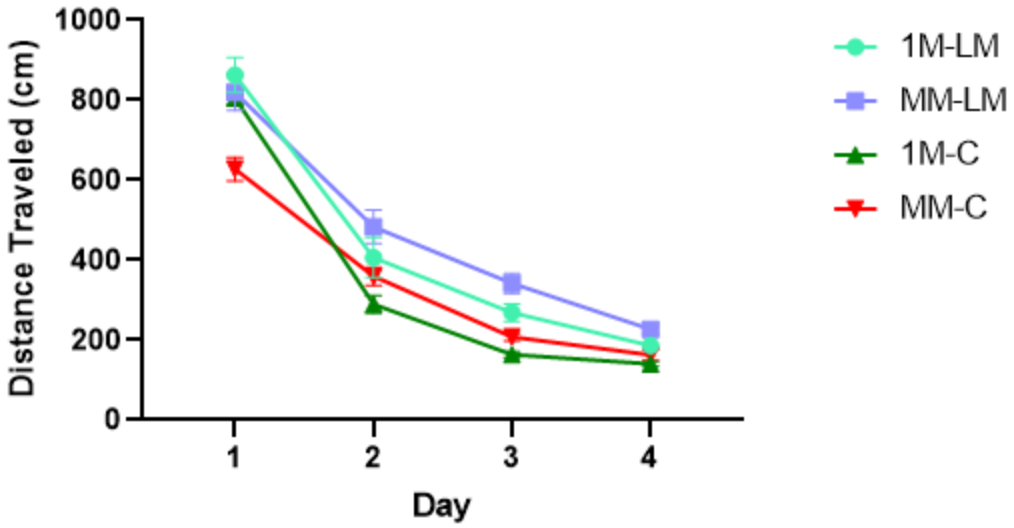

Figure 5. Average distance traveled (±SEM) on the Morris water maze over the four days of training for Experiment One.

