The Limits of the Circadian Clock Driving Food Anticipatory Activity

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Abstract

Much is known about circadian rhythms in regard to the Suprachiasmatic Nucleus (SCN) and light entrainment, however, little is known about Food Entrainable Oscillators (FEOs) and entrainment to food through time-restricted feeding paradigms. In this study, we investigated the limits of FEOs, as well as their interaction with the SCN when mice are entrained to a T18 light dark cycle. We found that mice lose their entrainment when entrained to a T18 light dark cycle under food-restricted conditions. Interestingly, the mice that were fed every 18 hours showed no evidence of Food Anticipatory Activity (FAA). However, mice that are entrained to T18 light dark cycle and are fed every 24 hours still lose their entrainment, but these mice show some evidence of FAA in response to 24 hour time-restricted feeding.

1. Introduction

Circadian rhythms drive much of our everyday activity and behavior. They drive behaviors such as our sleep-wake cycles, feeding behaviors, gene expression, hormone secretion, and many other daily rhythms. Circadian means “almost” (circa) “day” (dian), meaning that these rhythms are centered around clocks that are about a day, and thus result in rhythms that occur daily – i.e. 24 hours. The main pacemaker associated with circadian rhythms is the Suprachiasmatic Nucleus (SCN) – often referred to as the master clock; this brain region is located in the anterior part of the hypothalamus. Circadian rhythms are important as they help us be in tune with the environment that we live in. The light-dark cycle in our natural environment plays a large role in synchronization of behavior. Additionally, the hypothalamus plays a large role in maintaining the body's homeostasis, and our circadian rhythms have a strong influence on that. There are peripheral clocks in our brains and bodies that can be affected by other zeitgebers without shifting the SCN. Zeitgebers - meaning time giver - are environmental influences that

can act as circadian time cues (Lewy & Rough, 2009). There can be a large variety of zeitgebers that have influences on the body - cyclic food availability, drug administration, and more. The SCN plays a crucial role in synchronizing daily behavior, however, it shouldn't be discounted that other oscillators reside in the body and brain that respond to different zeitgebers that aid in synchronization of behavior as well.

A crucial element in circadian biology and research is the concept of entrainment. The SCN has a direct pathway with the eyes in which melanopsin indicates how much light is in our environment to relay time of day information. Melanopsin are non-image forming photopigments in the eyes that allow for the detection of irradiance in our environment – these photopigments allow for light entrainment of the SCN (Beaule et al., 2003). The SCN interacts heavily with the light dark environment and is therefore entrained by light. Entrainment is the synchronization or alignment of the internal biological clock rhythm, including its phase and period, to external time cues (Vitaterna, Takahashi, & Turek, 2000). While light is an important zeitgeber – as it entrains the SCN which can then entrain other oscillators – there can be non-photic zeitgebers as well. An example of a different zeitgeber is food. When animals are put on a time restricted feeding (TRF) schedule, they can reliably entrain to food due to its cyclic availability. This cyclic availability of food entrains liver cells, due to the role that it plays in feeding. Interestingly, this paradigm gives rise to a behavior known as Food Anticipatory Activity. This behavior persists in the absence of the SCN, suggesting that there are different oscillators at play – i.e. Food Entrainable Oscillators.

Food Entrainable Oscillators (FEO), found in our body and brain, are a system that is entrained by food rather than light and controls behavior in response to timed feeding cues. While it is known that FEOs exist, they are difficult to study as their location in the brain is still unknown. A crucial piece of evidence that FEOs exist is Food Anticipatory Activity (FAA). FAA

is the increase in locomotor activity prior to the cyclic availability of food. (reference). This means that animals that are put on time-restricted feeding schedules – that are approximately 24 hours – have the capability of showing food anticipatory activity as they can reliably predict when food will be made available to them. This is not possible if the feeding schedule is too far outside of the circadian range of 24 hours. For example, an animal would not reliably be able to anticipate food every 14 hours. Like most circadian research, this is a phenomenon that is seen in research in rats and mice, but can be extended to mammals in general. Current research points to the involvement of the hypothalamus, brainstem, and corticolimbic structures; however, lesion studies of those areas do not eliminate all food anticipatory rhythms. This suggests that control of this behavior may be a distributed, decentralized system of oscillators, or the existence of an essential oscillator in an unknown location (Mistlberger, 2011). While the dorsomedial nucleus of the hypothalamus' (DMH) involvement is still controversial, recent findings showed its role in disinhibition of behavioral expression (necessary for FAA) during the light phase (Carneiro & Araujo, 2012). From an evolutionary stance, it is speculated that this behavior of food anticipatory activity is a parallel to foraging behavior prior to when food is most readily available or safe to retrieve (Storch & Weitz, 2009).

FAA is an output of FEOs that we are able to study and use to better understand the mechanism and system as a whole. There are some key findings that help better understand Food Entrainable Oscillators. One way to investigate FEOs is to evaluate whether a well studied system like the SCN has similar circadian mechanisms that may be influential for FEOs. In a paper by Storch and Weitz, evidence is provided that the SCN and major clock components like Bmal1 are not necessary for the Food Anticipatory Activity. Bmal1 is a key component of the circadian mechanism, and a crucial clock component especially for mammals - Bmal1 is "the

batteries of the biological clock" (Takahashi, 2012). In the first study, with the use of an intact wildtype mouse and a mutant Bmal1 deficient mouse, both were able to exhibit food anticipatory activity when on a time-restricted feeding schedule. Another study in the same paper showed that SCN lesioned mice were also able to show FAA. Similar to the previous study, they used intact wildtype mice and wildtype mice with lesioned SCNs, and the results showed that both were able to exhibit FAA (Storch & Weitz, 2009). Additionally, in a review by Mistlberger, it was shown that anticipatory behavior in animals was not displayed when the feeding schedule was too different from 24 hours (bounds of 19hrs to 29 hrs) (Mistlberger, 2020) . This goes to show that food entrainable oscillators also function on a circadian basis. Lastly, what current research shows us is that food anticipatory activity is self-sustaining, and not just an output of a passive hourglass mechanism. In order to show that FAA is an output of an oscillator, it must be shown that it isn't simply due to an accumulation of hunger, or a reinforcement learning mechanism. To do this, animal subjects are put into constant conditions, which is either complete food deprivation or ad libitum feeding. With ad libitum feeding, it is evident that FAA disappears as food is available to the animal at all times and no 'anticipation' occurs. However, when animal subjects are completely deprived of food, the persistence of FAA would indicate that there is a timing mechanism involved. Bolles and Moot (1973) and then later Coleman (1982) showed that food anticipatory behavior still occurs during food deprivation tests in rats that had previously been fed in a cyclic time-restricted fashion. The persistence of the FAA for a brief window of time before *expected* timed feeding — in the absence of an entraining stimulus—implies that this phenomenon is generated by a self-sustained timing mechanism.

