UNIVERSITY OF CALIFORNIA SAN DIEGO

Mammalian Circadian Flexibility During Extreme Entrainment and Dim Light Exposure

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Biology

by

Maksat Idris

Committee in charge:

Professor Michael Gorman, Chair
Professor Stuart Brody, Co-Chair
Professor Susan Golden
Professor Sasha Kauffman

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The Thesis of Maksat Idris is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

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ABSTRACT OF THE THESIS

Mammalian Circadian Flexibility During Extreme Entrainment and Dim Light Exposure

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Professor Michael Gorman, Chair
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Simple dim green illumination (lux < 0.05) is sufficient for mice to entrain to extreme patterns of entrainment, such as T18, T30 and activity bifurcation. Chapter 1 depicts dim light as a transient facilitator of bifurcation to T18, through a significant improvement in the resetting capabilities of the mammalian circadian system. 3 weeks or more of concurrent dim light has significantly higher measures of entrainment to T18 than an absence of dim light for 3 weeks or more. The absence of dim light significantly lowers entrainment ability of mice to T18. However, after 3 weeks of dim light at night, mice exhibited an unprecedented Type 0 strong

phase resetting after being released into constant conditions. This provides concrete evidence of increased circadian flexibility and phase resetting in mammals. Chapter 2 takes dim light into bifurcation and analyzes *Per2* mRNA expression levels in the SCN. *Per2* in the SCN of bifurcated mice cycle on a similar 24h basis as unbifurcated mice, displaying a clear unimodal pattern of expression.

GENERAL INTRODUCTION

In mammals, the suprachiasmatic nucleus (SCN) in the anterior hypothalamus is a timekeeper and synchronizer of other cells in the body. Almost every cell has an autonomous circadian clock and can express rhythms in isolation [1,2,3,4]. The SCN uses an essential environmental cue, light, in order to coordinate other clocks in the body through a variety of autonomic outputs, such as neuronal networking, humoral connections and even body temperature [2,5]. Through these pathways, the SCN can organize various physiological rhythms, such as behavior, organ function, and sleep. These rhythms operate daily, set by external environmental cues, like the sun. This is where the term "circadian" comes from, a Latin-based term which means "approximately a day".

An important aspect of circadian biology is entrainment, which is the process of aligning an internal circadian clock with its environmental rhythms, such as the 24-hour daily rhythms of the sun. An animal is well-entrained if its internal rhythms are well matched with its external rhythms. Poor entrainment, on the other hand, comes with certain health risks [10,20]. As the master clock, the SCN entrains the body through stable, self-sustained circadian oscillations that are generated by strong coupling between its neurons. As a network, the SCN reinforces cellular rhythmicity through neural pathways that create redundancy, therefore becoming a very robust network of neurons that is resistant to noise and variance in its individual neurons [2]. Because of the resilience of this network, its properties become important in an organism's ability to entrain to external cues, such as light. In other words, the rigidity of the SCN neural network sets a specific range, within which an organism can entrain to. For our purposes, a mammal's range of entrainment is between 22h - 26h [35]; anything outside of this range, a wild type mammal under normal lighting conditions would have difficulty entraining to. Additionally, these limitations to

entrainment are well correlated with higher amplitudes and tighter coupling between the SCN neurons, which stems from the neurons' abilities to communicate as a network. Theoretically, these limits can be extended by decoupling SCN neurons, thereby reducing the amplitude of the oscillatory network [6].

In the past two decades, Dr. Michael Gorman's lab at the University of California, San Diego has discovered an unprecedented role for dim light (<0.05 lux) in making the circadian system in mammals more flexible [7,8,9]. The way in which dim light accomplishes this seemingly improbable task is through its administration during the night phase, or scotophase, of an animal's daily rhythms. The color of light used in these dim light experiments was green, due to the sensitivity of the circadian system to this wavelength. By turning on this dim green light during an animal's scotophase, the animal can adjust itself to various extreme patterns of entrainment. It is interesting that prior literature concluded the inability of such low light to shift the clock in any significant way [11,12,13]. However, newer literature has illustrated that dim light was effective in extending the range of entrainment in hamsters from 24h (T24) to 19h (T19) [14] and 30h (T30) [15]. Recent work by Dr. Gorman's lab illustrated an extension of the range of entrainment of mice to 18h (T18) – LD 13:5 – simply by exposing the organism to dim light (<0.1 lux). The convention, LD 13:5 during T18, denotes a pattern of entrainment consisting of 13 hours of daytime (photophase) and 5 hours of nighttime (scotophase). This extension of entrainment was achieved by progressively shortening the photophase down from LD 19:5 (T24) to LD 13:5 (T18). Due to the presence of dim green light during the scotophase of these mice, the shortening of the photophase by 6 hours did not lead to loss of entrainment in mouse activity patterns [16]. In addition, a bifurcated LDLD schedule was also possible with the addition of dim light. LDLD, another form of extreme entrainment, is when an animal's scotophase and

photophase are equally split in half, or bifurcated, leading to two separate but equal days and nights within a 24-hour period [16,17]. These studies have shifted away from the perspective that the circadian system is not very flexible and provided a non-pharmacological, non-genetic method of doing so.

An important aspect of the circadian system is its ability to reset itself and adjust to new light-dark cycles. This plays a role for animals that are adjusting to different seasons throughout the year, animals exposed to experimental lighting conditions in the laboratory and humans that travel across the globe and experience jetlag. The SCN neural network operates through extensive neural communication and synchronization, which creates strict limitations to how much an organism's clock can be shifted in response to a light pulse [2]. Having stricter limitations and smaller phase shifts is categorized as Type 1 phase resetting, or weak resetting of the circadian clock. Mice, and other mammals, usually exhibit Type 1 resetting.

The extent of an organism's resetting ability can be increased if the amplitude of clock gene levels are reduced and the stimulus presented is increased [18,19]. LDLD bifurcated mice under dim light had more flexible phase resetting due to reduced circadian amplitudes [20]. Other species routinely exhibit Type 0 (strong) resetting, such as cyanobacteria, which are highly sensitive to its environment and can be reset very easily [21]. In contrast, mammalian circadian systems find it difficult to exhibit Type 0 phase resetting behavior. Yet, certain environmental or physiological conditions that affect this critical coupling mechanism can be used to enhance the resetting capabilities of mammals. For example, hamsters exposed to dim light and novel wheel running stimulus in LD_{im} 14:10 experienced a significant increase in the magnitude of phase delays after a 7h light pulse, as opposed to LD_{ark} 14:10 mice not receiving dim light [22]. Additionally, mice bifurcated (T30) under dim light that were released into constant conditions

showed predictive behavior of its phase onset of activity based upon its previously bifurcated entrainment state, indicating increased flexibility of its resetting capacity [17]. Another perspective extends this notion: when exposed to T18 under the presence of dim light, mice that were released into constant conditions after a 9h light pulse (positioned after the last scotophase) as opposed to mice released immediately without a 9h light pulse, experienced a phase onset of activity in constant conditions that was 8.5 hours later than the latter group of mice [16]. Behaviorally, this raises the question as to whether this is Type 0 phase resetting. In contrast, mice exposed to light pulse durations ranging from 1h to 18h under standard conditions, administered in constant darkness and without dim light, experienced a maximum phase delay of 5h and maximum phase advance of 2h, exemplifying weak Type 1 phase resetting behavior [23]. Despite this, unique cases in mammals present themselves, such as in humans, where a strong resetting stimulus can lead to Type 0 resetting [24,25]. Therefore, the current literature does not present a strong case for Type 0 strong phase resetting in wild-type mice.

The circadian system in mammals operates as a negative feedback loop that cycles in daily 24-hour rhythms. These rhythms are present in most cells of the body and are manifested in various ways, through behavioral patterns and organ functions. *Per1/2/3* and *Cry1/2* are two important genes that are activated by a CLOCK:BMAL1 heterodimer at their promoter regions during the subjective day, or when the organism believes the sun is out. The protein products of these genes dimerize (PER:CRY) and translocate back into the nucleus during the scotophase, thereby repressing their own transcription. Therefore, *Per2* has peak mRNA expression during subjective day, while low mRNA expression during subjective night, due to the effect of the negative feedback loop. This makes *Per2* a particularly good marker that can be used to measure various aspects of tissue functions. Due to its modulatory nature, certain mutations in this gene

can lead to various effects, such as increased tumor progression and increased resistance to oxidative stress [26,27]. In humans, a mutation in the *Per2* gene leads to Familial Advanced Sleep Phase Syndrome (FASPS), where those affected continually experience 3-4h advanced sleep and wake times [28]. In general, changes in entrainment status are usually mirrored by changes in *Per2* rhythmicity.

The focus of the first chapter is on dim light's involvement in extreme entrainment. The first experiment looks at how different schedules of dim light administration affect a mouse's ability to entrain to an 18h schedule, or T18. The experiment attempts to analyze T18 entrainment by observing daily wheel running activity. These recordings are composed into graphs that display activity patterns across several days, called actograms. Using actograms and other analytical methods, the effect of dim light can be quantified and analyzed. The second experiment naturally extends from the first through a well-known phase resetting experiment. The goal is to see what effect T18 and dim light will have on the flexibility of the circadian system.

The second chapter transitions from T18 to bifurcation, which is a pattern of activity that is characterized by having two equal nights within one 24h cycle. Like T18, dim light is required for successful bifurcation in hamsters and mice [8,9,29]. Unlike the T18 study, a different approach is taken to study this phenomenon: using *Per2* as a marker to study bifurcation in C57 mice. *Per2* expression will be measured in the first and second scotophase of the cycle where both the presence of dim light and split lighting condition, LDLD, will be held constant. Therefore, the difference between the two groups, bifurcated and unbifurcated, is isolated to behavior. Through this lens, a splitting of activity between the two scotophases, an obvious indicator of bifurcation, can be analyzed separately regarding *Per2* rhythms in the SCN. In this way, variations in *Per2*

expression between the bifurcated and control conditions can be directly compared with behavioral bifurcation, in order to analyze how the SCN changes in response to an animal becoming bifurcated.

GENERAL METHODS AND MATERIALS

Animals

Male C57Bl/6J mice, aged 3-4 weeks in Chapter 1 and 5-6 weeks in Chapter 2, were ordered from Jackson Labs (Sacramento, CA). Upon receipt, mice were given food (Mouse Diet 5015; Purina, St. Louis, MO) and water ad libitum. At the beginning of the experiment, mice were weighed and housed in clear plastic cage boxes (length x width x height = 28 x 18 x 15 cm), with a running wheel attached (diameter = 13 cm). Cages were placed in large, light-tight chambers containing two sources of light: (1) white fluorescent lamps (300-350 lux) along the back wall during the photophases to represent daylight and (2) green LEDs (<0.01 lux) secured at the level of the cages during the scotophases (nighttime) to represent dim light. The University of California San Diego Institutional Animal Care and Use Committee approved these experiments.

Data Collection

Actograms were constructed from measurements of activity (wheel revolutions) from mice in each chamber. The activity rhythms were recorded using 2 magnets attached to the running wheel in each cage, with each half revolution producing 1 count stored in 6-minute bins (Vitalview Software v 4.2, Mini-mitter, Bend OR). Every 3-4 weeks, cage changes were performed during the photophase, with minimal disruption of the circadian clock.

