UNIVERSITY OF CALIFORNIA SAN DIEGO

Environmentally-Induced Entrainment Plasticity: Behavioral Adaptation to Extreme Conditions and its Consequences

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Experimental Psychology

by

Thijs Johannes Walbeek

Committee in charge:

Professor Michael R. Gorman, Chair
Professor Stephan G. Anagnostaras
Professor Christina M. Gremel
Professor Alexander S. Kauffman
Professor David K. Welsh

2019

The Dissertation of Thijs Johannes Walbeek is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

Chair

University of California San Diego

2019

Table of Contents

List of Abbreviations

Bmal1	Brain and Muscle ARNT-Like 1
BSI	Bifurcation Symmetry Index
CJL	Chronic Jetlag
CL	Corpus Luteum
Clock	Circadian Locomotor Output Cycles Kaput
Cry	Cryptochrome
Cq	Quantification Cycle
DD	Dark:Dark / Constant Darkness
DNA	Deoxyribonucleic Acid
E2	Estradiol
EQ	Entrainment Quotient
FRP	Free Running Period
GLA	General Locomotor Activity
GnRH	Gonadotropin-Releasing Hormone
HPG	Hypothalamic–Pituitary–Gonadal
ISH	In Situ Hybridization
LD	Light:Dark
LDLD	Light:Dark:Light:Dark
LH	Luteinizing hormone
N1	Night 1
N2	Night 2
RNA	Ribonucleic Acid
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RW	Running wheel
Per	Period
PRC	Phase Response Curve
SCN	Suprachiasmatic Nucleus
Tb	Body Temperature
TTFL	Translation-Transcription feedback loop
ZT	Zeitgeber time

List of Figures & Tables

Acknowledgements

I would like to thank my advisor, Michael Gorman, for his support and guidance through 5 years of grad school. It was a wonderful and enriching experience working in his lab that has shaped me as a researcher.

To Sasha Kauffman for his collaborative support for my work. Part of chapter 5 is a result of our collaborations. To the other members of my dissertation committee, Stephan Anagnostaras, Tina Gremel, and David Welsh, for their contributions and directions to my work.

Many thanks to all other Gorman lab members, including all undergraduate research assistants and volunteers, who all have had an important role in making it possible for me to complete this work. Special thanks to Liz Harrison, who is a great friend and has provided mentorship to many aspects of graduate school and research. Part of our work together is represented in chapter 3.

To the Executive Committee, trainees, and member of the Center for Circadian Biology for Providing a stimulating and intellectual community. Special thanks to Karen Tonsfeldt for being a great friend, mentor, and collaborator. Part of our work together is represented in Chapter 6.

Thanks to my family for all their boundaryless encouragements for all my adventures, and to my cohort in the Psychology graduate program for sharing this journey with me.

Finally, to my wife, Danbi Ahn, for always believing in me.

Chapter 2, in full, is a reprint of the material as it appears in *Walbeek, Thijs J., and Gorman, Michael R. 2017. "Simple Lighting Manipulations Facilitate Behavioral Entrainment of Mice to 18-h Days." Journal of Biological Rhythms 32 (4): 309–22. https://doi.org/10.1177/0748730417718347.* The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full has been submitted for publication of the material as it may appear in *Walbeek, Thijs J., Elizabeth M. Harrison, Robert R. Soler, and Michael R. Gorman. "Enhanced Circadian Entrainment in Mice and Its Utility under Human Shiftwork Schedules." Manuscript under Review.* The dissertation author was the primary investigator of Study 1 in the paper and author of this paper.

Chapter 4, in full is currently being prepared for submission for publication of the material. *Walbeek, Thijs J., Shannon B.Z. Stephens, Jennifer A. Yang, Takako Noguchi, Michael R. Gorman, Alexander S. Kauffman.* The dissertation author was the primary investigator and author of this material.

Chapter 5, in full is currently being prepared for submission for publication of the material. *Walbeek, Thijs J., Karen J. Tonsfeldt, Alexandra Mendez, Andrew Zhao, Konstancja Ziegert, Michael R. Gorman.* The dissertation author was the primary investigator and author of this material.

Vita

Education

2011	Bachelor of Science, University of Groningen
2013	Master of Science, University of Groningen
2019	Doctor of Philosophy, University of California San Diego

---

Publications

Walbeek, Thijs J., Deborah A.M. Joye, Ila Mishra, and Michael R. Gorman. 2019. "Physiological, Behavioral and Environmental Factors Influence Bifurcated Circadian Entrainment in Mice." *Physiology & Behavior* 210 (October): 112625. https://doi.org/10.1016/j.physbeh.2019.112625.