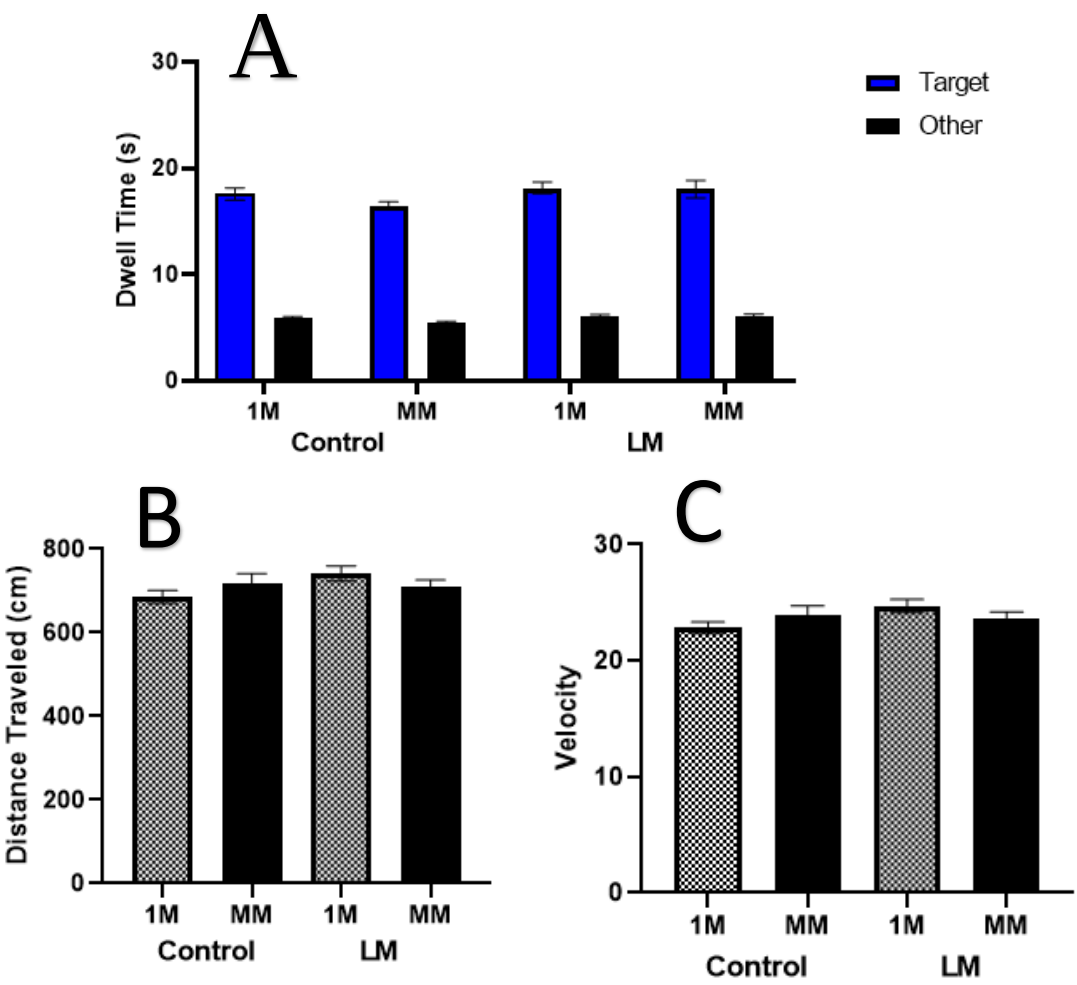

Figure 6. Results from the probe one trial on the Morris water maze task across the four groups for Experiment One. A) Time spent in the correct quadrant (±SEM) compared to average time spent in the incorrect quadrants in the first 30 seconds of the trial for each of the four groups. B) Total distance traveled (±SEM) in the first 30 seconds of the trial for each of the four groups. C) Average velocity (±SEM) in the first 30 seconds of the trial for each of the four groups.

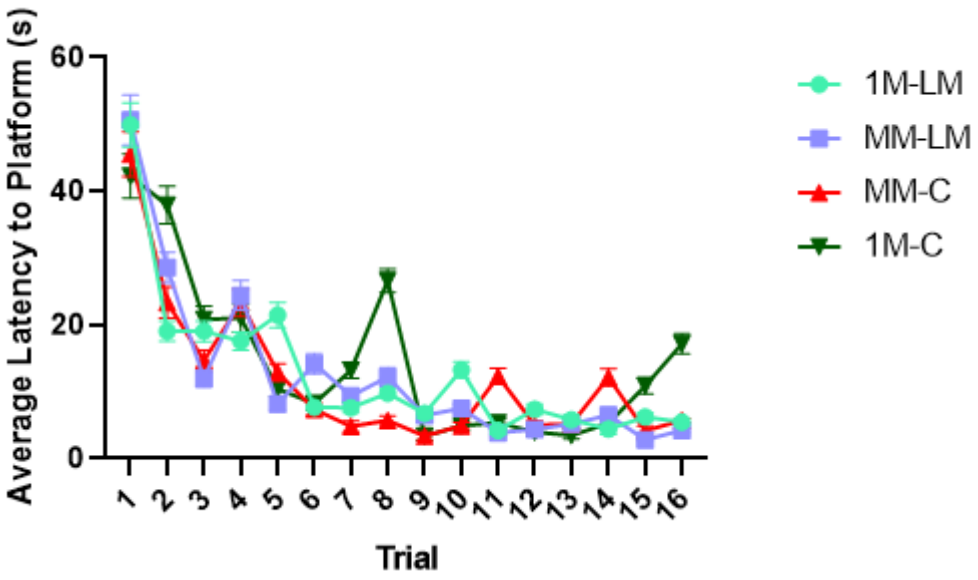

Figure 7. Latency to the platform (±SEM) during the Morris water maze task across the sixteen trials in one day for Experiment One.