CHAPTER 1: DIM LIGHT, T18 AND PHASE RESETTING

Introduction

To understand dim light, it is important to consider the critical elements of timing involved that give dim light its unique abilities. Prior studies have looked at dim light's involvement in extreme entrainment. Specifically, it has been established that dim light is necessary for bifurcation to occur in two hamster species and mice [29]. This combination of dim light and bifurcation permitted both animals the flexibility to entrain to 18h or 30h days, which is outside the previously described range of entrainment [17,30]. Yet, it was shown that continued dim light was not necessary for mice to entrain to T30 when they were previously induced into bifurcation with dim light at T24 LDLD. The same study showed that continued dim light was not sufficient for T30 LD entrainment when transitioned from T24 LD because of visible free-running components during the photophase [17]. On the other hand, T18 LD entrainment was possible in three separate scenarios: (1) dim light throughout the transition from T24 LD to T18 LD, (2) dim light throughout the transition from T24 LDLD to T18 LD, and (3) dim light discontinued after transitioning from T24 LDLD to T18 LD [16]. However, the study did not execute a scenario where dim light was discontinued when transitioning from T24 LD to T18 LD. Overall, the literature paints dim light as a necessity for both bifurcation induction and T18 entrainment, and shows that a history of inductive bifurcation is sufficient for T30 entrainment without dim light. Our hypothesis is that dim light would be more effective with its presence than its absence, and that a history of dim light would be sufficient for T18 entrainment. The first experiment explores certain variations in the timing and duration of dim light. The results show that dim light seems to have a transient effect on T18 entrainment.

Dim light has implications for increased resetting capabilities in rodents. When bifurcation is induced with dim light, there is a reduction in amplitude of important clock genes in the SCN and peripheral tissues. Faster resetting capabilities is a likely consequence of dim light bifurcation [20]. Additionally, bifurcation with dim light allows much faster re-entrainment to simulated time zone travel in hamsters, reducing a measure of jetlag by 71% [31]. Mice entrained to T18 LD had their phases advanced by 8.5h after exposure to a 9h light pulse at the beginning of release into constant conditions [16]. Although these findings point to a seemingly stronger resetting ability in the presence of dim light, there is still a lack of concrete evidence that mammals can experience Type 0 strong phase resetting when exposed to dim light, whether they are bifurcated or exposed to an extreme T cycle, like T18 or T30. Thus, the hypothesis of Experiment 2 is that mice will exhibit Type 1 resetting, typical of mammals. However, the findings indicate Type 0 strong phase resetting, and support the literature that dim light reduces the amplitude of circadian rhythms, thereby allowing more circadian flexibility.

Methods and Materials

Experiment 1

	Baseline T24			Phase 1 T18			Phase 2 T18
	week 1	week 2	week 3	week 4	week 5	week 6	week 7 & 8
$L:D_{im}$							
$L:D_{ark}$							
Pre-3							
Pre-1							
Post-3							
Post-1							

Figure 1. Framework for Testing Dim Light Effects
This figure represents the weekly schedule of dim light across 6 groups of mice (n=12, male). There is a distinct transition from T24 to T18 between week 3 and week 4, which characterizes the nomenclature of the four experimental groups. The last 2 weeks in each phase (weeks 2/3, 4/5, and 7/8) were analyzed using Student's T Test ($p<0.05$).

In order to understand when dim light has its facilitatory effects and how much is necessary for T18 entrainment, experimental conditions were set up in a way to allow a variation of dim light treatments in the 1 to 3 weeks before (Pre) or after (Post) the switch from T24 to T18. Specifically, six groups of mice (n=12, male, aged 3-4 weeks) were taken from the colony room (LD 14:10) and were assigned to four experimental conditions: Pre-3, Pre-1, Post-3, and Post-1. Two control groups were also added: (1) one with dim light in perpetuity (L:D_{im}), positive control) and (2) another completely without dim light (L:D_{ark}, negative control). The experiment was divided into Baseline (LD 14:10, 21 cycles), Phase 1 (LD 13:5, 28 cycles) and Phase 2 (LD: 13:5, 18 cycles). According to **Figure 1**, Groups Pre-1 and Pre-3 received dim light only during Baseline, before the T18 transition. Groups Post-1 and Post-3 received dim light only during Phase 1 after the T18 transition. At the end of Baseline + Phase 1 (6 weeks in total), all four experimental groups entered Phase 2 (2 weeks), in order to analyze the after effects of the administered dim light treatments among Pre-3, Pre-1, Post-3 and Post-1.

Experiment 2

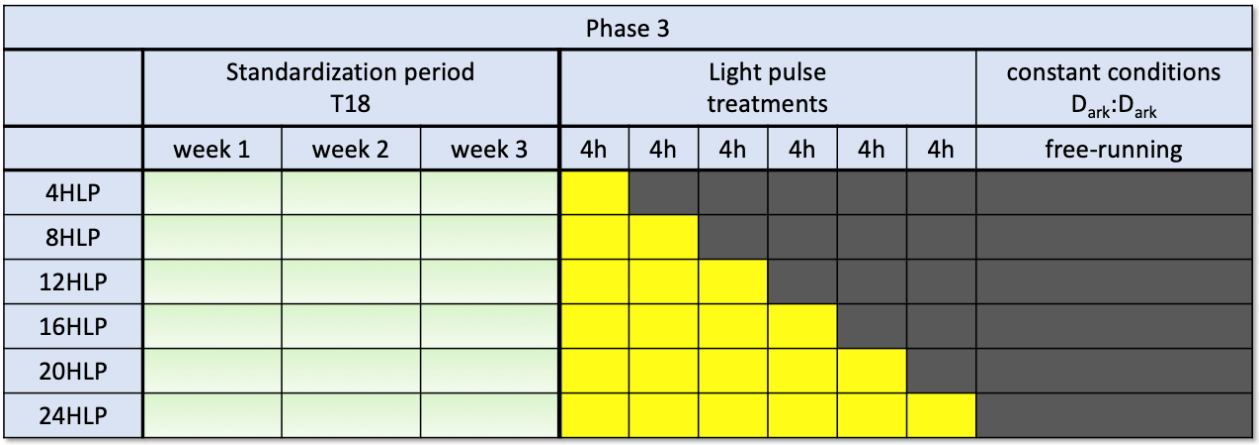


Figure 2. Framework for Testing Phase Resetting
Phase 3 was divided into 3 parts. All mice were standardized with dim light exposure and sequentially exposed to 6 different light pulse durations, meant to reset the mice's circadian system. Finally, all groups were released into constant conditions to quantify the extent of resetting achieved by the groups of mice.

The second experiment encompassed Phase 3: resetting the clock with varying light pulse durations. According to **Figure 2**, all the mice from Phase 2 were randomly reassigned into 6 groups and were exposed to 28 cycles of dim light to standardize the effect of dim light on every animal. Upon completion of this step, each group was exposed to a unique duration of light immediately after their last scotophase. 4h, 8h, 12h, 16h, 20h, and 24h light pulse durations were assigned to each group, after which all groups were released into constant conditions ($D_{ark}D_{ark}$, or DD) for 18 cycles. Wheel running was continuously recorded to assess each individual phase onset of activity. **Table 1** summarizes all the lighting schedules implemented in this study.

Table 1. Lighting Schedules

	Light Condition	T	No. of Cycles
Baseline	$L:D_{im}$ 14:10	24 h	21
Phase 1	$L:D_{im}$ 13:5	18 h	28
Phase 2	$L:D_{ark}$ 13:5	18 h	18
Phase 3 Standardization	$L:D_{im}$ 13:5	18 h	28
Phase 3 Light pulse treatments	4-24 h	-	1
Phase 3 Constant Conditions	$D_{ark}:D_{ark}$	-	14

Data Collection and Analysis

Entrainment quotients

To quantify entrainment of mice to a short T18 lighting cycle, a previously described entrainment quotient was used [16]. Lomb-Scargle periodograms were generated using 14-day intervals through ClockLab (Actimectrics, Evanston, IL) and two critical values were selected: power at T18 and peak power in the 22-26 h circadian range. Entrainment quotients were calculated based on these values:

$$EQ = \frac{power_{18h}}{power_{18h} + power_{24h}}$$

Values were generated from 0 to 1. If values approach 1, then the behavioral rhythm closely matches the period of the external T18 lighting cycle. If values approach 0, then the behavioral rhythm matches a free-running pattern and is not well entrained.

Percent activity in light (PAL)

To further quantify the degree of entrainment to T18, periodogram values were used:

$$\%\ activity\ in\ light = \frac{total\ activity\ during\ photophase}{total\ activity\ in\ both\ photophase\ and\ scotophase} \times 100$$

A smaller percentage of activity correlated with better entrainment, whereas larger percentages exemplified the opposite.

Onset of free-running phase in constant conditions ($D_{ark}D_{ark}$)

To determine the phase onset of activity of free-running rhythms in constant conditions, onsets were eye-fitted using ClockLab for 10 consecutive days, with the first 3-4 days skipped due to variability as the phase adjusts and stabilizes after release into constant conditions. The subsequent 10 days were selected and used to construct a least squares regression line fitted to the phase onsets of activity. This fitted line was used to project the phase of activity back to the first day of release into constant conditions, after the last scotophase. **Figure 5A** is an example of this process.

Results

Experiment 1: Various dim light treatments and its effects on entrainment

Baseline (LD 14:10, T24)

In this preparatory stage, five out of six total groups experienced similar PAL means when comparing them as independent sample means. L:D_{ark} had significantly higher levels of activity in the light (PAL) than the other five groups. A One-Way ANOVA between all six

groups yielded an F value of 3.31, $p<0.05$, supporting the Student's T conclusion that there is more variation than expected from the null hypothesis.

Table 2. Average EQ values

	Baseline	Phase 1	Phase 2
L:Dim	-	0.95 ± 0.01	0.98 ± 0.01
L:Dark	-	0.77 ± 0.07	0.82 ± 0.07
Pre-3	-	0.77 ± 0.07	0.80 ± 0.08
Pre-1	-	0.90 ± 0.02	0.85 ± 0.05
Post-3	-	0.94 ± 0.02	0.90 ± 0.04
Post-1	-	0.88 ± 0.04	0.82 ± 0.08

Table 3. Average PAL values

	Baseline	Phase 1	Phase 2
L:Dim	2.7 ± 0.6	7.9 ± 2.2	5.6 ± 1.4
L:Dark	10.2 ± 3.3	25.1 ± 5.7	21.1 ± 5.0
Pre-3	2.4 ± 0.6	23.9 ± 4.6	25.4 ± 4.7
Pre-1	4.4 ± 1.1	13.2 ± 3.2	14.2 ± 3.4
Post-3	5.0 ± 1.2	7.7 ± 2.8	13.3 ± 3.6
Post-1	3.8 ± 0.7	16.2 ± 3.8	17.3 ± 4.4

Phase 1 (LD 13:5, T18)

Mice with a constant supply of dim light (positive control, L:D_{im}), showed significantly higher EQ scores than mice in complete darkness (negative control, L:D_{ark}). The difference