These findings in current research still leave many questions unanswered. What are the relationships and mechanisms at play when looking at the interaction of the SCN and food

entrainable oscillators? Studies have mainly been done on normal 24 hours conditions (T24) and free-running rhythms, or in SCN-lesioned animals. A central question is how FAA is affected with the SCN intact but not entrained in a normal fashion. Instead of a 24 hour day, entraining animals to an 18 hours day (T18) allows us to study animals that are not entrained in a normal fashion. T18 entrainment should not be possible as it is significantly out of the circadian range, and because the SCN is a circadian oscillator, it best entrains to time periods that are roughly 24 hours. However, T18 entrainment *does* occur, and we see evidence of it in mice (Walbeek & Gorman, 2017). Studying the interaction of FEOs and SCN in T18 conditions raises interesting questions about FAA. Because T18 entrainment is a significantly short time period, it may be that the SCN has weaker oscillations. If FEOs do influence the SCN, it may be more evident when the SCN is dampened. While lesion studies are important, we aim to investigate which clock may dominate when the SCN and FEOs are miss-mismatched and when the SCN is not normally entrained. To see the effects, mice will have a light-dark (LD) cycle of T18, with a feeding schedule that is T24. Additionally, we expect that mice will be able to entrain and show FAA in T18 conditions.

In my experiment, there are three conditions. The control group is entrained to a 14:10 LD cycle, and fed every 24 hours for two hours. This means that animals get 14 hours of light, and 10 hours of darkness to make up their 24 hours day. Additionally, they are fed for two hours in the middle of their light phase in order to see the effects of FAA and have that be separate from night-time activity. This has been reliably evident in research. There is strong evidence that FAA should occur under these conditions, because FEOs are circadian oscillators as well, thus, feeding every 24 hours would allow for the optimal functioning of the FEO system, and we should see FAA. The 18:18 group was entrained to a 13:5 LD cycle, and fed every 18 hours for

two hours. This means that they receive 13 hours of light, and 5 hours of darkness to make up their 18 hour day. These animals are also fed for 2 hours during their light phase. This condition aims to investigate the lower limits of the food anticipatory activity. The 18:18 group, having been fed every 18 hours, we aim to investigate whether the food-entrainable oscillators involved in FAA can function on a T18 cycle and how it may influence entrainement to the LD cycle. Lastly, the 18:24 group was entrained to a 13:5 LD cycle, and were fed every 24 hours for two hours. This condition aimed to investigate the effects of having a mismatched light dark cycle and feeding schedule. Research has shown us that FAA is a product and evidence of food-entrainable oscillators that do not need the SCN to function. This was seen through studies in which the SCN was ablated. Nonetheless, it would be interesting to see when the SCN is intact but not functioning in the conventional manner (~24 hours), if it has impacts on the food entrainable oscillators responsible for FAA.

2. Methods

2.1 Subjects. All subjects were adult male C57BL/6J mice from Jackson labs; all mice were ~ 3 months old at the onset of the study. Mice were housed in individual cages, with up to 16 in a chamber together. Mice were kept on a 14:10 LD cycle, or 13:5 LD cycle based on the experimental group. During experiments, mice were on food-restricted feeding paradigms. Their weights were monitored on a weekly basis to make sure the weights remained 80% - 90% of their free-fed weight for optimal behavioral motivation and to maintain physical health.

*2.2 Apparat*us. Prior to time-restricted feeding, mice were housed in plastic shoebox cages (L × W × H: 28 × 18 × 15 cm). The cages had running wheels (13 cm diameter) attached to them and cage tops that allowed for ad libitum access to food and water. Food provided prior

to time restricted feeding was regular chow (Mouse Diet 5015; Purina, St. Louis, MO, USA) - mice would normally eat 3 grams a day.

2.3 Food Restriction Apparatus. For time restricted feeding, mice were housed in different cages (L × W × H: 31.5 × 14 × 15 cm) These cages were different in order for them to fit and be attached to the programmable feeders. The program used for the feeder is the ClockLab Chamber Control software. This software allows us to set specific durations of what the mice would be fed and specifically at what time. Additionally, the feeder allows for one pellet to be dropped every 10 mins, allowing for a maximum of 12 pellets to drop in the 2 hr feeding period. The feeder only drops another pellet if the mouse takes one, meaning that if a mouse is not feeding it will not pile up pellets. The usual range of pellets dropped is between 6-13 pellets. The feeders were monitored on a daily basis to ensure that all mice got sufficient food, and were not starved in case of malfunction. The food used during time-restricted feeding is different from the regular chow, the food (Bio-serv, Dustless Precision Pellets® Rodent, Purified) has a different nutritional composition and was used for its compatibility with the feeder system.

Sensors were attached to both the old and new cage tops which record wheel activity by activation of magnets on the wheels. These sensors send the wheel running data to a program called VitalView. This program allows wheel activity to be evaluated on a computer.

*2.4 Design***.** This experiment was a between groups design, in which there were three groups that differed based on feeding schedule and the light dark cycle. The control group (n = 9) had a 14:10 LD cycle, and food restriction to 2 hr every 24 hours during the middle of their light phase (see Figure 1A). The experimental group 1 (n = 6) had a 13:5 LD cycle, and food restriction to 2 hr every 18 hours during the middle of their light phase (see Figure 1B). The

experimental group 2 (n = 6) had a 13:5 LD cycle, and food restriction to 2 hr every 24 hours (see Figure 1C).