Walbeek, Thijs J., and Michael R Gorman. 2017. "Simple Lighting Manipulations Facilitate Behavioral Entrainment of Mice to 18-h Days." *Journal of Biological Rhythms* 32 (4): 309–22. https://doi.org/10.1177/0748730417718347.

Harrison, Elizabeth M, **Thijs J Walbeek**, Jonathan Sun, Jeremy Johnson, Qays Poonawala, and Michael R Gorman. 2016. "Extraordinary Behavioral Entrainment Following Circadian Rhythm Bifurcation in Mice." *Scientific Reports* 6 (December): 38479. https://doi.org/10.1038/srep38479.

Geerdink, Moniek, **Thijs J Walbeek**, D.G.M. Domien G M Beersma, Vanja Hommes, and M.C.M. Marijke C M Gordijn. 2016. "Short Blue Light Pulses (30 Min) in the Morning Support a Sleep-Advancing Protocol in a Home Setting." *Journal of Biological Rhythms* 31 (5): 483–97. https://doi.org/10.1177/0748730416657462.

Walbeek, Thijs J., Elizabeth M. Harrison, Robert R. Soler, and Michael R. Gorman. "Enhanced Circadian Entrainment in Mice and Its Utility under Human Shiftwork Schedules." *Manuscript under Review*.

Harrison, Elizabeth M, **Thijs J. Walbeek**, Dominick G. Maggio, Andrew A. Herring; Michael R. Gorman. "Circadian profile of an Emergency Medicine Department: scheduling practices and their effects on sleep and performance." *Manuscript under Review*.

Abstract of the Dissertation

Environmentally-Induced Entrainment Plasticity:
Behavioral Adaptation to Extreme Conditions and its Consequences

by

Thijs Johannes Walbeek

Doctor of Philosophy in Experimental Psychology

University of California San Diego, 2019

Professor Michael R Gorman, Chair

The mammalian circadian system is regulated by an internal oscillator that has evolved to keep time in a very predictable rhythmic environment, and is generally considered not flexible enough to adjust to rapidly changing sleep schedules in shiftworkers. This inability to quickly adjust leads to circadian disruption, which is associated with increased risk for chronic disease. Increasing the flexibility of the circadian system could enhance adaptation to irregular cycles, and thereby alleviate negative consequences. This dissertation examines a mouse model for enhanced circadian entrainment, its mechanisms, and its utility for human shiftworkers.

Chapter 2 describes that with the addition of dim night time illumination, mice can behaviorally adapt to 18 hour days (T18). T18 entrainment is remarkable and unprecedented in any mammalian system, which typically can only entrain to a narrow range of day-lengths (i.e 22-26 hours). Chapter 3 expands the characterization of this state of enhanced circadian plasticity by describing oscillator characteristics. After demonstrating the lack of circadian oscillator-typical behavior in T18, we conclude that control of behavior in this condition must not derive from entrainment of a conventional circadian oscillator as explained by classical entrainment theories. In Chapter 4, clock gene expression rhythms in the suprachiasmatic nucleus -- a small area in the hypothalamus that orchestrates rhythms in mammals -- and liver and kidney further support the hypothesis that canonical circadian drivers are not involved in control of behavior in T18. While behavior displays 18 hours rhythms, clock gene expression is 24 hours in all tissues. Lastly, Chapter 5 demonstrates that the temporal organization of control of female reproductive function is altered in T18, without any negative impact on reproductive efficacy.

Together, this work demonstrates that the rodent circadian system can be markedly more flexible than traditional circadian entrainment theory predicts, but that this flexibility might rely on mechanisms that do not fit with the classical understanding of entrainment systems. Flexible entrainment has translational potential for human shiftworkers to adapt to irregular and sometimes unpredictable work schedules; and does not lead to negative health consequences seen in other non-24h paradigms.

Chapter 1. General Introduction

Every organism on our planet has evolved to live in a world that rotates around its axis every 24 hours and revolves around the sun every year. As a result, many species have evolved biological clocks that regulate behavior and physiology to anticipate predictable rhythmic changes in the environment. For example, sleep-wake cycles, metabolism, alertness, and body temperature all show daily rhythms that help conserve energy, reduce predation risk, or optimize fitness (Mohawk et al. 2012). Our modern 24-hour society, however, exposes the circadian system to an environment it did not evolve to operate in. Working night shifts, especially, exposes the circadian system to irregular sleep-wake cycles that it was long believed to be incapable of adjusting to. This type of circadian disruption has been associated with impairments in physical and mental health (Evans and Davidson 2013; Moreno et al. 2019). A more flexible internal clock could potentially aid shiftworkers in adjusting to their work schedules and thereby alleviate these negative consequences. Limited work so far, however, has focused on circadian flexibility. My work combines novel techniques that markedly enhance circadian plasticity with rodent models of negative consequences associated with shiftwork. It offers significant insights on the mechanistic basis of flexible adaptation and its potential translational application.