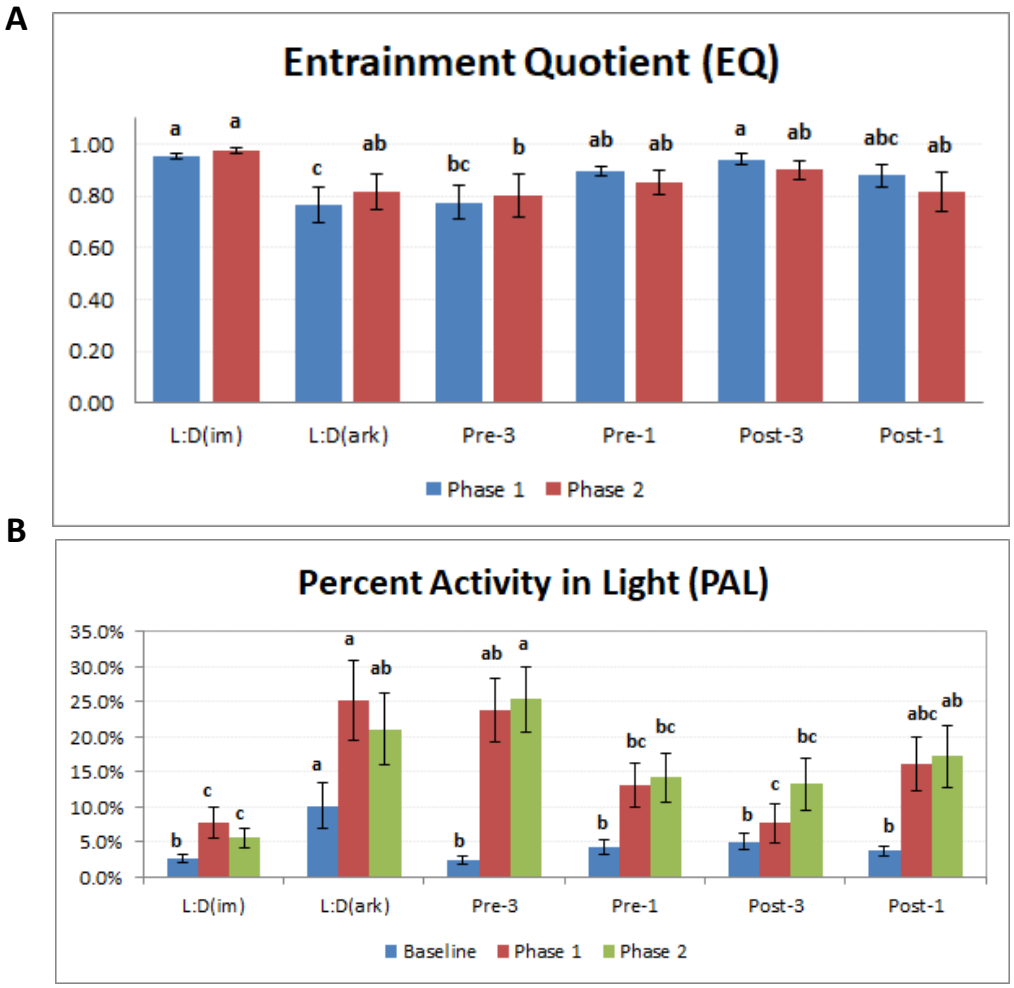


Figure 3. Quantitative Analysis of Entrainment to T24 and T18
(a) Entrainment quotient (EQ) values represent the degree of entrainment experienced under T18, as previously described. As EQ values approach 1, entrainment to T18 gets quantitatively better. (**b**) Percent activity in light (PAL) is another measure of entrainment ability, except this can also be applied to both T24 and T18. A higher percentage of activity during the photophase denotes poor entrainment, and vice versa. Significant differences are based on Student's T-Test all pairs comparisons; differences are represented as letters above each bar.

between L:D_{im} and L:D_{ark} is indicative of having good control groups and becomes visually clear by looking at the actograms in **Figure 4A and 4B**, L:D_{im} and L:D_{ark} respectively. Minimal

activity during the photophase (PAL) and activity maintained within the scotophase (EQ) can be seen in L:D_{im}, whereas L:D_{ark} has evident free-running rhythm during the photophase that slowly drifts to the right, similar to its activity patterns during T24 Baseline. Its activity is also not sustained within the confines of the scotophase, suggesting more power within the 24h range and a lower overall EQ score.

Those with only 1 week of Baseline dim light exposure (Pre-1) had similar EQ scores as L:D_{im} and Pre-3, but not L:D_{ark}. Evidently, Pre-3 and L:D_{ark} were similarly entrained, and this similarity can be visualized by looking at their respective actograms in **Figure 4B** and **4C**, and seeing significantly more activity during the photophase than **4A** and **4D**. Both EQ and PAL scores exhibited similar statistical outcomes for these relationships.

For groups Post-3 and Post-1, the timing of dim light occurred during Phase 1 instead of Baseline. In other words, dim light was administered immediately upon induction to T18, whereas with Pre-3 and Pre-1, dim light was administered immediately prior to T18 induction (**Figure 1**). When mice were given dim light during the first week of Phase 1 (Post-1), there was no difference in T18 entrainment compared to either controls. However, those mice given a constant supply of dim light during Phase 1 (Post-3) were significantly different from L:D_{ark} mice, and statistically similar to both L:D_{im} and Post-1 (EQ and PAL). These differences can be seen by looking at a representative Post-3 actogram (**Figure 4D**) and contrasting it to L:D_{ark} (**Figure 4B**) and L:D_{im} (**Figure 4A**). The actogram for Post-3 has minimal PAL and seemingly good entrainment (EQ), which is mirrored by the actogram for L:D_{im}. However, a stark difference in activity within the photophase is evident when comparing Post-3 with L:D_{ark}.

When comparing Pre-3 to Post-3, the main difference is whether mice received dim light before or after T18 induction. Both groups have significant differences in both PAL and EQ

scores, showing that dim light presence is better for T18 entrainment than its absence. These differences can also be visualized when comparing **Figure 4C** to **4D**, where Pre-3 (**4C**) has significantly more activity and visible free-running components within the photophase than Post-3 (**4D**). However, the timing of dim light - before or after T18 induction - does not seem to play much of a role when administering only 1 week of dim light (Post-1 or Pre-1), as EQ and PAL scores are both very similar.

One-way ANOVA results support the Student's T test results for Phase 1, showing a significant F value that suggests large variations in EQ and PAL values in response to the experimental dim lighting conditions.

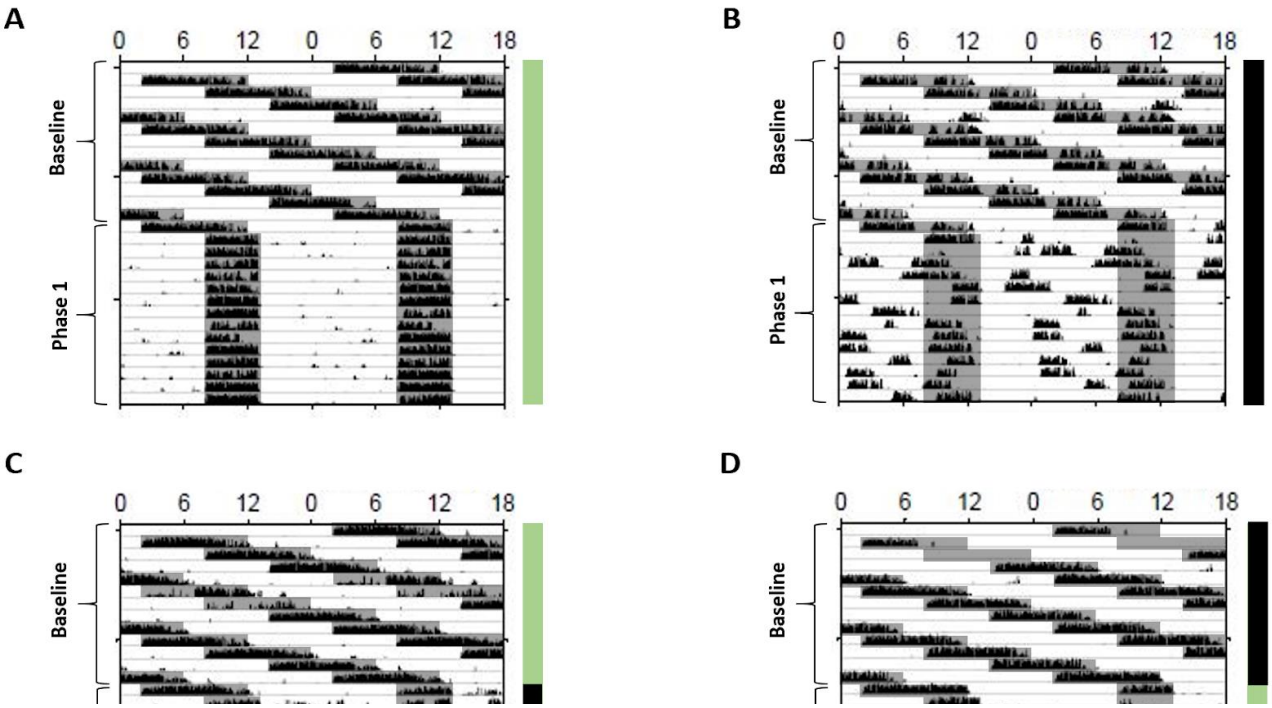


Figure 4. Representative Actograms for L:D_{im}, L:D_{ark}, Pre-3, and Post-3
All four actograms span the transition from T24 (Baseline) to T18 (Phase 1). Colored bars on the right illustrate lighting conditions: green depicts dim light and black depicts dark/no dim light. The L:D_{im} **(a)** and Post-3 **(d)** actograms show minimal PAL, as seen by very few and small bits of activity during the photophase. Although both actograms are well-entrained during Baseline T24, both L:D_{ark} **(b)** and Pre-3 **(c)** mice have decreased EQ scores and increased PAL scores upon T18 induction. Evident free-running rhythms persist during the photophases of these actograms, further indicating higher PAL scores. All actograms are available in Supplementary Figures S1-S6.

Phase 2 (LD 13:5, T18)

During Phase 2, L:D_{im}, L:D_{ark}, Pre-3, Pre-1 all experienced a continuation of their respective treatments from Phase 1 (**Figure 1**). However, there was a rise in EQ for L:D_{ark} (thus making it similar to L:D_{im} ($p>0.05$)) and a consequent decrease in PAL for L:D_{im} (yet difference from L:D_{im} was maintained ($p<0.05$)).

After the additional 2 dark weeks in Phase 2, Pre-3 and Pre-1 showed slight increases in PAL, while only Pre-1 consequently decreased in EQ. This led to non-significance between the means of L:D_{ark} and both Pre-1 and Pre-3 (EQ and PAL, $p>0.05$). Besides a novel statistical difference between Pre-1 and Pre-3 in Phase 2 for PAL, the rest of the statistical relationships were maintained from Phase 1.

When comparing Post-3 and Post-1 during Phase 2, an additional two weeks of absent dim light created two differences in statistical relationships from Phase 1: Post-3 and L:D_{ark} means became similar across EQ and PAL estimates, while Post-1 PAL vs L:D_{im} PAL means became significantly different. The elimination of a significant difference between Post-3 and L:D_{ark} in Phase 1 into Phase 2 further shows that the absence of dim light leads to a decrease in overall entrainment.

Although PAL scores remained statistically significant in Phase 2 between Pre-3 and Post-3, their EQ scores did not. It can be concluded as further evidence that in the absence of dim light, a measure of entrainment (EQ) decreases, even though PAL scores remained marginally significant. Just like in Phase 1, Post-1 and Pre-1 again failed to realize any meaningful statistical analysis, which serves to suggest that having dim light for a week does not provide enough of an effect to warrant any significant differences in T18 entrainment.

Similarly to Baseline and Phase 1, the One-Way ANOVA results were significant and supported the results from the Student's T test comparisons.