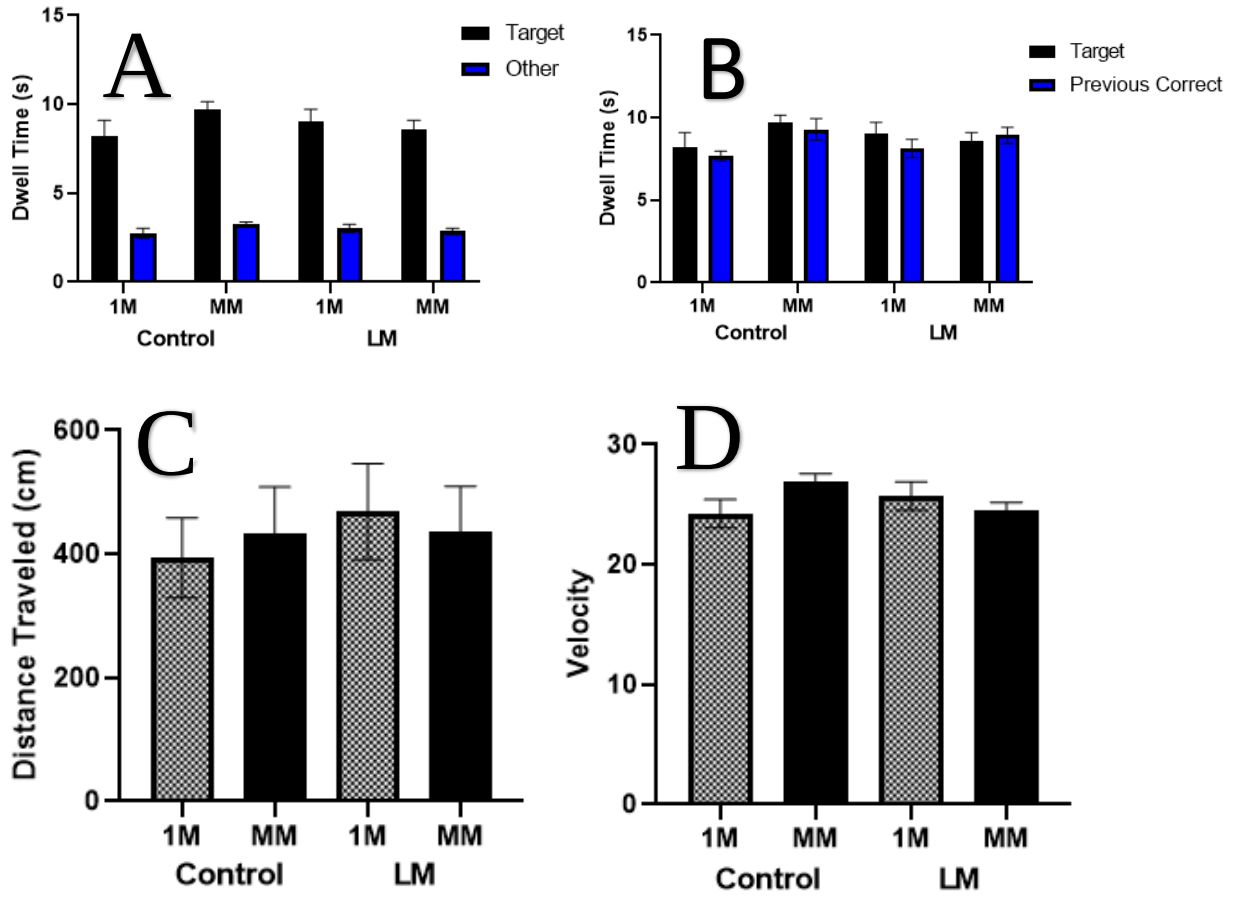

Figure 8. Results of the massed training probe trial of the Morris water maze task across the four groups for Experiment One. A) Time spent in the correct quadrant (±SEM) compared to average time spent in incorrect quadrants (±SEM) in the first 30 seconds of the trial for each of the four groups. B) Time spent in previously correct quadrant (±SEM) compared to time spent in the correct quadrant (±SEM) in the first 30 seconds of the trial for each of the four groups. C) Total distance traveled (±SEM) in the first 30 seconds of the trial for each of the four groups. D) Average velocity (±SEM) in the first 30 seconds of the trial for each of the four groups.