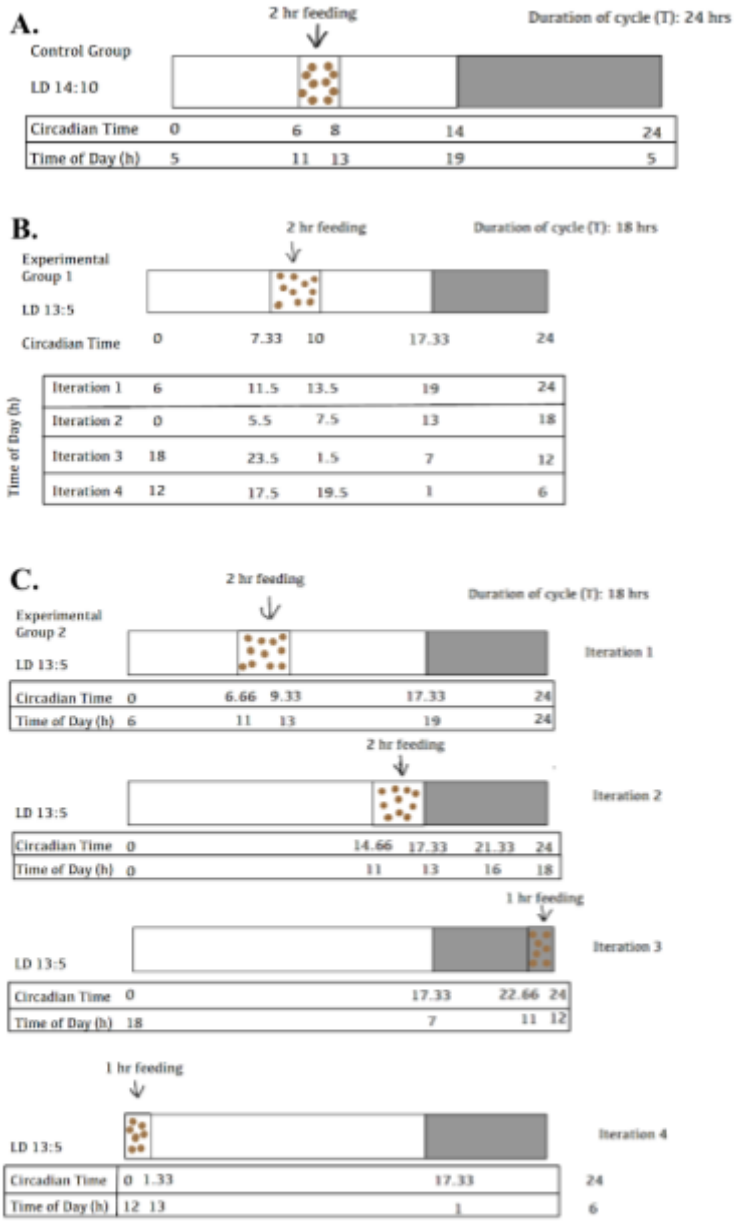

Figure 1: Design of the experiment
(A) Control group; (B) Experimental Group 1; (C) Experimental Group 2

2.5 Procedure. All mice experienced a pre-treatment period of 23 days after being moved from the colony, in which they were housed in individual cages, with exposure to 14:10 LD cycle, and ad libitum access to food and water. After the 23 days, the entrainment period for all three groups of mice began. Experimental animals (18:18 group and 18:24 group) were exposed to a T18 LD cycle in a manner previously shown to facilitate entrainment of the activity rhythms. Control animals remained in T24 during this time period.

After 21 days, all of the control mice were moved to a chamber attached to the feeders, which was on a 14:10 LD cycle. For the two experimental groups, out of the 16 mice, the 12 best entrained mice were moved to a separate chamber attached to the feeders, which was a continuation of the 13:5 LD cycle. Once moved to new cages, into the chambers with feeders, all of the mice were weighed. This was a baseline to ensure that mice did not get too underweight during time-restricted feeding. The first three days of being in the new cages, all mice had ad libitum access to the new food pellets, in order for them to get accustomed to their new environment.

After three days of ad libitum feeding, mice were put on time-restricted feeding. This was programmed into the feeder program to ensure that all mice were fed during the correct window. Checks happened once every day, to ensure that the timer that controlled the lighting was set correctly. Additionally, a manual record of daily feeding of the mice was kept. The records show how many pellets dropped for each mouse, how many excess pellets there were (uneaten pellets), and how many pellets they were given (if any). This check was to make sure that each mouse got at least 5-7 pellets during every feeding period to avoid starvation of the mice. During this time, their wheel running activity was also monitored, and any issues seen were recorded and fixed.

Additionally, there were cage changes every 3 weeks, to ensure sanitation. Weight was monitored on a weekly basis to ensure that mice maintained a weight of 80% - 90% of their free-fed weight. Mice that fell below 20% of their starting weight were closely monitored and provided with extra food.

2.6 Analysis. All data analysis was conducted on ClockLab and Microsoft Excel.

First, once data was collected through VitalView, it was converted to allow ClockLab to create actograms. Actograms are graphs depicting the activity of individual mice over a set time period. Actograms were created for the control group (in T24), 18:18 group (in T18), and the 18:24 group (T24 and T18). Once the actograms were created, Preview was used to input the LD cycle and the time-restricted feeding period, as well as other annotations for a clear visualization.

Next, activity profiles were created using ClockLab, and that information was then converted to an Excel sheet. In ClockLab, the dates for the activity profiles were manipulated in order to get a week by week understanding of what is happening with their activity. Additionally, ClockLab allowed for the average activity within an hour to be calculated. Once converted to Excel, all the activity was normalized and a graph was created to show the change in activity throughout the day for each week. The LD cycle and time-restricted feeding period were overlaid on the activity profile for better understanding. It should be noted that activity profiles were only created for the control group and the 18:18 group. Activity profiles for the 18:24 group were too noisy, and didn't allow for valuable extraction of data.

Periodograms were also created in order to better understand how entrainment changed over the course of the experiment. Periodograms were created in two week intervals – two weeks of baseline in their old cages, first two week of TRF, and second two weeks of TRF. Once these periodograms were created using ClockLab, they were converted to Excel. This allowed for the

calculation of the Entrainment Quotient (EQ = power at 18h/(power at 18 h + power ~24h). Once this was calculated, averages were calculated for each cohort and then plotted on a graph. This allowed the visualization of how entrainment changed over the course of the experiment. It should be noted that EQ was only calculated for the 18:18 group and the 18:24 group, as the control group was very well entrained.

3. Results

The activity profiles, actograms, and entrainment quotient of the various groups were analyzed in order to evaluate whether entrainment sustained over the course of the experiment, and if the various groups reflect food anticipatory activity. We found that while entrainment to T24 was maintained over the course of the experiment, T18 entrainment to light dark cycle worsened and then slightly recovered towards the end of the experiment, as can be seen in Figure 4. Additionally, actograms and activity profiles combined were used to evaluate the occurrence of FAA – which seems to be present in all groups to some degree.

3.1 Defining FAA.

In order to evaluate whether FAA was present among the different groups, we used activity profiles from the control group to set the parameters and intervals. We defined the threshold of activity to be >0.01 normalized mean counts/hr. This threshold was chosen because when lights are on, prior to the learning of when food is available (food entrainment), mice have an average baseline activity that is below this threshold but not 0 – this can be seen in Figure 2A. As the mice continue to entrain to the food availability, day time activity surpasses the threshold of 0.01, particularly around the time of feeding – this can be seen in Figures 2B - 2E. When comparing each individual animal, the normalized mean counts per hour met this threshold, allowing us to consider it FAA.