Experiment 2: Type 0 strong phase resetting

Mice from *Experiment 1* were randomized into six novel groups (n=12). One mouse in group 24HLP died. After three weeks of dim light at night for all six groups, subsequent

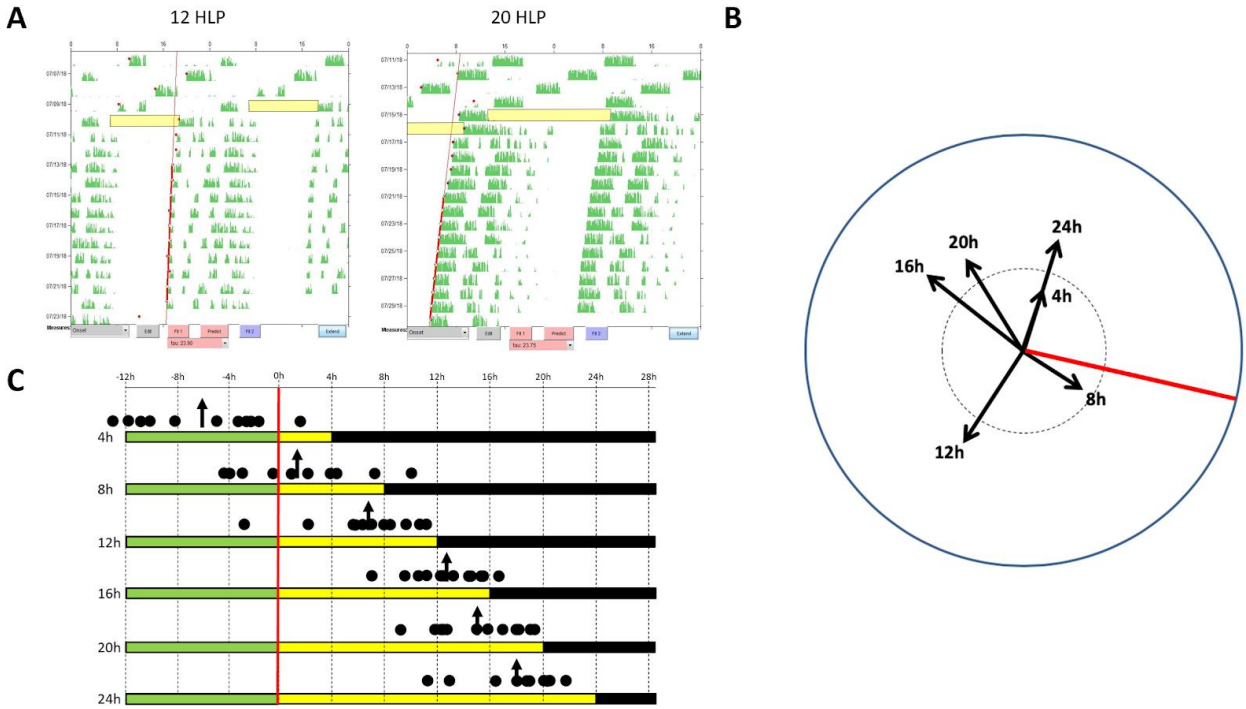


Figure 5. Type 0 Strong Phase Resetting by Six Light Pulse Durations
(a) Representative actograms from the 12h and 20h light pulse duration groups depict how phase onset values were obtained to predict the phase of activity upon release into constant conditions. Yellow bars represent each respective light pulse duration. All six light pulse groups were plotted **(b)** on a circular graph. Dark vectors represent the average phase onset of each group. The magnitude of the dark vectors illustrates clustering, while significance is indicated by the dotted circle ($p<0.05$). The red line represents the end of the last scotophase, as well as the beginning of the light pulse for each group **(c)** All groups have been re-plotted on a linear graph with black dots representing individual mice and the red line corresponding to the beginning of the light pulse, as in the circular graph in (b). The yellow bars represent the length of each respective light pulse duration and each group's upright bold arrow represents the mean clustering, which mirrors the mean vectors from the circular graph in (b). The green bar before each light pulse indicates the prior standardization period for all mice under dim light, which also includes the last scotophase before release into DD.

exposure to various dim light durations and release into constant darkness, the data in **Figure 5** was obtained.

For the group 4HLP, mice began free-running after being exposed to a 4h light pulse. The mean projected phase of the first onset in constant conditions (DD) was approximately 6h earlier than the beginning of the 4h light pulse. This can be seen by the mean vector being about 6 hours before the 4h light pulse in **Figure 5B** and **5C**, from both a circular and linear perspective. Although there was a trend in projected phase onsets of mice in the group, the clustering was not significant. The same statistical outcome - trend but no significant clustering - was obtained for 8HLP. After release into DD, the 8HLP group had a mean projected phase of first onset in DD that was ~1.5h later than the beginning of the 8h light pulse.

12HLP mice were released into DD and significantly clustered about 7h after the beginning of the 12h light pulse. A representative actogram of a mouse from this group is depicted in **Figure 5A**. The yellow bar indicates the light pulse, and it is evident that the mouse begins its first phase onset of activity right around the end of the 12h light pulse. The 16HLP mice were significantly clustered around 13h after the beginning of their 16h light pulse, 20HLP mice were significantly clustered around 14.5h after the beginning of their 20h light pulse (representative actogram in **Figure 5A**), and 24HLP mice were significantly clustered around 18h after the beginning of their 24h light pulse. (**Figure 5B**). There is a general trend in the projected phase onsets in DD, in that the longer the light pulse duration, the further away the projected phase of first onsets in DD are. This is indicative of strong, Type 0 phase resetting.

Discussion

The results from Experiment 1 provide statistical evidence that dim light has a transient effect on T18 entrainment. In other words, dim light exerts its effects when it is present, and

these effects wear off in its absence. From a bird's eye view, L:D_{im} always had dim light and L:D_{ark} never had dim light. It should logically follow that L:D_{im} would consistently have the highest EQ scores and one of the lowest PAL scores and L:D_{ark} would have one of the lowest EQ scores and one of the highest PAL scores. This was the final outcome and provided new insight into the characteristics of dim light.

Prior experiments have concluded the necessity of dim light for T18 entrainment [16]. These results extend the findings of this study because not only does dim light need to be present at all times for successful T18 entrainment, as also seen in L:D_{im} vs L:D_{ark}, but a history of dim light alone is not sufficient for such extreme entrainment as T18. The latter claim is supported by the results from Phase 2 when looking at Pre-3 versus Post-3. When using EQ and PAL data from weeks 7 and 8 of Phase 2 (**Figure 1**), it was evident that Pre-3 has not had dim light since before T18 induction, while Post-3 has had consistent dim light since T18 induction. Therefore, the only difference between the two groups was whether or not dim light was present during weeks 7 and 8. Additionally, both groups had similar means as the positive control group (L:D_{im}) during Baseline (T24), which meant that both groups were well entrained before T18. However, only Pre-3 had significantly decreased EQ scores and increased PAL scores relative to the positive control group. Post-3 maintained strong EQ and PAL scores during Phase 2 due to the presence of dim light. Together, these results provide new evidence that a history of dim light alone is not sufficient for full entrainment to T18, and they support prior literature that dim light is necessary for T18 entrainment [16].

One aspect of Experiment 1 that could be improved for future research is phase duration, studied at 3 weeks or longer. Results from Pre-1 and Post-1 provide insight as to why. While Pre-3 and Post-3 provided meaningful statistical results, Pre-1 and Post-1 had EQ and PAL

scores that were non-significant and did not reveal meaningful information about dim light. It seems that one week of dim light is not enough to provide any significantly better entrainment to T18 than a dark night would. Therefore future research should focus on executing experimental methods that look at 3 weeks or more, to better understand how the timing of dim light plays a role in extreme entrainment. Furthermore, it is interesting that when mice were exposed to 4 weeks of dim light before entrainment to T30, dim light was not sufficient [17]. However, 3 weeks of dim light before entrainment to T18 was sufficient [17]. There might be precedent to examine the unique effects of having 3 weeks of dim light versus 4 weeks of dim light in entrainment to either T18 or T30, separately.

It has been theorized that reducing the amplitude of the SCN oscillatory network may allow an extension of the entrainment range set by the SCN neural network [6]. By the same token, when inducing bifurcation with the necessary dim light treatment, there is a reduction in the amplitude of the circadian network [20]. Consequently, the current study confirms that dim light is necessary for extending the range of entrainment to T18. By connecting the dots, it can be posited that dim light reduces the amplitude of the SCN network, thereby allowing extreme entrainment, like T18 and bifurcation [16.17]. Furthermore, a reduction in the amplitude most likely leads to faster resetting of the mammalian circadian clock [20]. This has been shown by T18 entrained mice that were exposed to a 9h light pulse and were phase shifted by 8.5h [16]. Experiment 2 provides substantial evidence that T18 entrainment with dim light possibly reduces the circadian amplitude by increasing the resetting capabilities of the mammalian circadian system from Type 1 to Type 0. Simply by looking at **Figure 5A**, it becomes clear that the mice depicted in the actograms have reset their phase to follow the end of their respective light pulse, 12h or 20h. This contrasts with a previous study that showed maximum phase delays of 5h and

phase advances of 2h when mice under standard conditions - no dim light, no T18 entrainment - were exposed to light pulse durations varying from 1h to 18h. These results indicated typical Type 1 phase resetting seen in mammals in the literature [23]. However, the current study proposes that dim light increases the flexibility of the mammalian circadian system from Type 1 to Type 0, likely from reducing the amplitude of the SCN network. This provides fuel for an array of experiments involving dim light and Type 0 resetting, in attempts to explore the internal clock components as well as other extreme schedules that can be facilitated by dim light.

CHAPTER 2: UNIMODAL *PER2* RHYTHMS IN SCN OF BIFURCATED MICE

Introduction

Bifurcation is a bona fide entrainment state facilitated by the presence of dim light [29]. This splitting of activity has been extensively analyzed, dating back to theories from Pittendrigh and Daan about two separate oscillators being responsible for such phenomena. Several postulations have been made about separate and distinct oscillators within the SCN neural network that have been derived from Pittendrigh and Daan. Such theories describe two separate oscillators that couple together when under an LD cycle, and decouple when exposed to an LDLD cycle [21,26]. These studies postulate that mice have two oscillators, dorsomedial-like (DM-like, or shell) and ventrolateral-like (VL-like, or core) oscillators, that operate in anti-phase, with higher *mPer1* expression in the DM-like during the first scotophase and higher *mPer1* expression in the VL-like during the second scotophase, therefore exhibiting overall bimodality in *mPer1* expression [32]. There have also been noted differences in PER1 protein expression rhythms in core versus shell regions of the SCN of hamsters [33]. These studies have provided a strong background for the multi-oscillator theory and have focused specifically on subsections of the SCN to locate these oscillators. However, a key step is missing that the present study provides:

disentangling the independent effects of a bifurcated pattern of activity and the external lighting condition, LDLD. This study takes a step back and measures *Per2* mRNA levels of the entire SCN, bifurcated or unbifurcated. Both groups are treated with dim light and placed under 4:8:4:8 LDLD. Therefore, the only visible difference between the two groups is whether mice are behaviorally bifurcated or not. By isolating bifurcation status in this way, direct variations in SCN rhythmicity as a result of bifurcation status can be analyzed. The hypothesis, based on the current literature, was that the SCN would show bimodal *Per2* rhythms in bifurcated mice and unimodal *Per2* rhythms in unbifurcated mice. However, there was significantly higher *Per2* expression in the second scotophase for both groups, indicating a 24h unimodal rhythm in the SCN, regardless of bifurcation state.