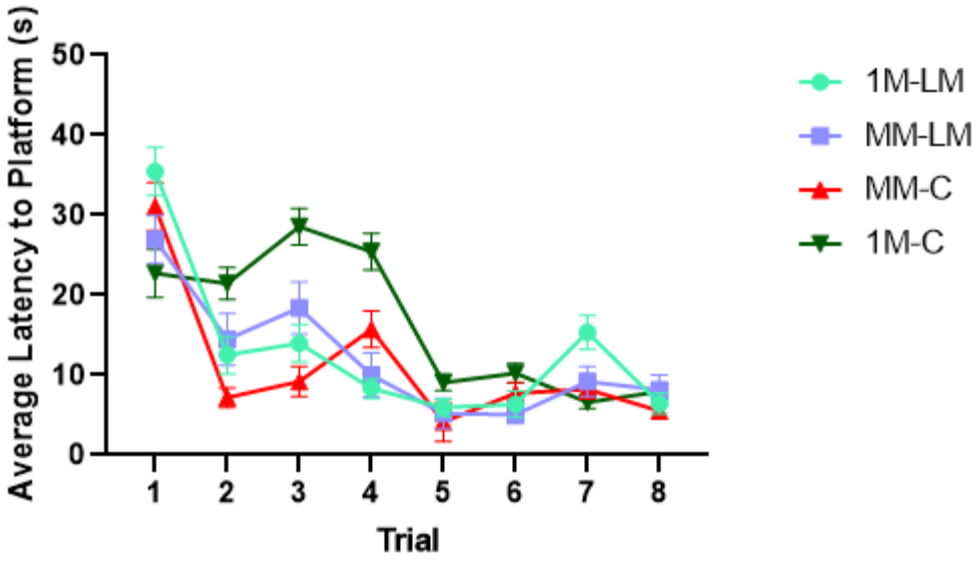

Figure 9. Average latency (±SEM) to the platform during the Morris water maze task across the eight trials in one day for Experiment One.