3.2. FAA and FSA

Additionally, as can be seen from Figure 2B - 2E, there is a greater increase of wheel running activity during the hour prior to food availability (hour 12 - 13). Over the course of the weeks of time restricted feeding, the hour prior to food availability reached an average normalized mean counts/hr of 0.046. This is higher than the threshold set (0.01). In addition, there seems to be elevated wheel running activity while food is made available to them. We defined this period of wheel running activity as Food Simultaneous Activity (FSA). Similar to FAA, looking at Figures 2B - 2E, we can see that there are elevated levels of wheel running activity when food is made available to the mice, between hours 13 - 15. There is a decline in activity following hour 15, after food is no longer available to the mice. During FSA, the average normalized mean counts/hr was 0.025, which surpasses the threshold previously set. Even when looking at the hours 13 - 15 individually, all seem to surpass the threshold, meaning that FSA is consistent among the animals in the control group. Defining the threshold, and what FAA and FSA look like in the control allows us to use these parameters to gauge whether the experimental groups showed evidence of FAA and FSA. Finally, when animals were put on ad libitum feeding after four weeks of time-restricted feeding, we can see that FAA is diminished, but some lingering effects of FSA still are exhibited - this can be seen in Figure 2F.

When looking at the activity profiles for experimental group 1, we can compare its findings to the control group and the defined parameters to determine whether FAA and FSA were exhibited. We found that the 18:18 group does not require the same ~4 day period to learn food availability, and shows evidence for FAA and FSA starting from day 2-4 of time-restricted feeding, seen in Figure 3A. This is evaluated by comparing the same threshold of 0.01 normalized mean counts/hr determined by the control group. Additionally, over the four weeks

of time-restricted feeding, there is evidence that FAA occurred. In Figures 3B-3E, we see that wheel running levels are elevated prior to food restriction and stay elevated when food is available to the animals. However, it should be noted that these levels are elevated for 4 hours prior to food availability. Additionally, during refeeding of ad libitum access, FAA and FSA evident in the 18:18 group do not disappear. This may suggest that the elevated activity is not due to FAA but rather an element of a loss of entrainment and the mice free running.

When evaluating whether the 18:24 group showed FAA and FSA, it was important that we did that by comparing actograms as the activity profiles of these animals were not telling due to the excessive activity. When looking at Figure 7, we can see in the actograms there is some evidence of FAA and FSA. By determining that 0.01 normalized mean counts/hr requires the activity on each line of the actogram to surpass ⅛ of the height. Therefore, especially in actogram 7B, we can see that there is FAA and FSA during the middle of the experiment. Additionally, as the experiment continues, the animal begins to free run, but we can still evaluate the activity that does not coincide with the free running to see that there is still evidence of FAA and FSA. Considering that these animals had two conflicting zeitgebers, it is very interesting that FAA was so strongly exhibited over the four weeks of time-restricted feeding.

3.3 EQ.

Entrainment to the light-dark cycle was impacted over the course of the experiment. While the control group remained well entrained to a T24 light dark cycle, the two experimental groups were not able to maintain entrainment to the T18 light dark cycle. In Figure 3, we can see that during their baseline, these animals were strongly entrained. During the first two week, we see a sudden decline in their entrainment. Finally, during the second two weeks of TRF, we see that entrainment recovers slightly, but not back to baseline levels. This suggests that either

moving to the new cages impacts their entrainment, causing that initial dip, and recovery once they are used to it. Or, TRF itself may play a role in interfering with their entrainment, due to a conflict between the SCN and FEOs.

3.4 Night-time Activity.

Additionally, another phenomenon exhibited by the control group is that night time activity grew shorter over the course of the four weeks of time-restricted feeding when FAA and FSA were present. Looking at Figure 2A, prior to FAA being exhibited, we can see that activity dips below the threshold of 0.01 normalized mean counts/hr around hour seven. Using this as baseline, when comparing the following weeks, we can see that night time activity ends earlier each week. For instance, comparing Figure 2B - 2D, we see the night time activity decreases below 0.01 normalized mean counts/hr around hour six, then the following week around hour four and a half, then during week 3 it decreases around hour four, and finally in Figure 2E, it decreases below 0.01 around hour four as well. Contrastingly, when looking at the night time activity during when allowed ad libitum feeding, seen in Figure 2F, we can see that night time activity recovers as night time activity decreases below 0.01 normalized mean counts/hr at around hour seven.