Methods and Materials

Behavioral Bifurcation

Table 4. Bifurcation Timeline

	Phase 1 (4 weeks)	Phase 2 (>4 weeks)
Bifurcated (L:D:L:D)	8:4:8:4	4:8:4:8
Non-Bifurcated (L:D)	16:8 (week 1)	
(L:D:L:D)	4:8:4:8 (week 2,3,4)	4:8:4:8

The aim of this study was to compare levels of expression between a bifurcated group of mice to a non-bifurcated, or control, group of mice. Mice were aged 5-6 weeks (n=52). The challenge was to figure out a way to create a good control group that differed from the experimental group by only one variable: bifurcation. A recent bifurcation study investigated various LDLD schedules that can maintain bifurcation and found that 4h and 8h photophases had the worst and best bifurcation, respectively. However, a history of successful bifurcation with an

8h photophase is sufficient for bifurcation with a 4h photophase [34]. In other words, mice with a history of 8:4:8:4 bifurcation were successfully able to stay bifurcated when switched to 4:8:4:8. The goal of Phase 1 of the experiment was to set up a good control group that remained unbifurcated in 4:8:4:8 and an experimental group that remained bifurcated in 4:8:4:8, thereby limiting the number of variables. To accomplish this, the experimental bifurcated group was subjected to four weeks of 8:4:8:4 (Phase 1) in order to maintain stable bifurcation in 4:8:4:8 (Phase 2), while the control unbifurcated group was subjected to one week of (L:D) 16:8 and then 4:8:4:8 for the rest of Phase 1 and Phase 2, to remain unbifurcated. **Table 3** summarizes this schedule. All groups were given dim light during both phases of the experiment. Out of 28 mice in the bifurcated group, 18 mice had splitting of activity between scotophases and were chosen for further analysis. Out of 24 mice in the unbifurcated group, 14 were chosen with activity isolated to only the second scotophase of the cycle.

Tissue Collection

After several weeks in Phase 2, the most well-bifurcated and most un-bifurcated mice were selected from each group and proceeded with tissue collection and genetic analysis. Brains were collected from each animal and immediately frozen on dry ice and stored in a -80C freezer. Dr. Alexander Kauffman, School of Medicine, kindly allowed the use of his cryostat to slice extracted brains into 20 micrometer coronal slices spanning the entire SCN region and mount them onto SuperFrost Plus slides stored in -80C freezer.

Single-Label *in situ* Hybridization

Upon completion of work with the cryostat, SuperFrost Plus slides with brain tissue were subjected to single-label *in situ* hybridization (ISH) using RNA probes for *Per2* and *BMAL1*. Upon removal from the -80C freezer, slides were racked and lightly thawed for about 15 seconds

before being fixed in cold 4% paraformaldehyde for 15 minutes. The slides were then washed with two separate 5 minute 1X phosphate buffer treatments and dipped in Milli-Q water and 1X triethanolamine (TEA) sequentially. This was followed by a 10 minute acetic anhydride treatment (500 mL 1X TEA + 625 microL acetic anhydride) and then washed in 2X saline-sodium citrate (SSC) for 3 minutes. The next three steps involved ethanol (EtOH) dehydration in concentrations of 70%, 95% and 100% respectively, followed by a 5 minute chloroform wash and two final sequential 3 minute EtOH dehydration steps of 100% and 95%. Slides were left to air dry for 90 minutes before applying 100 μL of either the *Per2* or the *BMAL1* probe-tRNA mix to each individual slide, coverslipping and allowing overnight hybridization in a 55C humidity chamber. The probe-tRNA mix was created by adding 0.4 pmol/mL *Per2* or *BMAL1* probe to tRNA, denaturing it, and adding it to a hybridization buffer. The following day consisted of removing the coverslips and washing the slides in two separate 15 minute 4X SSC tubs followed by 30 minutes of RNase A treatment at 37C and 30 minutes in an RNase buffer at 37C. Then, at room temperature, the slides were washed in 2X SSC for 30 minutes and moved to a shaking 62C hot bath for 60 minutes in 0.1X SSC wash. The washes concluded with three, 3 minute EtOH dehydration steps of 50%, 85% and 100%, in order. Slides were left to air dry for 90 minutes, before being dipped in Kodak NTB emulsion and left to air dry for another 90 minutes. Finally, slides were packed and stored at 4C to be developed after 12-15 days.

After fixing and developing the slides, levels of gene expression were measured using a computer-assisted microscope and a counting software (Grains; Dr. Don Clifton, University of Washington) in Dr. Kauffman's lab. The software automatically detected silver grains against a dark field microscope, thereby counting the number of *Per2* or *BMAL1* cells in the SCN relative to background cells. This method separated those mice that had higher levels of expression from

those that had lower, or indistinguishable, levels of expression. Dr. Clifton's software output was in the form of GPC's, which represented absolute levels of expression in each SCN slice. The higher the GPC count, the more *Per2* was expressed in that particular coronal SCN slice.

Data Collection and Analysis

Bifurcation Symmetry Index (BSI)

A bifurcation symmetry index (BSI) quantifies the degree to which an organism evenly splits its activity between two scotophases within one 24-hr cycle. Averaged over 14 days, the activity in the scotophase with the least amount is doubled and consequently divided by the total activity over 24 hours.

$$BSI_{14} = \frac{(average\ of\ daily\ activity\ in\ least\ active\ scotophase) \times 2}{total\ activity\ over\ 24\ hours}$$

A score of 0 indicates non-bifurcation; as the BSI value approaches 1, the animal exhibits a more equal splitting of activity between two scotophases within one 24-hr cycle. This provides a quantitative way of analyzing the level of activity bifurcation an organism is experiencing.

Results

Figure 6 displays the behavioral differences between bifurcated and unbifurcated groups. These actograms illustrate the division of activity between two scotophases within a 24-hour cycle. This division of activity is quantified through a Bifurcation Symmetry Index (BSI). The bifurcated group does split its activity between the scotophases, but it does so unequally, as does the rest of this group (**Figure 6A**). An equal splitting of activity bouts would produce a BSI value of 1. Yet, the bifurcated group has an average BSI of 0.48 (**Figure 6C**), which quantitatively supports the actogram in **Figure 6A**. On the other hand, the average BSI of the

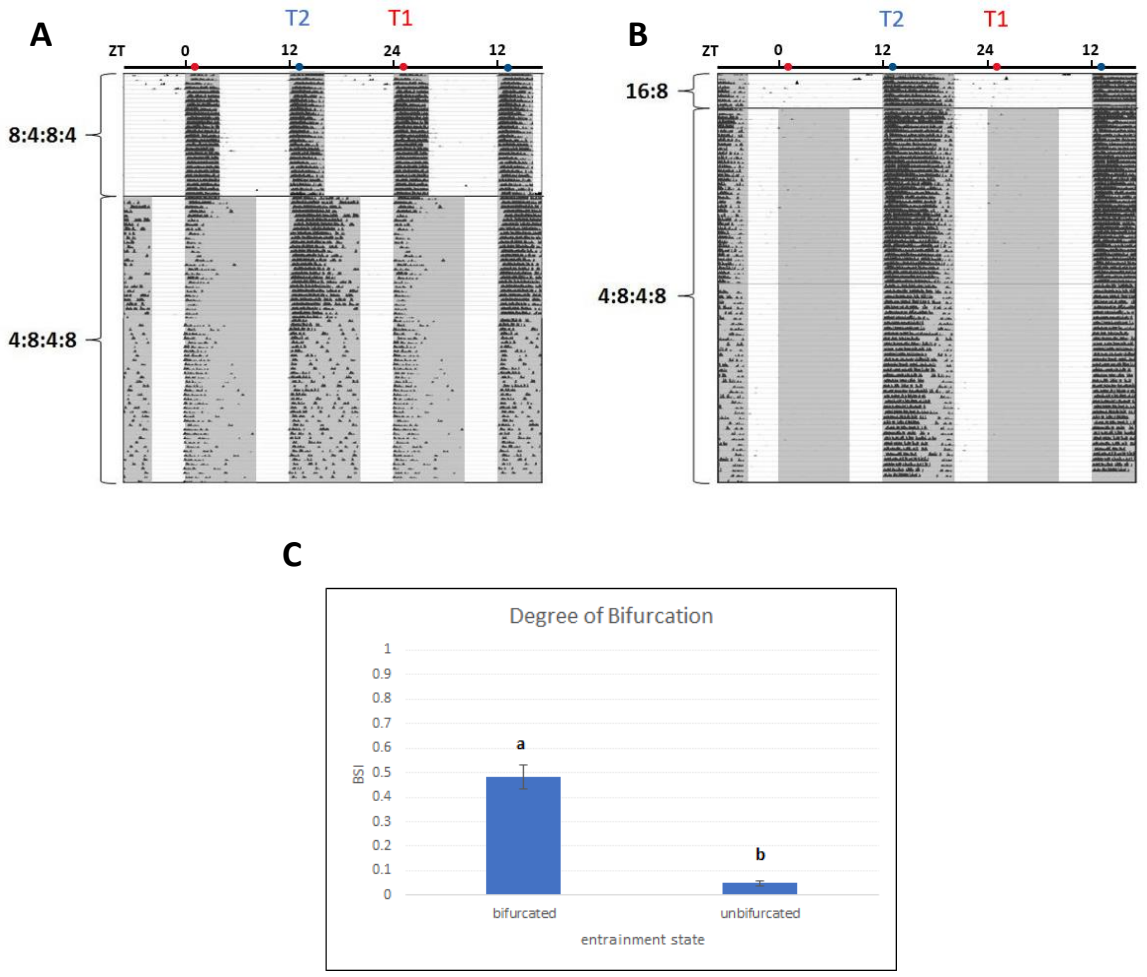


Figure 6. Distinguishing bifurcation Between Experimental and Control Groups
Based on **Table 3**, two distinct groups of mice differed only on their bifurcation abilities, not on other factors, such as lighting schedule. (**a**) Bifurcated mice had 4 weeks of LDLD 8:4:8:4 to allow bifurcation and then transferred to LDLD 4:8:4:8, while (**b**) unbifurcated mice were initially given 1 week of LD 16:8, then kept in 4:8:4:8 for the rest of the experiment. The graph (**c**) displays bifurcation symmetry index (BSI) values, with letters representing significance ($t(30) = -7.72$, $p<0.05$).

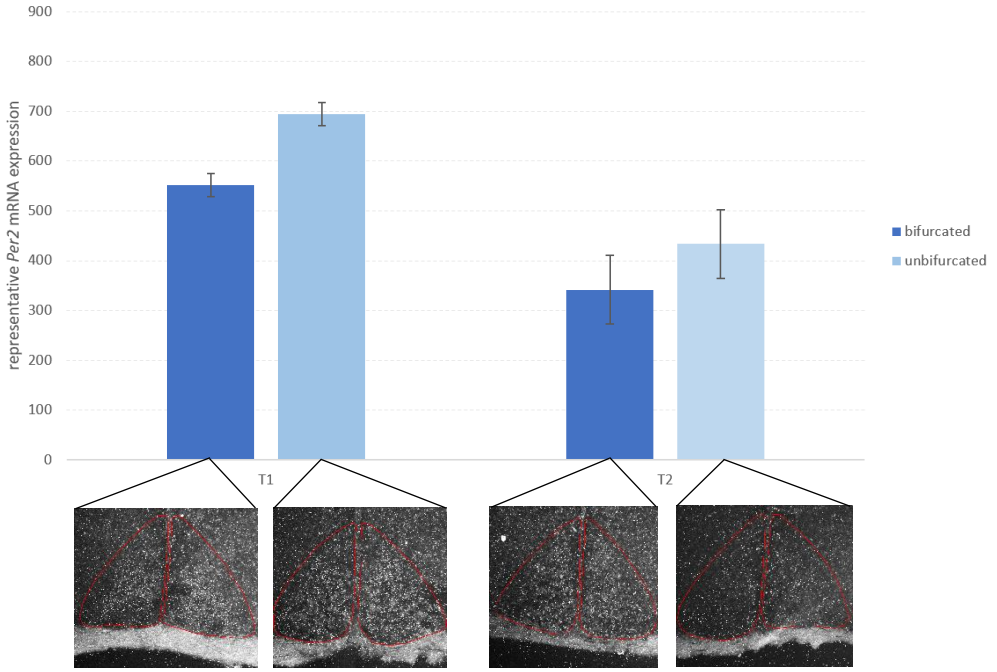
unbifurcated group is 0.05, which is represented by **Figure 5B**, where activity is localized to only one scotophase.