In mammals, the circadian system is hierarchical with rhythms at all levels of the biological system from cellular to organismal (Reppert and Weaver 2002). Each cell has an intrinsic circadian rhythm generated by the transcription-translation feedback loop (TTFL) between clock genes and their protein products (Mohawk and Takahashi 2011). In short, BMAL1 and CLOCK are transcription factors that promote transcription of period (*Per1*, *Per2*, *Per3*) and cryptochrome (*Cry1*, *Cry2*) genes. In the nucleus, CRY and PER dimerize, inhibit the activity of BMAL1 and CLOCK and repress their own transcription. Regulated by at least a dozen known and potentially more unidentified genes, the TTFL takes about 24 hours to

complete a full cycle. Within each organ, cells are often synchronized, causing organ function to be rhythmic as well. The core mammalian internal time-keeper is the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, a network of ~10,000 oscillating neurons. The SCN is synchronized by light input coming from the eye, and keeps all the peripheral clocks in organs synchronized (Reppert and Weaver 2002; Welsh et al. 2010).

The endogenous rhythms in an intact mammalian system are close to, but not exactly 24 hours. A mouse in constant darkness (DD), for example, typically has an activity-rest cycle of about 23.5h (Pittendrigh and Daan 1976a), meaning it will start its active phase about 30 min earlier every day relative to clock time. In a light:dark cycle, this near 24h internal rhythm adapts to the exact 24h environmental rhythm; this process is called entrainment. For a mouse with a free running period (FRP) of 23.5h (τ = 23.5h) to entrain to a 24h light:dark (LD) cycle (T = 24h), the internal rhythms need to be delayed by 30 minutes every day. Conversely, for a mouse with τ = 24.5h, a daily 30 min phase advance would be required to entrain to T24. Light affects the circadian rhythm of a mouse in a very predictable way. A phase-response curve (PRC) represents the phase shifting effect (response) of a given light stimulus, depending on when (phase) the stimulus is presented (Daan and Pittendrigh 1976). A light-PRC has very consistent shape across species, diurnal or nocturnal alike, with phase advances at the beginning of the day, phase delays at the end of the day and a period of relative unresponsiveness in between. For an organism with a FRP of less than 24h, light is needed late in the afternoon (the phase-delaying part of the PRC), while an organism with a long FRP needs light early in the morning (phase advancing part of the PRC) to entrain to T24. This results in different but stable phase angles of entrainment (the phase relation between internal rhythms and the light:dark transitions) between animals depending on τ, T and the PRC. The process of entrainment by daily phase advances or phase delays of the internal clock to match the external rhythm is called non-parametric entrainment (Daan 2000).

Just like light entrains the mammalian SCN, peripheral organs can be entrained by physiological signals like temperature, melatonin, glucocorticoids or metabolic stimuli. Because body temperature, melatonin secretion, and activity and feeding behavior are regulated by the SCN, the SCN has the ability to entrain peripheral organs such as the liver (Mohawk et al. 2012). Harmony in rhythmicity among all oscillators in an organism is important for optimal health.

Since the industrialization, artificial light has extended the human day to 16-18 hours, regardless of season. Moreover, in many occupational settings such as hospitals, transportation and emergency response, work must go on at every hour of the day and night. Living in a 24-hour society with a biological clock that evolved in a world without artificially extended days leads to many behavioral, cognitive and medical deficits. In the US, yearly costs of consequences of shiftwork exceed $200 billion (Kerin and Aguirre 2005). Two types of negative consequences of shiftwork can be distinguished. First, acute effects of being awake at night and sleep deprivation lead to loss of alertness, which leads to decreased productivity and increased frequency of mistakes and accidents. Second, chronic exposure circadian disruption is associated with many medical problems (Evans and Davidson 2013), including increased mortality rates (Knutsson et al. 2004), breast cancer incidence (Haus and Smolensky 2013), prevalence of metabolic syndrome (De Bacquer et al. 2009), risk factors of cardiovascular disease (Ha and Park 2005), prevalence of common infections (Mohren et al. 2002), reproductive dysfunction in women (Mahoney 2010), and impaired cognitive functioning (Cho et al. 2000; Wright et al. 2006).