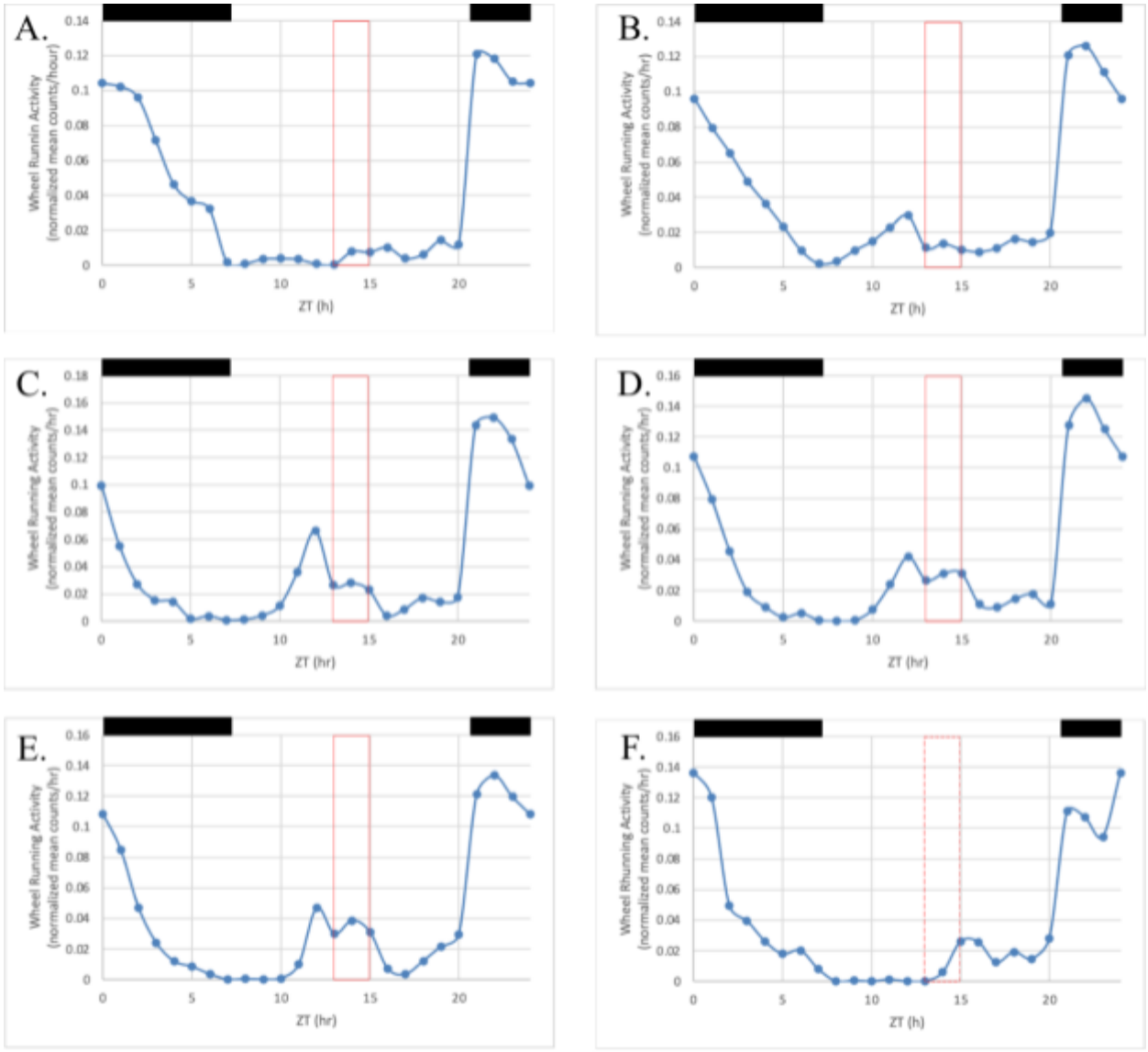

Figure 2: Activity profiles of the average of the control group

(A) Day 2-4; (B) Week 1; (C) Week 2; (D) Week 3; (E): Week 4; (F) ad lib

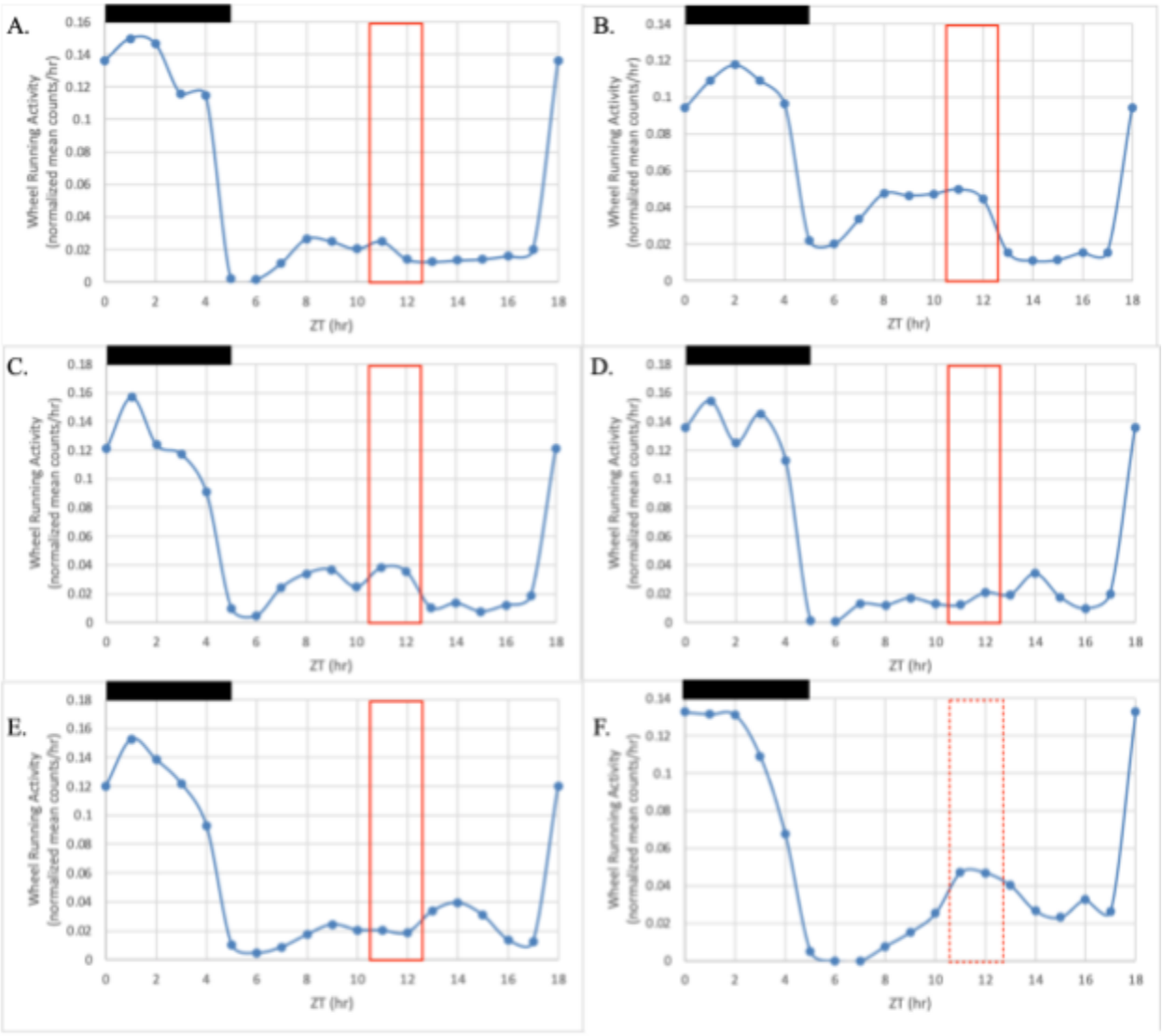

Figure 3: 18:18 group activity profile
(A) day 2-4; (B) week 1; (C) week 2; (D) week 3; (E) week 4; (F) ad lib - refeeding