Having a bifurcated activity pattern is an interesting result from dim light and LDLD manipulations (see Methods and Materials). However, *Per2* was used as a marker to further study bifurcation beyond the physical changes in activity. **Figure 7A** is a graphical

representations of *Per2* mRNA expression at T1 and T2 in the bifurcated and unbifurcated groups. The actual values are GPC counts, which is the output of Dr. Clifton's software to count grains from *in situ* hybridization experiments in Dr. Kauffman's lab. It is evident that, although bifurcation creates differences in activity patterns visible in **Figure 6**, the SCN still cycles on a 24-hour basis, with peaks during the photophase (T1) and troughs during the scotophase (T2). **Figure 7B** display SCN photographs taken at each time point with Leica Firecam, with special thanks to Dr. Kauffman and his lab equipment. Visibly brighter grains correlate with higher GPC count and *Per2* mRNA expression. Statistical analysis via a Two-Way ANOVA multifactorial design (**Figure 7C**) depicted a significant main effect of entrainment status (bifurcated or unbifurcated) and a significant main effect of scotophase (T1 or T2) on *Per2* mRNA expression levels in the SCN. However, there was no interaction effect between scotophase and entrainment status. Therefore, I opted not to run any post-hoc analysis. The effect size of each (partial eta squared), indicated that 22.5% of the variance in *Per2* mRNA expression was due to the mouse's entrainment state, 52.1% of the variance in *Per2* mRNA expression was due to the scotophase (T1 vs T2), and less than 1% of the variance was due to the interaction effect, reaffirming the non-significant interaction effect.

When performing a Two-Way ANOVA using raw GPC values, the test failed the homogeneity of variance assumption (Levene's Test of Equality of Error Variances; $F = 6.96$, $p = 0.001$). Therefore, the natural log transformation of GPC values was performed, in order to meet the homogeneity of variance assumption and allow the use of the multifactorial ANOVA test.

A



B

C

	F value	Significance	Partial Eta Squared
entrainment	8.118	0.008	0.225
scotophase (T1 v T2)	30.409	0.000	0.521
entrainment*scotophase	0.025	0.875	0.001

Figure 7. *Per2* mRNA Expression Between Bifurcated and Unbifurcated Groups
(a) The bifurcated group and the unbifurcated group had similar *Per2* mRNA expression profiles between T1 and T2. GPC counts are represented on the y-axis, and independent variables are represented on the x-axis. In both groups, the difference in means between T1 and T2 was statistically significant ($t(14) = 0.02$, $p<0.05$). Their respective SCN tissue **(b),** exposed to *in situ* hybridization, showed a similar decrease in grain counts from T1 to T2. A multifactorial Two-Way ANOVA was performed, and the results are displayed in **(c)**.

Furthermore, a linear regression model was calculated in order to predict *Per2* mRNA expression levels (GPC) as a function of BSI, across both bifurcated and unbifurcated groups in each time point (T1 vs T2). In T1, there was a significant negative correlation between GPC and BSI ($F = 6.45$, $p<0.05$). T2 saw a similar significant negative correlation between GPC and BSI ($F = 4.76$, $p<0.05$). However, when bifurcated and unbifurcated group GPC values were individually predicted as a function of BSI, there were no significant correlations found.

Discussion

Bifurcated mice were shown to have two separate oscillators that were localized in the VL-like and DM-like areas of the SCN. Each oscillator was functionally different based on analysis of each scotophase [32]. Hamsters had differential PER1 expression in their core and shell regions of their SCN, each of which had an oscillator that was responsible for the unique activity profiles in its respective scotophase [33]. When mice and hamsters both were bifurcated and then released into constant conditions, the two unique bouts of activity, each with their own amount of activity and phase angles of entrainment, rejoined quickly, indicating the presence of two separate oscillators [30]. These studies all pointed to a bimodal expression pattern of canonical clock genes in the SCN, due to the mere presence of two separate oscillators that each controlled a respective scotophase during bifurcation. Therefore, we initially hypothesized that the SCN as a unit would have a bimodal expression profile for *Per2*. However, this was not the case, as *Per2* expression of bifurcated mice matched the control group of mice.

Although both groups experienced two scotophases, all mice showed high *Per2* mRNA expression during the scotophase that would have correlated with day and showed low *Per2* mRNA expression during the scotophase correlating with night. Unlike prior studies where decoupled oscillators led to high expression in each scotophase, the current study shows that, although there are two distinct oscillators, they still seem to be coupled, because of the significantly higher level of expression in one scotophase, indicating a 24h rhythm.

Upon observation of the total average GPC count, or representative *Per2* expression levels, the unbifurcated control mice have higher GPC counts than their bifurcated counterparts at their respective scotophases (T1 vs T2). Although this was not statistically significant in terms of an all pairs Student's T test comparison, there was a significant main effect of entrainment

status (bifurcation status) on *Per2* mRNA expression. This points to the possibility that, within the SCN, there is a degree of decoupling where the oscillators may be sharing or consolidating *Per2* expression, which could dilute the overall *Per2* expression in the SCN. Additionally, the linear regression analysis showed a significant negative correlation between GPC count and BSI. The more bifurcated a mouse was, the lower their *Per2* expression levels. This corroborates the theory that the two oscillators may be diluting *Per2* expression. A similar study with analysis of individual subsections of the SCN is necessary to separate the resulting 24h *Per2* rhythm from independent effects of multiple potential oscillators. The suggested study requires more animals, to see if the difference in overall *Per2* expression between bifurcated and unbifurcated mice can be statistically significant, and whether this difference in overall expression is manipulated by distinct expression profiles of various oscillators in the SCN. Furthermore, a larger population of mice can be used to increase the number of time points within which SCN genetic expression can be analyzed more discretely.

There are apparent differences in bimodality of circadian clock genes in the SCN of bifurcated animals in the literature compared to the current study. Bifurcated hamsters with dissociated PER1 rhythms in core and shell regions of the SCN are primarily studying similar clock components as *Per2*, yet they are studying the protein product, not the mRNA expression levels [33]. Therefore, the fact that *Per2* is unimodal can be a result of studying a different gene at a different level of translation. Additionally, the hamsters were bifurcated to an 8:4:8:4 LDLD schedule, which is the opposite of 4:8:4:8 that the current study implemented. This difference in LDLD schedule is significant because prior studies have shown that an 8h photophase has better bifurcation outcomes than a 4h photophase [34]. This may be another factor that can explain the unimodality in *Per2* mRNA expression that differed from the PER1 bimodality seen in hamsters.

When studying mice bifurcated to 7:5:7:5 LDLD, bimodality was also observed, but of *mPer1* and *FOS* [32]. This paradigm has more similarities to the current study than hamsters, in that both studies used mice. However, there is still the obvious difference of LDLD condition; a 7h photophase is more optimal for bifurcation than a 4h photophase. Since a 4h photophase LDLD schedule is not sufficient to induce bifurcation, it is closer to unbifurcation than an 8h or a 7h photophase is. Therefore, a 4h photophase may be the cause of unimodality, due to its relative proximity to unbifurcation. This may be a more trivial explanation for the unimodality seen in mice in the current study. Due to this, the lighting condition may play a critical role in bimodality of the SCN in bifurcated organisms.

Based on the results, *Per2* has a clear unimodal pattern of rhythmicity in the SCN, regardless of whether the mouse has a bifurcated activity pattern or not. This brings the concept of bifurcation into further scrutiny. Is there an instantaneous flip from bimodality to unimodality as an organism shortens their photophase from 8h down to 4h? It is known that bifurcation is selective based on certain LDLD conditions [34]. However, it is important to study other clock genes in the circadian pathway during bifurcation to ascertain if all the clock genes behave in a similar fashion. Based on the literature and results of the study, bifurcation may be acting in a way that it rapidly switches between bimodal and unimodal expression of its clock genes in response to reduced amplitudes, higher sensitivities to resetting and bifurcated activity patterns. More directed studies at other genes and lighting conditions must be done to paint a better picture of the SCN during bifurcation.