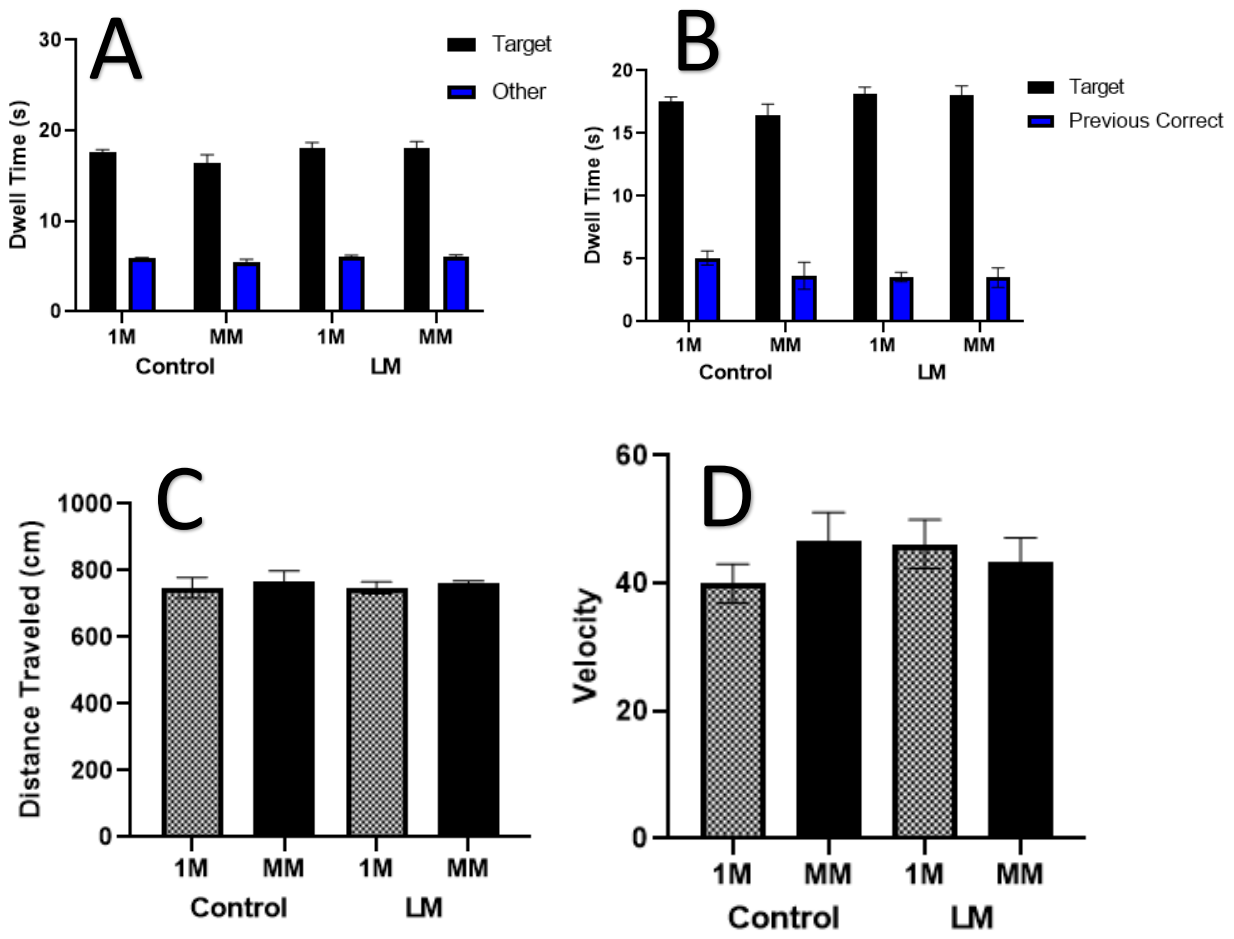

Figure 10. Results from the competition training probe trial on the Morris water maze task across the four groups for Experiment One. A) Time spent in the correct quadrant (±SEM) compared to average time spent in incorrect quadrants (±SEM) in the first 30 seconds of the trial for each of the four groups. B) Time spent in previously correct quadrant (±SEM) compared to time spent in correct quadrant (±SEM) in the first 30 seconds of the trial for each of the four groups. C) Total distance traveled (±SEM) in the first 30 seconds of the trial for each of the four groups. D) Average velocity (±SEM) in the first 30 seconds of the trial for each of the four groups.

Figure 11. Timeline for Experiment Two.

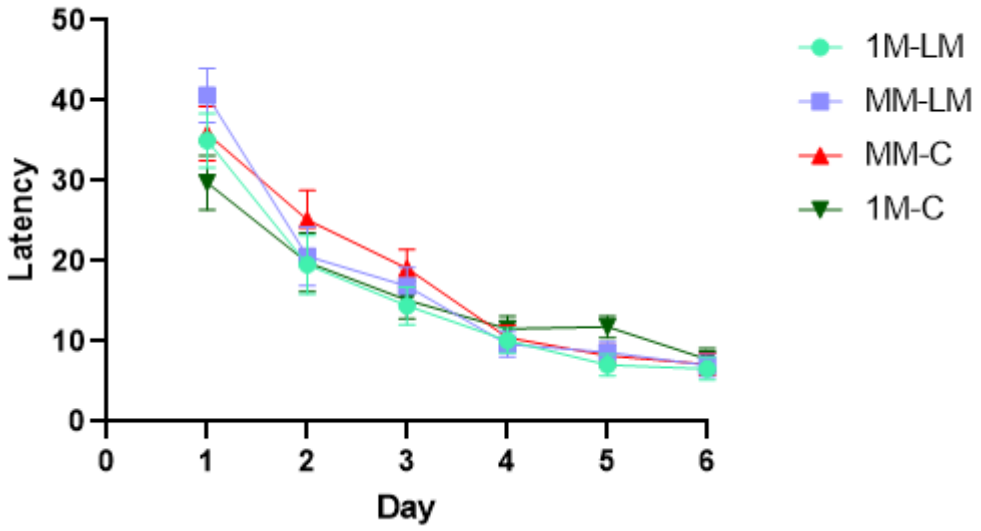

Figure 12. Average latency (±SEM) to the platform on the Morris water maze task blocked into six days of eight trials per day for Experiment Two.