Many of these symptoms can be reproduced in animal models by inducing circadian disruption (Penev et al. 1998; Filipski et al. 2004; Davidson et al. 2006; Castanon-Cervantes et al. 2010; Loh et al. 2010; Karatsoreos et al. 2011). Two common experimental manipulations to induce circadian disruption in the lab are chronic jetlag and T-cycles. Chronic jetlag is induced

by repeated exposure to 6h phase advances (i.e. equivalent to eastward travel across 6 time-zones). In mice, for example, this leads to reduced successful completion of pregnancy by 70% compared to non-jetlagged controls (Summa et al. 2012). In T-cycles, animals are exposed to days that are beyond conventional limits of adaptation. For example, mice housed in 20h light:dark cycles (T20) show markers of metabolic syndrome such as body weight gain and increased levels of insulin and leptin (Karatsoreos et al. 2011). These types of paradigms demonstrate the consequences of exposing the circadian system to schedules it can not adapt to.

Until recent discoveries, the circadian system was thought to be too inflexible to deviate from its 24h basis by more than 1-2 hours per day. Two surprising, but simple, environmental manipulations in rodents, however, nearly eliminate jetlag and allow rest/activity cycles to be timed at any hour of the day or night. First, illuminating the nights with light comparable to starlight, as opposed to complete darkness, allows the circadian clock to be much more easily adjusted. For example, hamsters exposed to completely dark nights failed to entrain to light cycles longer than 26 hours, while animals exposed to dimly lit nights successfully entrained to cycles as long as 30h (Gorman et al. 2005). Second, with the use of dim light, mice and hamsters can bifurcate to a double light:dark cycle with two ‘days’ and ‘nights’ every 24h. Following rhythm bifurcation, jetlag following a phase shift was reduced by 71% (Harrison and Gorman 2015) and mice were able to entrain to 30h LDLD cycles, in some cases without continuation of dim light (Harrison et al. 2016). Combined, this work highlights unusual conditions in which the circadian system can be remarkably more flexible than traditionally believed.

Given the similarities between biological clocks among mammalian species, rodent models of circadian adaptability provide a unique approach toward solutions for negative health consequences of shiftwork. For example, living on an 18 or 30h schedule would allow efficient

rotating between day- and night-time work shifts. In addition to the potential application, novel techniques for altering circadian rhythms provide innovative methods to characterize the dynamics and interactions of physiological, neurological and molecular rhythms.

The work presented in this dissertation uses entrainment models in mice to study extraordinary circadian plasticity, its mechanisms, potential application for human shiftworkers, and consequences for reproductive health. These aspects were studied using T18 entrainment, and are presented in 4 empirical chapters.

Chapter 2: "What is the separate contribution of dim light at night and bifurcation after-effects in facilitating T18 entrainment?"

The work in this chapter extended prior research on circadian flexibility induced by dim light at night and bifurcation to 18 hour rhythms. Successful entrainment to 18 hour rhythms by a mammalian species is highly unusual, as it would require shaving off nearly 6 hours of the endogenous free-running period, while even the strongest light pulses cause advances up to 2 hours. Yet, behavioral entrainment in T18 can be induced rapidly, robustly and repeated without the need of genetic, pharmacological or invasive manipulations, making T18 entrainment an attractive model to study circadian plasticity. Furthermore, 13 hour long light phases would allow for sufficiently long 'work days' if translated to diurnal human shiftworkers. In prior work on flexible behavioral entrainment in T-cycles, the use of dim light at night and rhythm bifurcation after-effects have been confounded. In chapter 2, I tested the separate contributions of these factors on T18 entrainment.

Chapter 3: "Does behavior in flexible entrainment display characteristics of a strong underlying circadian oscillator?"

One of the unique characteristics of flexible entrainment in T18 is the lack of large phase angles (deviation between activity onset and lights offset) seen in other T-cycle entrainment

context (Pittendrigh and Daan 1976b). This led to the hypothesis that under T18-entrainment behavior might not be controlled by a strong circadian oscillator but is more directly light-driven while the internal clock is dampened or uncoupled. To test this, in Chapter 3, behavior in T18-mice was scored for other characteristics of a strong circadian oscillator. Specifically, mice in T18 were challenged with phase shifts and observed for transient misalignment (i.e. jetlag) following each of these shifts.

Chapter 4: "What is the molecular basis for circadian entrainment in T18?"

In Chapter 4, clock gene expression patterns in the SCN, liver, and kidney were measured. Period, amplitude and phase of rhythmically expressed genes were compared between T24 and T18 to test the involvement of the canonical circadian TTFL in entrainment in T18.

Chapter 5: "How is reproductive function in female mice affected by T18 entrainment?"

In rodents, ovulatory rhythms, or estrous cycles, are controlled by the hypothalamic-pituitary-gonadal axis (HPG) and are tightly linked to the circadian system. Every 4-5 days, on the day of ovulation, a circadian gating window allows hypothalamic neurons to release a bolus of Gonadotropin-releasing hormone into the pituitary, which causes a surge in release in luteinizing hormone (LH), which triggers ovulation. Altered circadian rhythms have the potential to disrupt the temporal organization in the HPG axis and lead to subfertility. In Chapter 5, estrous cycles, LH surges, ovulation, and mating behavior were characterized, and fecundity was quantified in mice entrained to T18.