SUPPLEMENTAL FIGURES

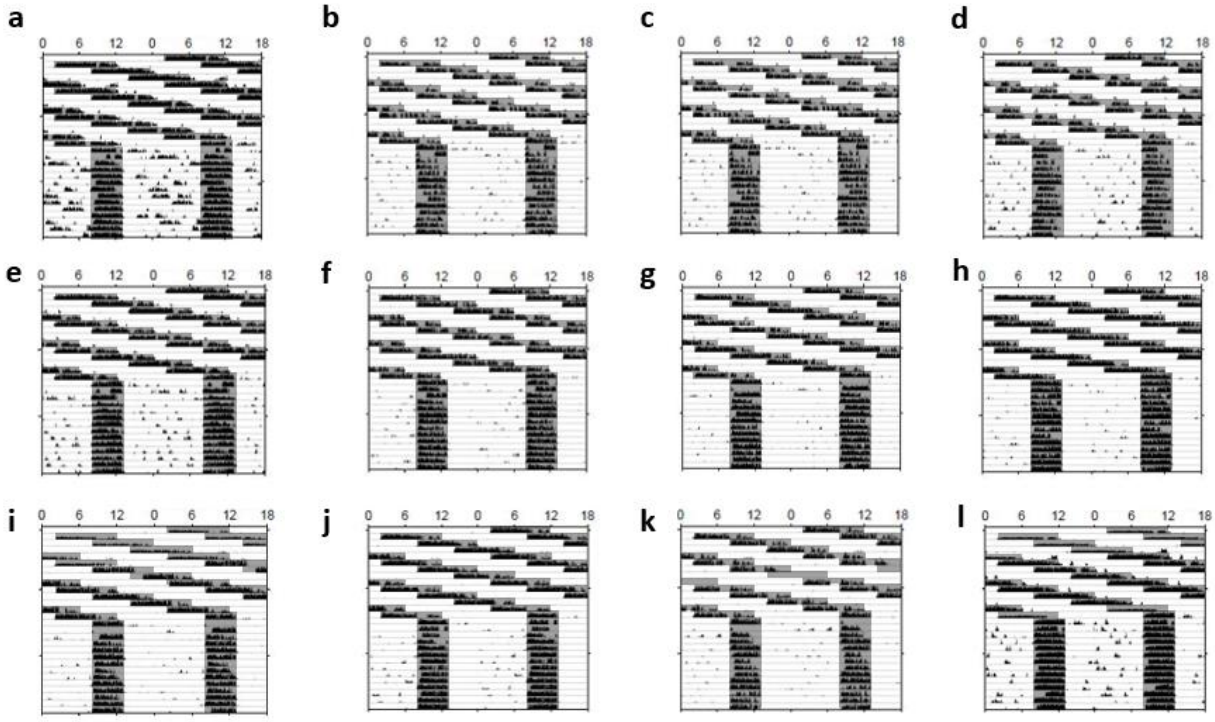


Figure S1. L:D$_{im}$ Actograms in T18

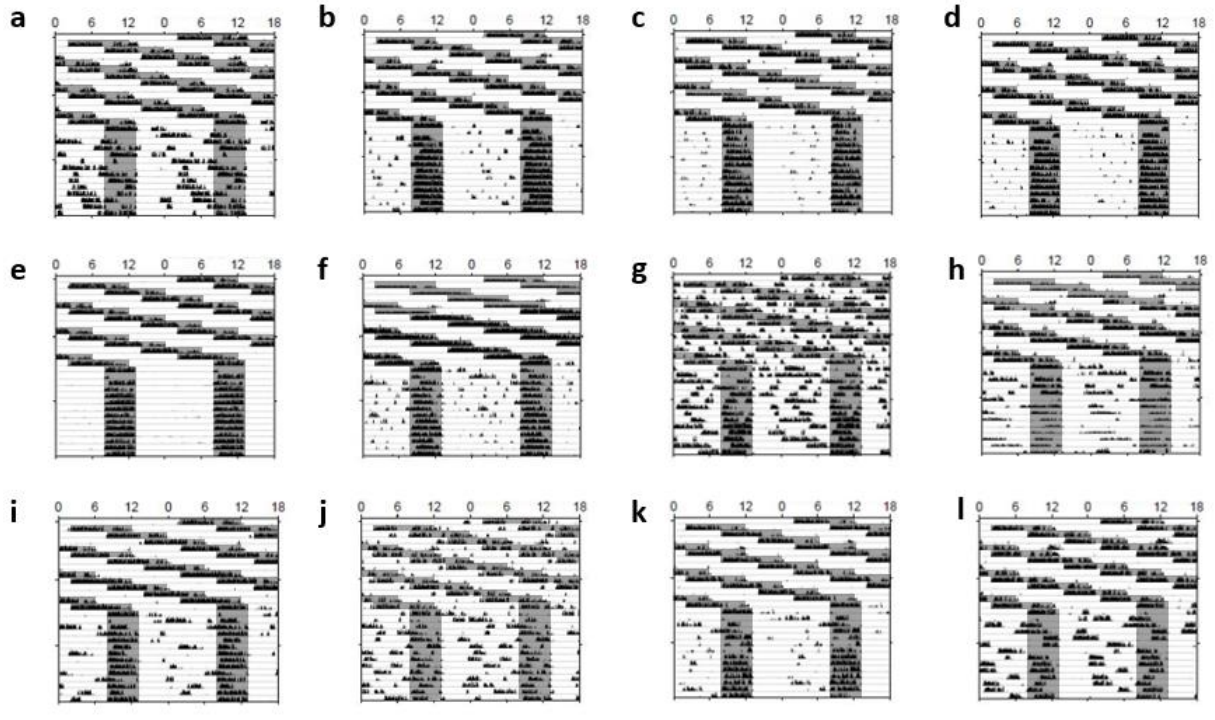


Figure S2. L:D$_{ark}$ Actograms in T18

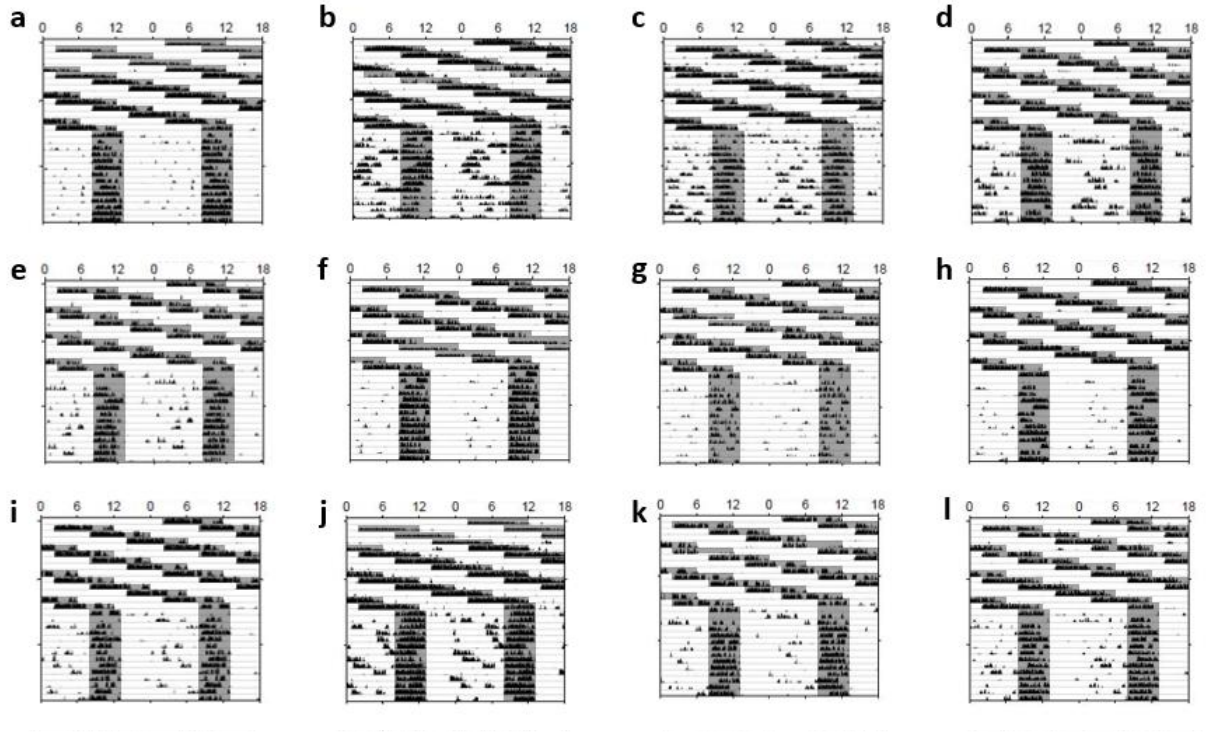


Figure S3. Pre-3 Actograms in T18

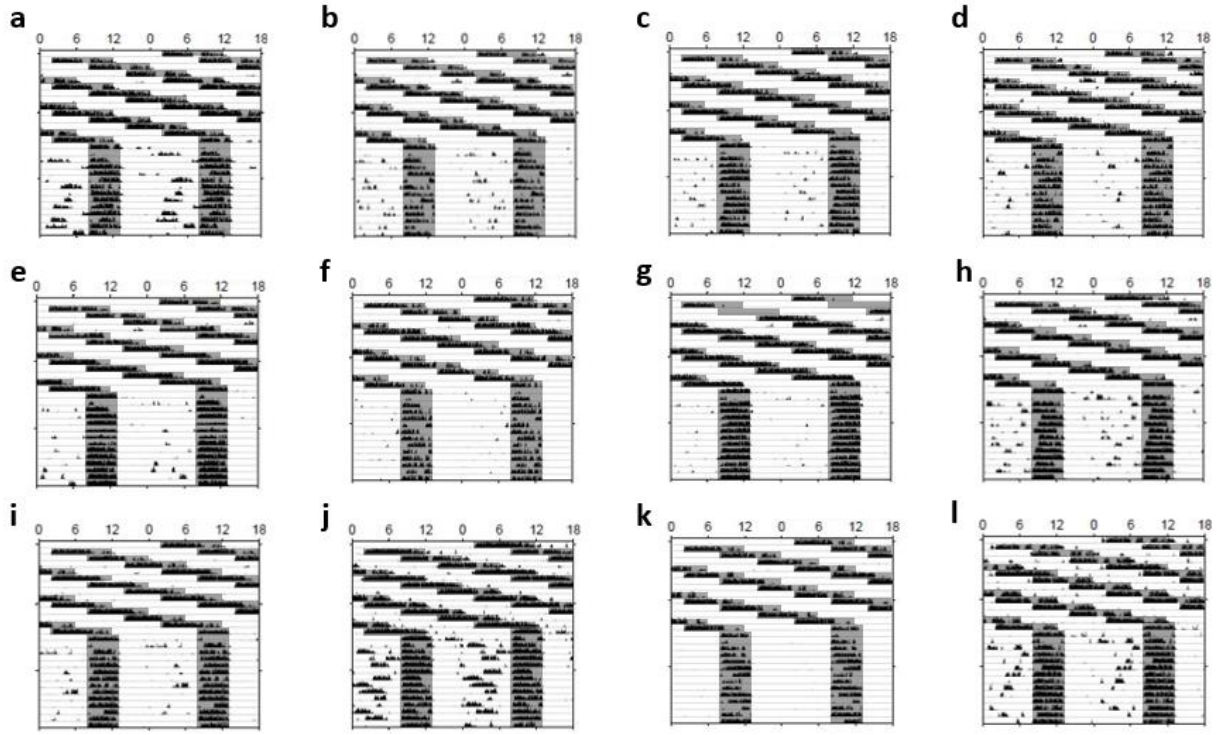


Figure S4. Pre-1 Actograms in T18

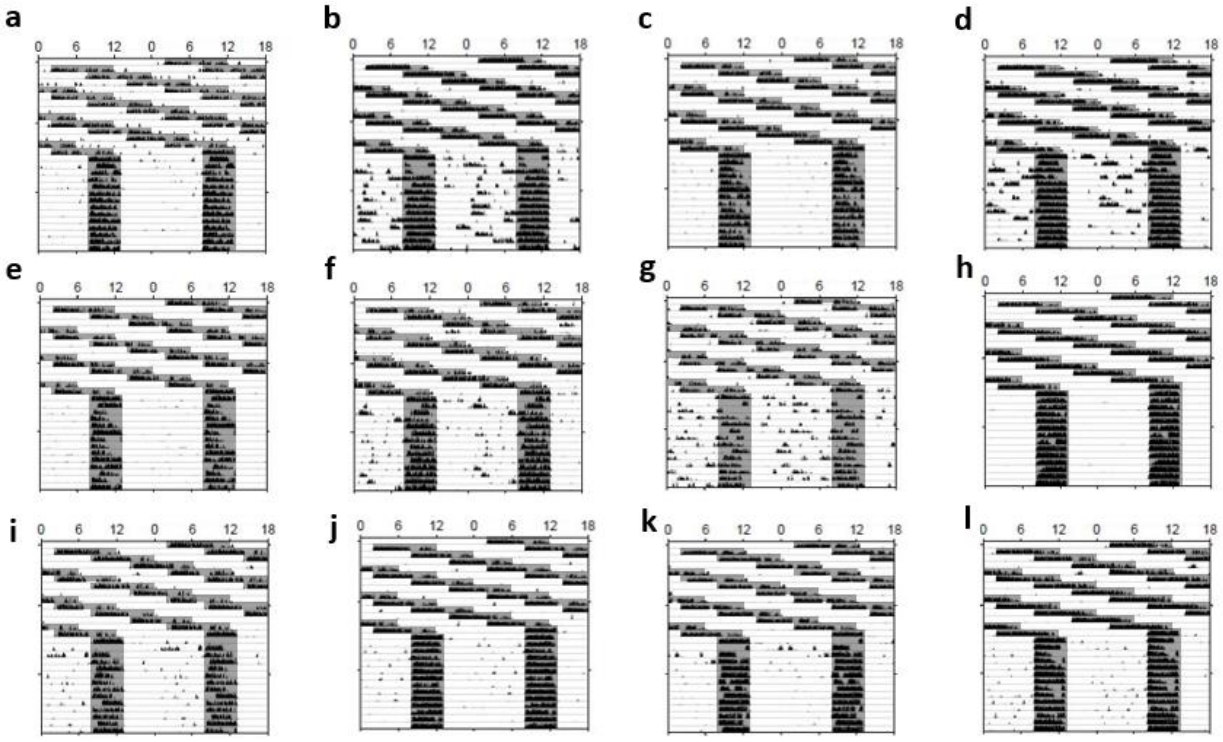


Figure S5. Post-3 Actograms in T18