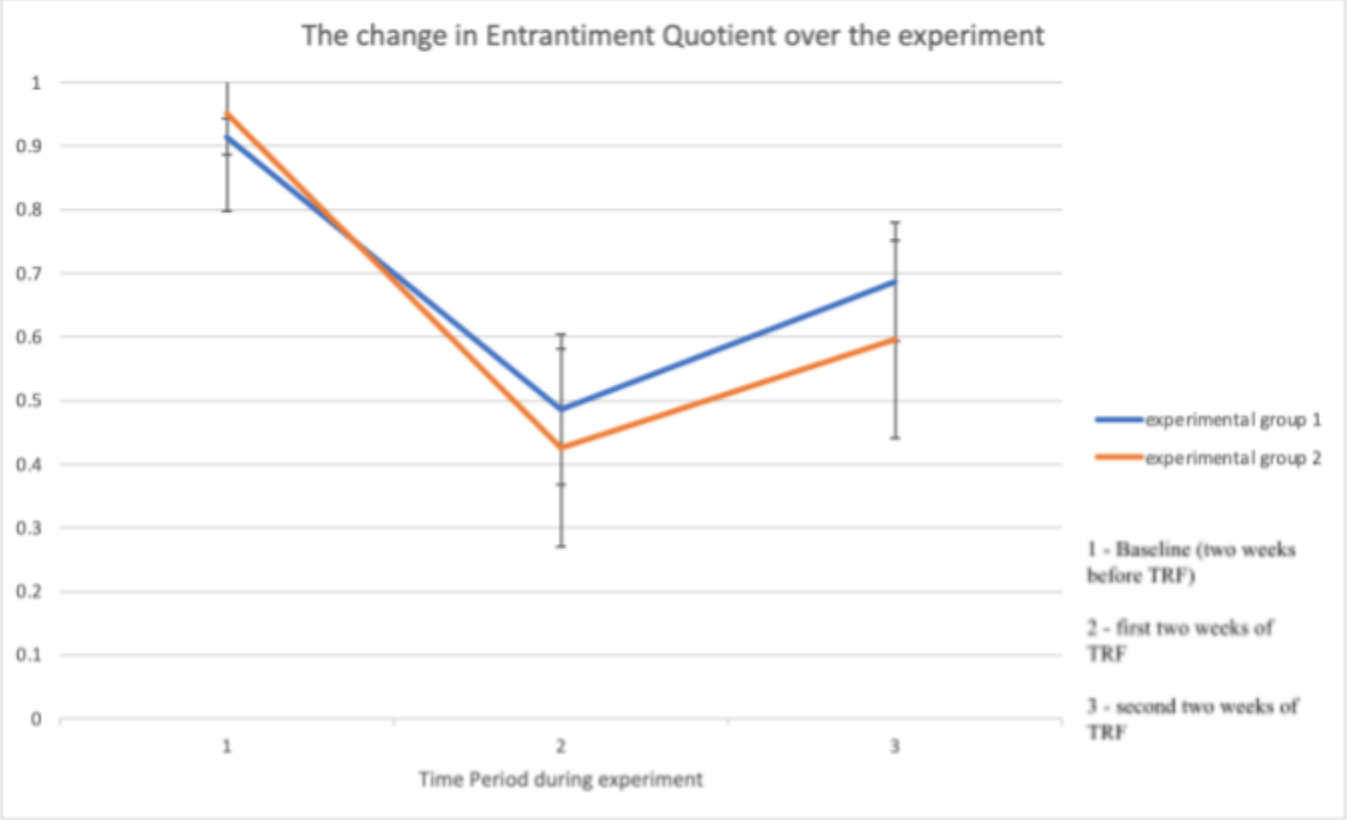

Figure 4: the change in entrainment quotient over the course of the experiment

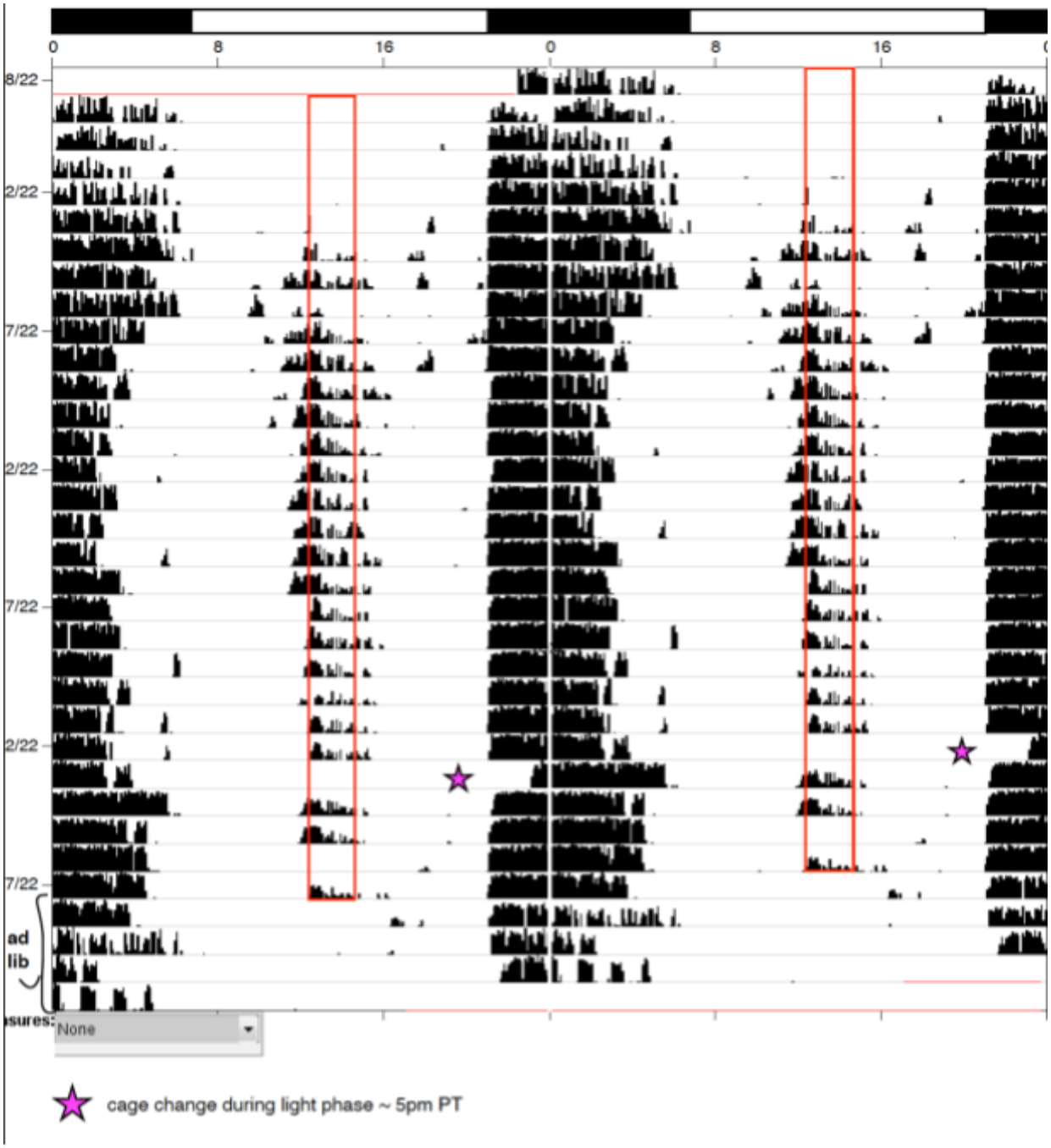

Figure 5: Actogram of an animal in the control group

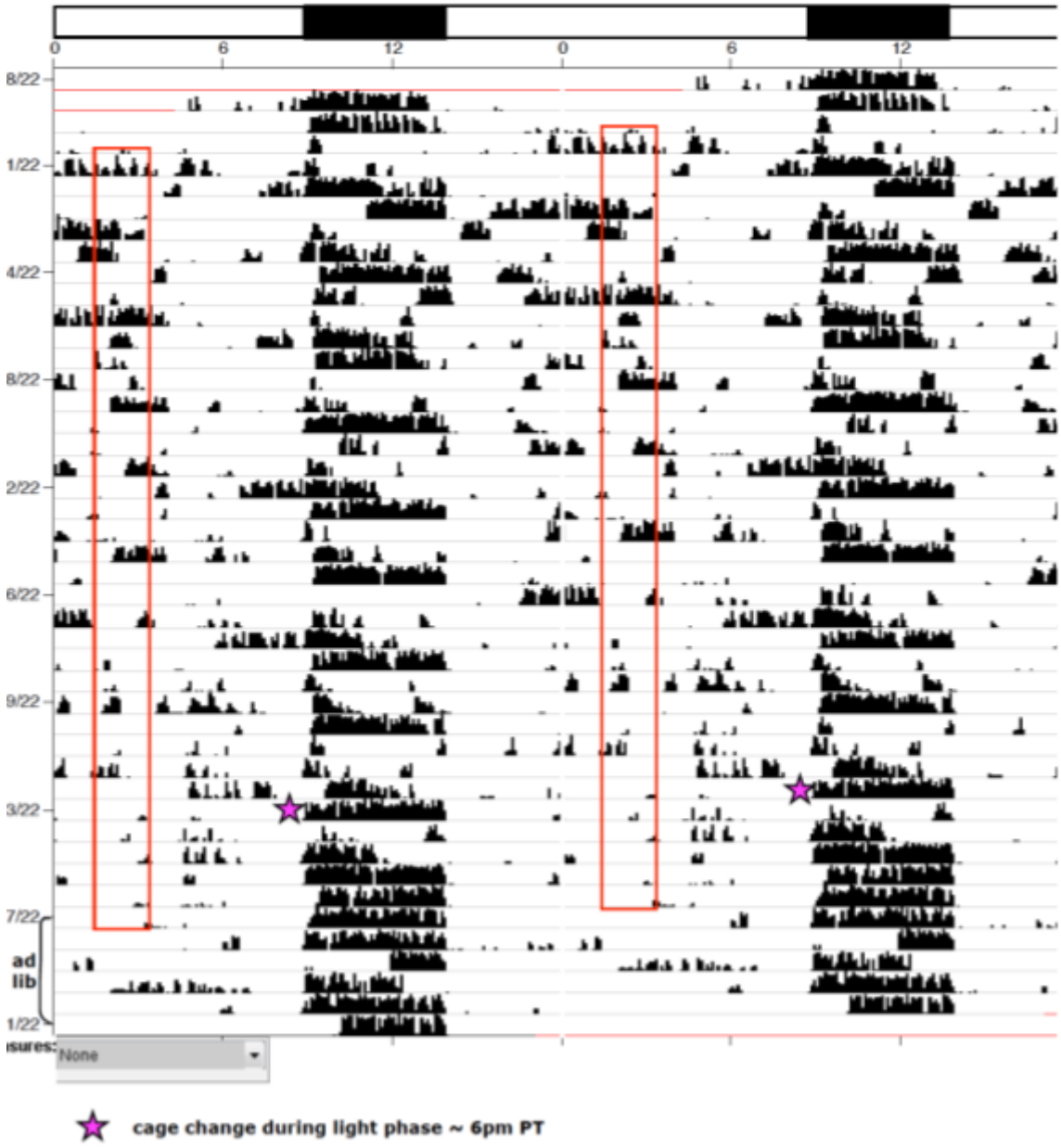

Figure 6: Actogram of an animal from 18:18 group

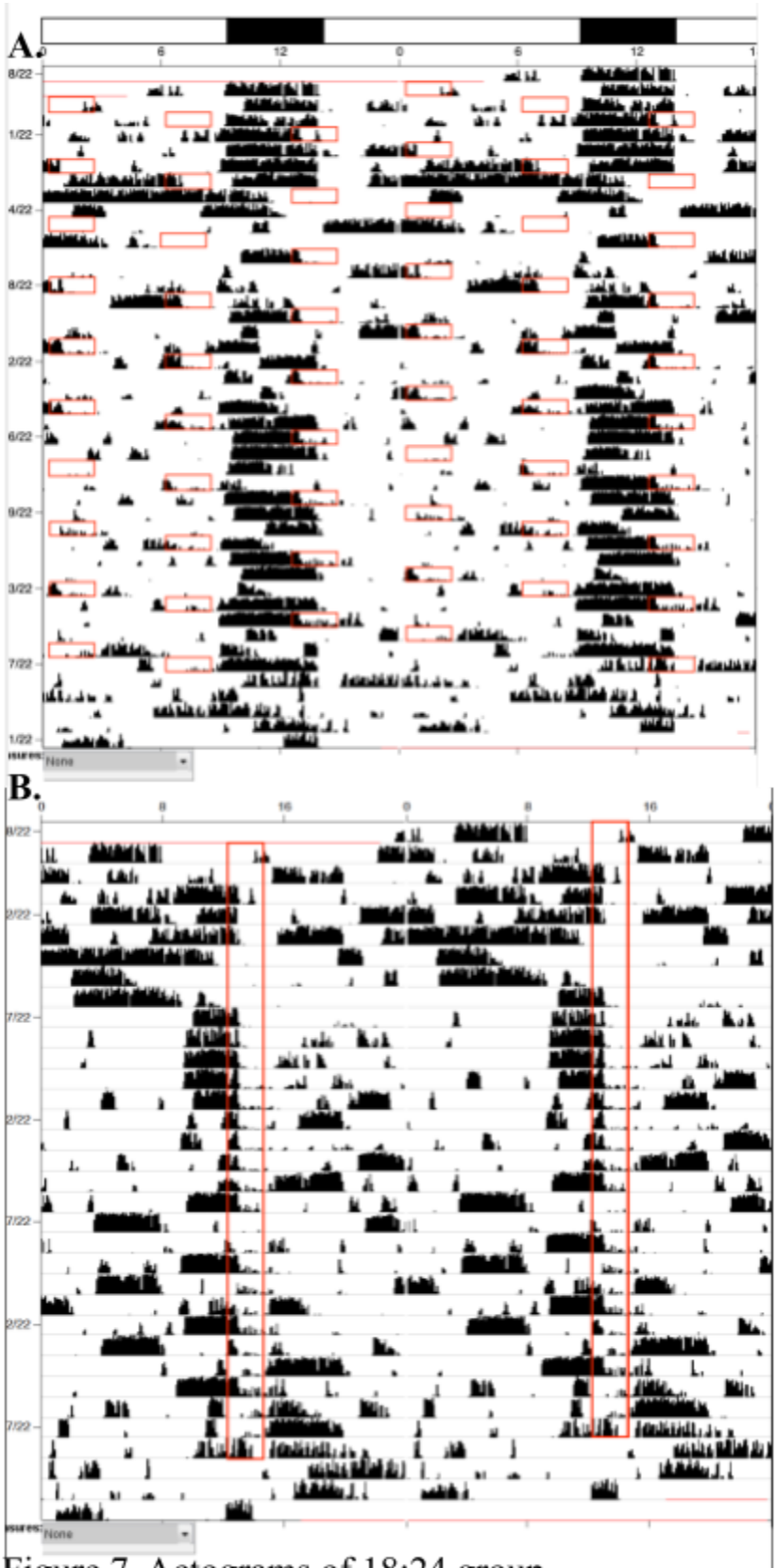

Figure 7. Actograms of 18:24 group
(A) actogram of an animal from 18:24 group set to 18 hours; (B) actogram of an animal from 18:24 group set to 24 hours;

4. Discussion

4.1 Discussion of Results

For the control group, it is in line with current research that FAA was exhibited under the conditions. Interestingly, in Figure 5, we can see that the elevated activities during FAA and FSA have a component where there is spike prior to food availability, and a smaller spike in activity towards the end of food availability. While this has been seen in research before (Caneijo & Araujo, 2012), it is not clear why there is a spike after food availability as well. Additionally, we can see clearly in Figure 5, as FAA continues, there is a slow decrease in night time activity. Another point of discussion is that in Figure 5, on the day of the cage change, there was an interesting impact on the animal. When cages are usually changed, animals have an increase in wheel running activity due to arousal, however, there was a phase shift in their activity that day. It could be possible that they had some light exposure during their dark phase, causing this phase shift, however, we do not know for sure.

For the 18:18 group, when looking at the actogram (Figure 6) and the activity profiles (Figure 3), it is difficult to determine whether FAA and FSA really occurred. While activity levels did surpass the threshold determined by the control group, it does not look like true FAA. This is because activity levels were elevated for a significant amount of time prior to food availability. Additionally, as we can see in Figure 4, the 18:18 group lost their entrainment and only slightly regained it. Therefore, it is fair to say that this elevated activity may be due to

disrupted entrainment to the LD cycle, causing excessive activity during the light phase as a result of free running.

For the 18:24 group, the actograms were the main tools to determine whether FAA and FSA were evident in this cohort. While this means that there was no statistical data used to determine this, we were able to do so visually by looking at different iterations of the actograms and comparing them to the threshold set by the control group. Nonetheless, it is interesting to see in Figure 7B, how strongly FAA was exhibited. Even when