Chapter 2. Simple lighting manipulations facilitate behavioral entrainment of mice to 18 hour days

Abstract

In an invariantly rhythmic world, a robust and stable mammalian circadian clock is presumed to confer fitness advantages. In shift-work or after rapid trans-meridian travel, however, a stable clock might be maladaptive and a more flexibly resettable clock may have advantages. The rate at which rodents can adjust to simulated time zone travel and the range of entrainment can be markedly increased through simple light manipulations, namely, exposing animals to extremely dim light (<0.01) at night or by bifurcating rhythms under 24h light:dark:light:dark (LDLD) cycles. Here we investigated the separate effects of dim light and bifurcation on the ability of mice to entrain to 18h days (LD13:5; T18). Incorporating dim light at night, mice in Experiment 1 were exposed either to LD cycles with photophases that were progressively shortened from LD19:5 to LD13:5, or to bifurcating LDLD cycles with photophases that were lengthened from LDLD7:5:7:5 to LDLD13:5:13:5. In both cases, wheel-running rhythms were robustly synchronized to T18 and the phase of the free-running circadian rhythm was controlled by the timing of release into constant conditions. In Experiment 2, either dimly illuminated nights or a history of bifurcation without continuing dim light was sufficient to allow behavioral entrainment to T18 whereas previously unbifurcated mice under dark nights failed to entrain to T18. Additionally, concurrent measurement of body temperature rhythms in T24 LDLD revealed them to be bimodal. These studies suggest that the circadian system is markedly more flexible than conventionally thought, and that this can be achieved in a non-invasive and non-pharmacological way. Facilitation of behavioral entrainment to extreme light:dark cycles may have translational potential for human shift-workers.

Keywords: Circadian, Bifurcation, Dim Light, Behavioral Entrainment, After-effect, T cycles, Mouse

Introduction

The hypothalamic suprachiasmatic nucleus (SCN) generates an approximately 24h neural rhythm that synchronizes clocks throughout the body to orchestrate daily rhythms in physiology and behavior (Reppert and Weaver 2002; Welsh et al. 2010). The phase of the SCN is set by light, which synchronizes it with the environmental light-dark cycle. However, the circadian timing system is generally slow to shift, and organs shift at different rates (Kiessling et al. 2010). During shift-work requiring altered rest/activity cycling, or after trans-meridian travel, the circadian timing system may be disturbed inducing a state of temporarily desynchronized oscillators (i.e jetlag) (Kiessling et al. 2010). Chronic circadian disruption is associated with elevated risk of obesity, impaired immune function, increased cancer risk, and impaired cognition (Costa 1996; Hansen 2001; Karatsoreos et al. 2011; Voigt et al. 2013; Colwell and Matveyenko 2014; Marquié et al. 2014). A more flexible circadian system might be expected to decrease clock disruption in these contexts. The current study explores two different light manipulations that increase flexibility of activity rhythms in mice, and which therefore have potential to reduce negative effects of circadian disruption.

Under traditional laboratory conditions, most mammals have been shown to exhibit stable entrainment to a narrow range (e.g. ±2h) of periods around 24h (Daan and Aschoff 2001). Syrian hamsters (*Mesocricetus auratus)*, for example, failed to entrain to T-cycles longer than 26h (Boulos et al. 2002; Gorman et al. 2005). This range of entrainment, however, can be markedly extended by exposure to dim light at night (as compared to complete darkness) at an illuminance comparable to starlight or dim moonlight (Boulos et al. 2002; Gorman et al. 2005; Gorman et al. 2006), even though such low light levels have been reported to be too weak to shift the clock (Brainard et al. 1982; Brainard et al. 1984; Nelson and Takahashi 1991a; Brainard et al. 2001). With the incorporation of dim nighttime illumination, hamsters were

successfully entrained to T-cycles up to 30h (Gorman et al. 2005) or as low as 19h (Chiesa et al. 2005).

Additionally, in 24h light:dark:light:dark (LDLD) cycles, a *bifurcated* entrainment pattern, characterized by nearly equal amounts of running wheel activity in each of two nights every 24 hours, is facilitated by addition of dim light at night in both mice and hamsters (Gorman 2001; Gorman et al. 2003; Gorman and Elliott 2003; Gorman and Elliott 2004; Evans, Carter, et al. 2012). Bifurcation itself was recently shown to exert surprising effects on enhanced adaptability of activity rhythms of rodents. Bifurcated hamsters, for example, more than doubled speed of re-entrainment to new light cycles after travel across virtual time zones (Harrison and Gorman 2015). Additionally, even without dim light, bifurcated mice were capable of entraining to an LDLD cycle lengthened from 24h to 30h, whereas unbifurcated mice free-ran under these conditions (Harrison et al. 2016).