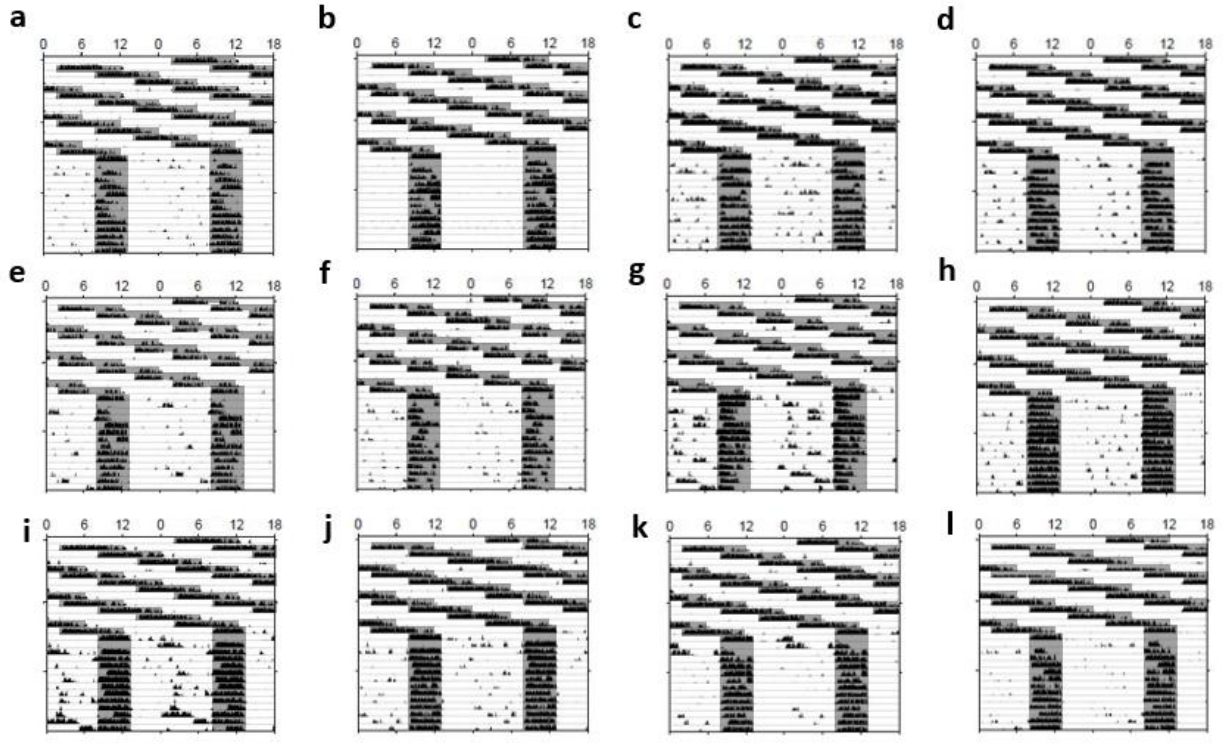


Figure S6: Post-1 Actograms in T18

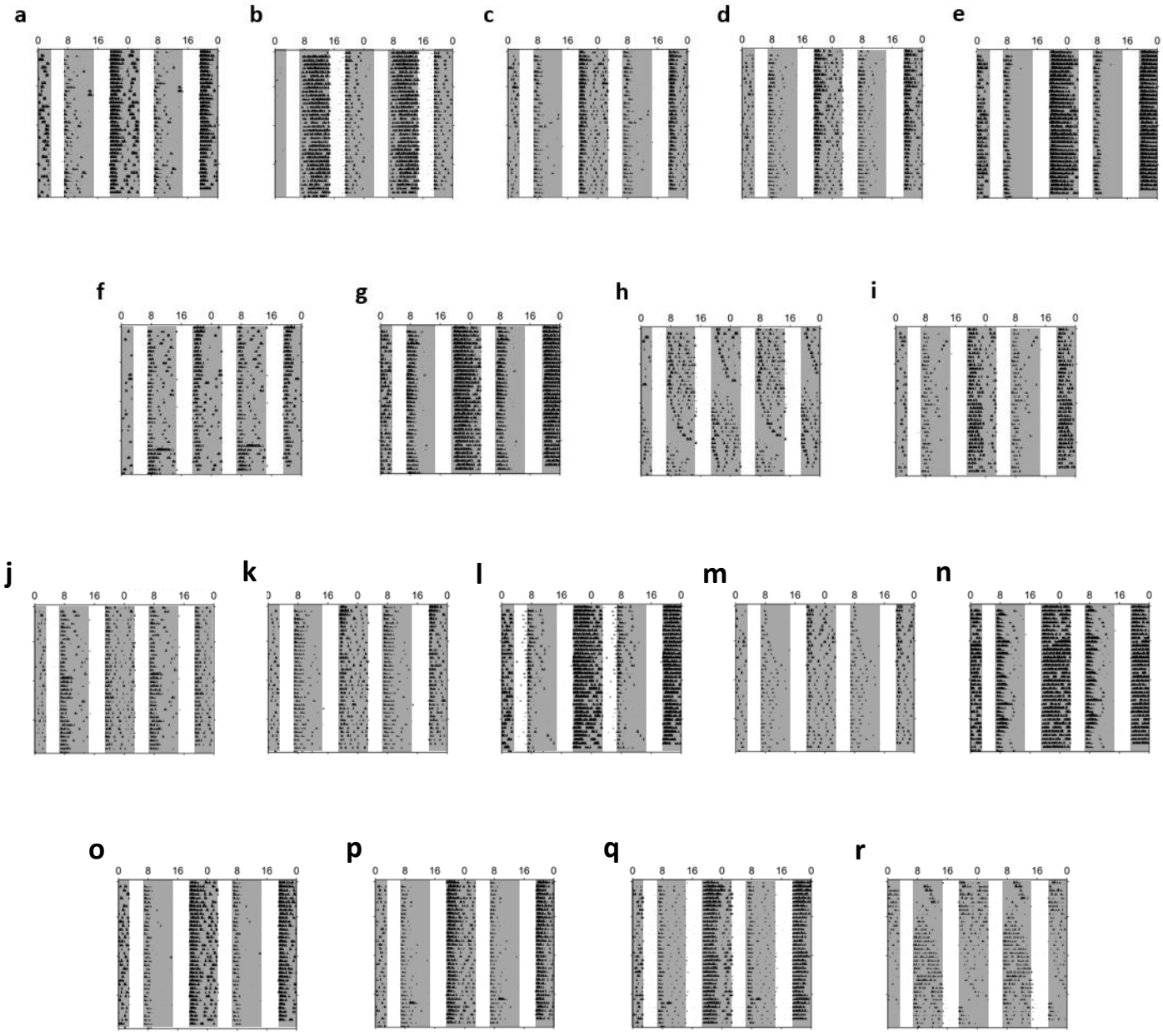


Figure S7. Bifurcated Mice Actograms in 4:8:4:8

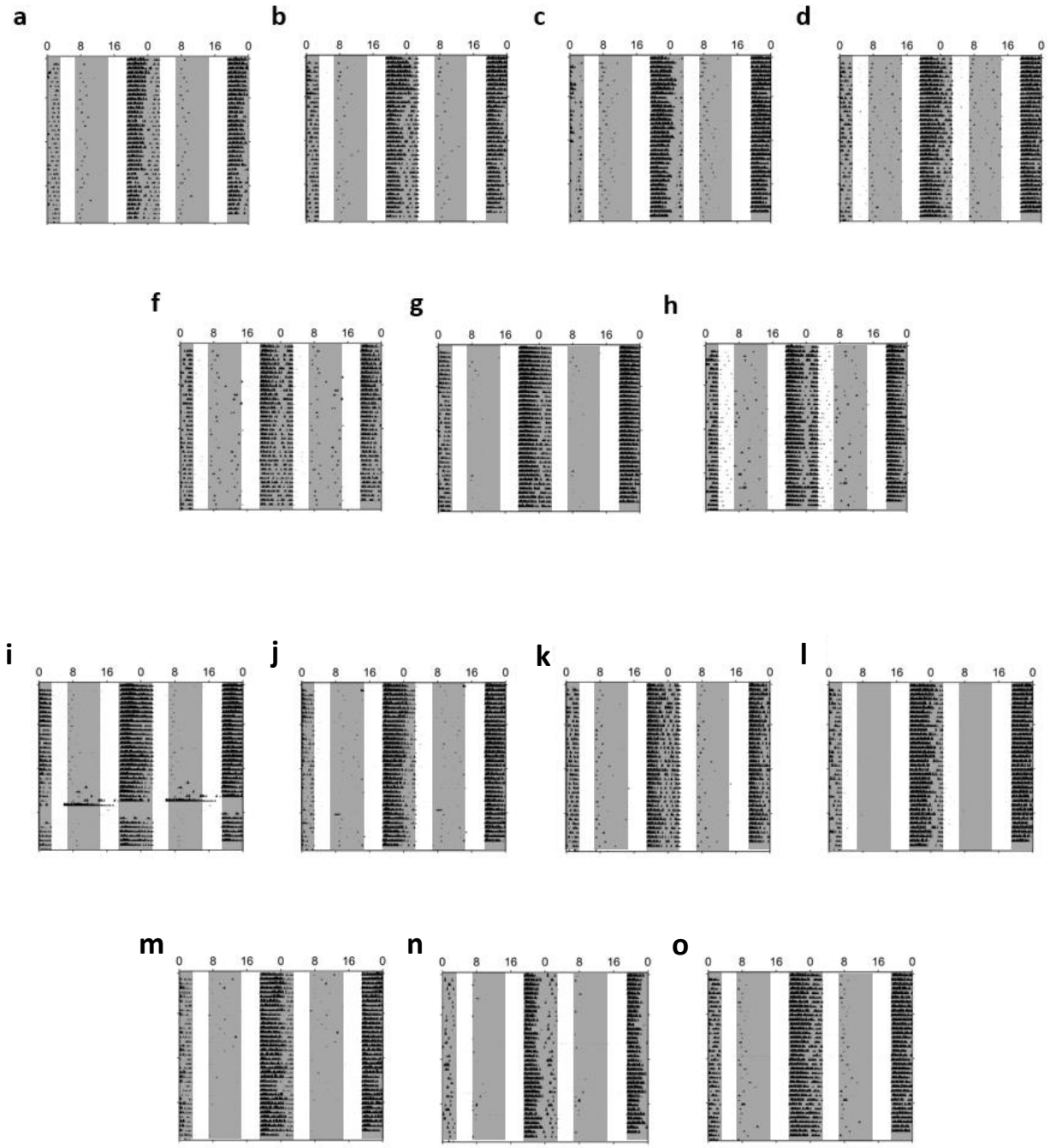


Figure S8. Unbifurcated Mice Actograms in 4:8:4:8
The mouse depicted in figure **(i)** experienced a glitch in the software that recorded daily activity.

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