the animals began to free run, we can see FAA still exhibited when disregarding the free running period. This suggests that during that period of time, the FEOs were able to better entrain the animal to food availability than the SCN was able to do for the light dark cycle. This points to the idea that due to the SCN being dampened by entrainment to a LD cycle out of the circadian range, FEOs had stronger oscillations and were better able to entrain the animal. This is an interesting finding, as the interaction between the SCN and FEOs shows that FEOs can have a very strong influence, one that dominates over the SCN - the master clock.

Lastly, it is interesting to see the trend of the entrainment quotient (EQ) over the course of the experiment. The EQ are values that determine how well an animal is entrained to their respective LD cycle. When looking at Figure 4, we can see that all the animals entrained to a T18 LD cycle were very well entrained at baseline. However, during the first two weeks of time-restricted feeding their EQs worsened. This may be due to the fact that animals were moved to the new cages that attach to the feeder. However, it could also be due to the interference of TRF with their entrainment. This is difficult to determine as the cage changes, and TRF occurs simultaneously. Additionally, it is interesting that their EQs recover slight during the second half

of being in time-restricted feeding. This may be due to the fact that the animals were better acclimated to their environment and TRF, allowing them to regain their entrainment.

4.2 Limitations

In our study, we were able to draw conclusions about Food Anticipatory Activity, by defining the time period ourselves by looking at the data and seeing when mice were active and inactive based on time-restricted feeding. However, it must be noted that during all data collection, wheel blockage (because of excess bedding and nesting), or a loss of signal to the VitalView program (due to chewing of wires, or disconnection) may result in some degree of noisy data. This is because the aforementioned reasons result in a loss of signal, so no data would be collected even if mice were active. While wheel running is definitely the preferred method of monitoring activity, because it is non-invasive, alternative methods are also used in research.