The effects of previous states of entrainment on properties of the biological clock are termed *after-effects*. Best known are *period* after-effects - lengthened and shortened free running period in constant darkness following entrainment to long versus short light cycles (Pittendrigh and Daan 1976a) and *waveform* after-effects – lengthened and shorted active period (alpha) in free-run after short versus long photoperiod. Less well known are after-effects on *entrainment* – different patterns of entrainment in identical conditions in T22 depending on lighting history (Chiesa et al. 2006) and after-effects on phase *resetting* - larger light-induced phase shifts in hamsters entrained to short compared to long photoperiods (Pittendrigh et al. 1984; Evans et al. 2004; Glickman et al. 2012; Glickman et al. 2014). The ability to entrain to 30h LDLD cycles following bifurcation, but not other conditions, represents another type of circadian entrainment after-effect.

In studying modulation of circadian flexibility (Harrison and Gorman 2015; Harrison et al. 2016), our prior work studied the effects of dim light in close conjunction with bifurcation so that

the relative contributions of each on entrainment have not been clearly distinguished. The current study, therefore, aimed to separate these contributions on the capacity of mice to entrain behaviorally and physiologically to non-24h cycles. Further, to test whether extraordinary entrainment plasticity was limited to lighting cycles lengthened from 24 to 30 h, the current study explored behavioral entrainment to short, 18 h, T-cycles and employed telemetry to determine whether entrainment extended to daily rhythms in body temperature. Together with earlier work, the current results establish that photic manipulations permit extraordinary entrainment to a diversity of non-24 h conditions and identify dim light, without prior bifurcation, as a sufficient stimulus for this capacity.

Methods

Nomenclature

The study of bifurcation in non-24h conditions strains conventional circadian nomenclature. In this manuscript the zeitgeber period will be specified by T (e.g. T18 or T24). Zeitgeber cycles may be additionally described as unimodal (LD) or bimodal (LDLD). Although T12 LD and T24 LDLD are formally equivalent, the latter description is preferred here, since the bifurcated entrainment state is *not* generally a 12h rhythm, but rather a 24h rhythm comprised of two active periods. For example, bifurcation was first demonstrated in LDLD cycles that were not equivalent to T12 LD (e.g. LDLD9:5:5:5) (Gorman and Steele 2006), and bifurcation appears to have a 24h organizational basis in the SCN (Watanabe et al. 2007; Klett and Allen 2017). In contrast, prior entrainment studies under LDLD10:5:10:5 (Harrison et al. 2016), where T15 LD was formally equivalent to T30 LDLD, suggest either a 15h oscillation or a driven rhythm. Pending information about SCN rhythmicity, these cycles are referred to as T15/T30 cycles. This paper reports on cycles that could be considered T18 LD cycles or T36 LDLD cycles. Because the native 24h period is much closer to 18h than to 36, we generally avoid reference to

T36 LDLD cycles in favor of the simpler T18 LD designation. Moreover, no analysis revealed any suggestion of behavioral rhythmicity with a period of 36h.

Animals and Housing

C57BL/6J mice (Jackson, Sacramento, CA), 6-9 weeks at the start of the experiments, were individually housed in plastic shoebox cages (LxWxH: 28x18x15 cm) furnished with a running wheel (13 cm diameter). Food (Mouse diet 5015, Purina, St. Louis, MO) and water were available *ad libitum*. Cages were placed in light-tight chambers to ensure full control of light exposure. During the photophases, chambers were lit by white fluorescents lamps providing 300-350 lux at the cage level. Scotophases were either complete dark (LD_{ark}) or illuminated with green LEDs (555 ± 30 nm) mounted approximately 25 cm outside of the cage generating illuminance <0.01 lux (irradiance of 3.90×10^{-5} W m^{-2}) measured within the cage (LD_{im}). For details on lighting see Evans et al (2012). Cages were changed at least every 21 days on a schedule to minimize disruption of activity rhythms. Animals were weighed at the beginning and end of experiment. Experiments were conducted with prior approval of the University of California San Diego Institutional Animal Care and Use Committee.

Experiment 1: Entrainment to T18 cycles.