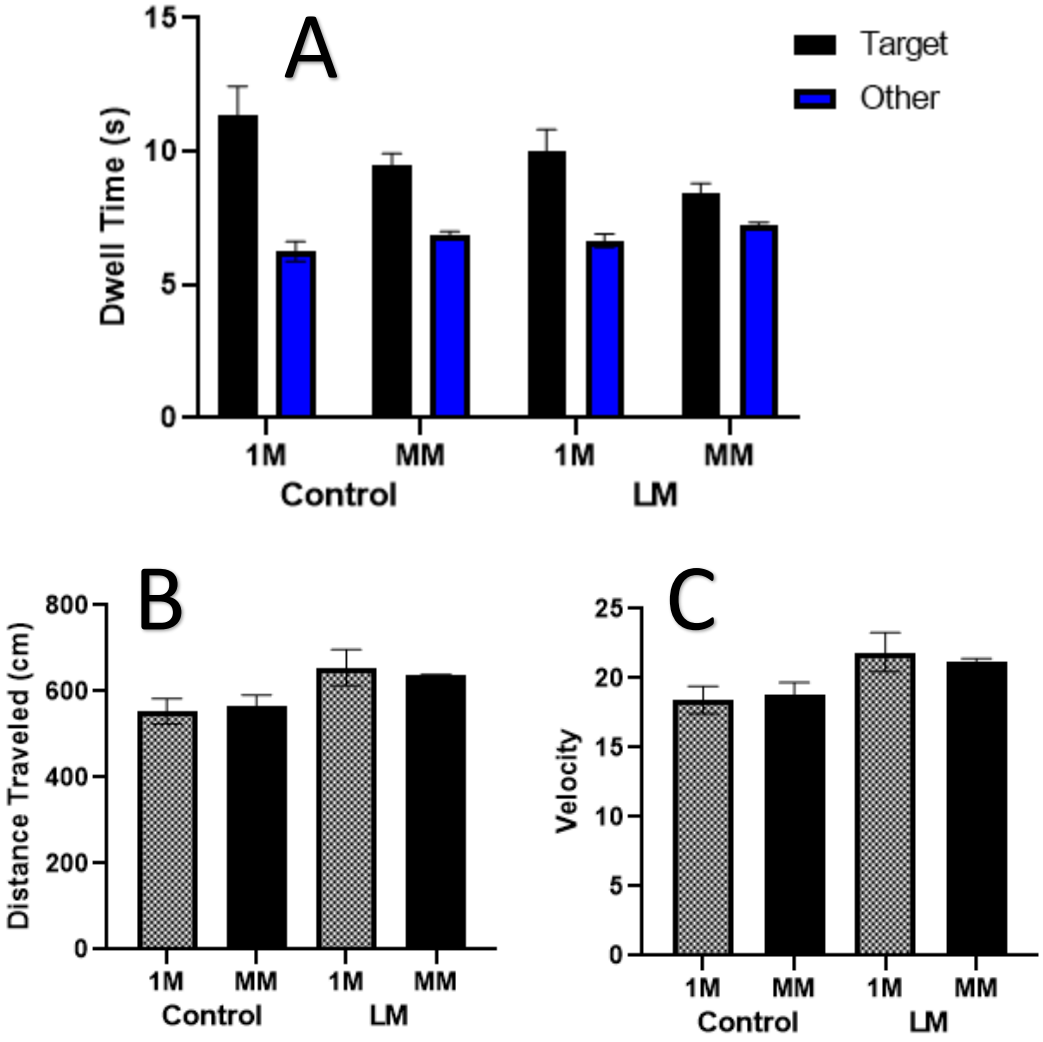

Figure 13. Results from the retention probe on the Morris water maze task from the four groups for Experiment Two. A) Time spent in the correct quadrant (±SEM) compared to average time spent in the incorrect quadrants (±SEM) in the first 30 seconds of the trial for each of the four groups. B) Total distance traveled (±SEM) in the first 30 seconds of the trial for each of the four groups. C) Average velocity (±SEM) in the first 30 seconds of the trial for each of the four groups.