In addition, another important limitation is in the interpretation of the loss of T18 entrainment. It is not known to us what may have caused this loss of entrainment. One possibility could be that due to being moved to the new cages and being attached to the feeder system, it disrupted their entrainment. This could be due to different cage size, a change in wheel type, light diffusion, or even the change in food composition. Another possibility is that there was an interference of food timing or TRF in general with their entrainment. Because the cause of loss of entrainment is unknown, it raises many questions. Would results be different if the T18 entrainment remained robust?

Additionally, the sample size of each experimental group was intended to be n = 6, and a larger sample size for the control group, n = 9. However, early on in the experiment, less than one week into time-restricted feeding, two mice died; one was part of experimental group 1 and the other was part of the control group. All mice were monitored on a daily basis, and they were

found dead with food and water available to them. This altered the sample size of the experimental group 1, n = 5, and the control group, n = 8.

Lastly, two of the animals in experimental group 2 had stiff wheels when they first moved into the new cages attached to the feeders. This lead to them having less activity, along with less frequent activity. Once the wheels were replaced, with new well functioning wheels, the mice still had a lack of activity. This may be due to them learning and associating that the wheels were stiff. Or it could be individual differences, in which these mice did not want to run. However, this once again impacts sample size as the data retrieved from them was not very telling.

4.3 Further Studies

It is possible that ketone bodies and circulating ghrelin, along with other metabolic signals, may play a role in the results that we observed. Ketone bodies provide a source of energy to the brain and body in the absence of glucose or glucose scarcity - i.e. during fasted states. Gastric ghrelin is secreted during fasted states as a signal to the brain and body that an individual is hungry. Measuring ketone body and ghrelin levels to see their impact on FAA and how they may play a role in timing the phase of FAA would be a powerful tool in understanding their roles and interaction with FEOs. They may not be direct clock signals, but they are metabolites that play an integral role in feeding. While some studies have been done on liver-derived ketone bodies and their role in food anticipation (Chavan et al., 2016), more support for the evidence is still necessary. In terms of gastric ghrelin, while studies have been done indicating that it reduces food anticipatory behavior (Blum et al., 2009; Gunapala et al., 2011), it would be interesting to see what role it plays when there are conflicting zeitgebers (light cues and feeding cues).

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