To test the hypothesis that prior bifurcation would uniquely enable entrainment to short T-cycles, mice from the colony (LD_{ark} 14:10) were assigned to one of two groups. One was housed in $LD_{im}LD_{im}$ 7:5:7:5 to induce bifurcation (Bif; n= 12, 6 female, remainder male), while a second group was housed in LDim 19:5, which does not allow rhythm bifurcation (nBif; n = 11, 6 female). In 3 phases the photoperiods were progressively lengthened from 7 to 10 to 13 hours in the Bif group and shortened from 19 to 16 to 13 hour in the nBif group (Table 2.1; Figure S1). In both groups, scotophase duration was fixed at 5 hours. Fifty days after the start of the experiment, light conditions were identical between groups, LD_{im} 13:5, but are denoted with

regard to whether mice had a bifurcated history (LD_{im} LD_{im} 13:5:13:5 – Bif) or not (LD_{im} 13:5 – nBif; Table 1).

***Table 2.1**. Complete schedule of light cycles in Experiment 1.*

	Bifurcated History (Bif, n = 12)			**Non-Bifurcated History (nBif, n = 11)**		
	Light condition	**T**	**# cycles**	**Light condition**	**T**	**# cycles**
Recovery	LD_{im} 14:10	24 h	7	LD_{im} 14:10	24 h	7
Phase 1	$LD_{im}LD_{im}$ 7:5:7:5	24 h	20	LD_{im} 19:5	24 h	20
Interphase	$LD_{im}LD_{im}$ 8:5:8:5	26 h	2	LD_{im} 18:5	23 h	2
	$LD_{im}LD_{im}$ 9:5:9:5	28 h	2	LD_{im} 17:5	22 h	2
Phase 2	$LD_{im}LD_{im}$ 10:5:10:5	30 h	18	LD_{im} 16:5	21 h	28
Interphase	$LD_{im}LD_{im}$ 11:5:11:5	32 h	2	LD_{im} 15:5	20 h	2
	$LD_{im}LD_{im}$ 12:5:12:5	34 h	2	LD_{im} 14:5	19 h	2
Phase 3	$LD_{im}LD_{im}$ 13:5:13:5 =	36 h	24	LD_{im} 13:5	18 h	48
	LD_{im} 13:5	18 h	48			
Constant conditions	$D_{im}D_{im}$	-	-	$D_{im}D_{im}$	-	-

After 48 unchanged T18 LD cycles, animals were released in constant condition ($D_{im}D_{im}$) at one of two different time points. For half the animals (n = 12), photophase lighting was extinguished permanently at the beginning of a scheduled scotophase; for the other half, 9h later, after an abbreviated, 4h, photophase. After 20 days in constant conditions, the experiment was ended.

Body temperature and general locomotor activity were continuously recorded using surgically implanted telemeters. To permit characterization of the endogenous body temperature rhythm independent of masking-effects of running wheel activity, wheels were blocked with a pin for three full LDLD cycles during all 3 phases with at least 3 full cycles between each time being blocked. With the exception of the days of wheel-blocking, wheel running was continuously recorded.

Experiment 2: Cues for induction and maintenance of T18 LD entrainment

Because Experiment 1 yielded unexpected robust entrainment to LD_{im}13:5 in both previously bifurcated and non-bifurcated groups, Experiment 2 assessed the roles of dim light and bifurcation history for their separate contributions to T18 entrainment. Adult mice from a

separate cohort were assigned to each of 4 groups (n = 8, 4 female per condition). All groups were first entrained to T24 cycles for 3 weeks before exposure to T18 cycles. The first group was bifurcated in $LD_{im}LD_{im}$ 7:5:7:5 and subsequently transferred directly to LD_{im} 13:5 (Bif-Dim). A second group bifurcated in $LD_{im}LD_{im}$ 7:5:7:5 was moved to LD_{ark} 13:5 (Bif-Dark). A third group went straight from non-bifurcating LD_{im} 14:10 into LD_{im}13:5 (nBif Dim), while the last group was exposed initially to LD_{ark} 14:10 and then to LD_{ark} 13:5 (nBif -Dark). Running wheel behavior was continuously recorded.

To assay T18 entrainment under potentially weaker zeitgebers (i.e. with lower L:D ratio), after 3 weeks in LD_{im}13:5 or LD_{ark}13:5, all groups were exposed to days that were shortened and nights lengthened by 2h increments every 15 days (i.e., 20 18h cycles), resulting in the following light:dark or light:dim cycles; LD_{im} 11:7, LD_{im} 9:9, LD_{im} 7:11, LD_{im} 5:13, LD_{im} 3:15, LD_{im} 1:17 and constant conditions. Light offsets were kept constant, and light onsets were delayed two hours for every new phase.

Data Collection and Analysis

Running wheel activity (RW, half revolutions, Experiment 1 and 2), general locomotor activity (GLA, Experiment 1), and body temperature (Tb, Experiment 1) were collected using VitalView (MiniMitter, Sun River, OR). All data were stored in 6 min bins. In Experiment 2, two out of eight animals in both Bif-Dark and Bif-Dim groups were excluded from analyses because they failed to meet objective and subjective criteria for bifurcation in the initial LDLD 7:5:7:5.

Entrainment Quotients.

To quantitatively assess entrainment to T18 light cycles, Lomb-Scargle periodograms were generated, and power at 18h and the peak power value in the circadian range (22h-26h) were each determined. Entrainment Quotients (EQ) were calculated as the ratio between power at 18h and ~24h ($power_{18h}$ / ($power_{18h}$ + $power_{\sim 24h}$)) and logit-transformed using base e

(log(*EQ*/(1-*EQ*))). EQs were separately calculated for wheel running (EQ_{wheel}) and body temperature (EQ_{Tb}) data.

Phase angles of entrainment.

Activity onsets were eye-fitted. Phase angles in $LD_{im}LD_{im}$ 7:5:7:5 were calculated separately for each of the alternate dark episodes. For all other light cycles, a single phase angle was calculated averaging onsets from all dark phases. Only animals that met criteria of entrainment (i.e., EQ > 0.75) were included in the analysis.

Period of free-running animals.

For animals that were poorly entrained to T18 ($EQ_{wheel} < 0.75$), periods of the free-running components in T18 were determined from periodograms calculated over 20 cycles as the peak power in the range from 22-26h.

Phase of free-run in $D_{im}D_{im}$.

Skipping the first 3 days in $D_{im}D_{im}$, eight consecutive eye-fitted onsets of activity were used for a least-squares regression to project the phase of activity onset at the day of release (Figure S2A). Watson-Williams test was used for differences in mean phase between the two times of release, and the Rayleigh test was used to test for uniformity in the distribution of projected activity onsets relative to onsets of $D_{im}D_{im}$.

Activity-independent temperature.

To calculate body temperature independent of activity a non-linear regression method was adapted from previously described techniques (Weinert and Waterhouse 1998; Damaggio and Gorman 2014) using only data collected during blocking of the running wheels. General locomotor data were convolved with a delay function (Equation 1) to create a smoothed activity measure that takes into account the delayed effect it has on temperature. Parameters were fit to get the highest correlation with GLA data. Next, the non-linear relationship between temperature

and activity was estimated (Equation 2). All parameters were fit using the non-linear least squares function in R (Rstudio - Version 0.98.1062, Boston, MA; R - version 3.1.1, Vienna, Austria). To estimate endogenous body temperature (Tb'), the activity-induced temperature was subtracted from the measured temperature signal with equation 2 fitted parameters.

$$A * e^{(-\frac{x}{B})} \quad (1)$$

$$\text{Temperature} = K * e^{(-1 * e^{(-L * Activity)})} + M \quad (2)$$

Surgery / Telemetry

Miniature radio telemeters (< 2.0 g; G2 E-Mitter; Respironics, Inc., Bend, OR) were implanted intraperitoneally (I.P.) under isoflurane anesthesia. Animals received a single dose of 0.05 mg/kg buprenorphine (S.C.) inter-operatively for pain relief. After the animals were ambulatory they were returned to colony conditions (LD_{ark} 14:10) and allowed 7 days for recovery before experiments started. Prior to surgery and during recovery mice were housed in the same-sex groups of 3-4. Cages were placed on an ER-4000 Energizer/Receiver (Respironics, Inc., Bend, OR) to communicate with the telemeters. Over the course of the experiment, four telemeters gave unusable data, so that body temperature (Tb) and general locomotor activity (GLA) analyses were conducted with n = 9 and n = 10 for Bif and nBif respectively.

Results

Experiment 1: Entrainment to T18 cycles.

Figure 2.1 depicts representative patterns of running-wheel activity (RW) over 12 weeks of mice initially bifurcated in T24 $LD_{im}LD_{im}$ 7:5:7:5 and exposed to cycles lengthening to T36 $LD_{im}LD_{im}$ 13:5:13:5 (A) and of mice initially non-bifurcated in T24 LD_{im}19:5 and exposed to cycles shortening to T18 LD_{im} 13:5 (B). In both cases and in all phases of the experiment, most activity occurs during the dimly lit nights, and there is no visually salient free-running periodicity.

Qualitatively, general locomotor activity (GLA) and body temperature (Tb) showed similar patterns as RW, with greater activity and higher temperatures in the scotophases (Figure S3). When uninterrupted wheel running of the same two mice is plotted modulo 18 (Figure 2.1C&D), both animals showed activity strictly aligned with the dark periods with activity onsets nearly coincident with lights off.

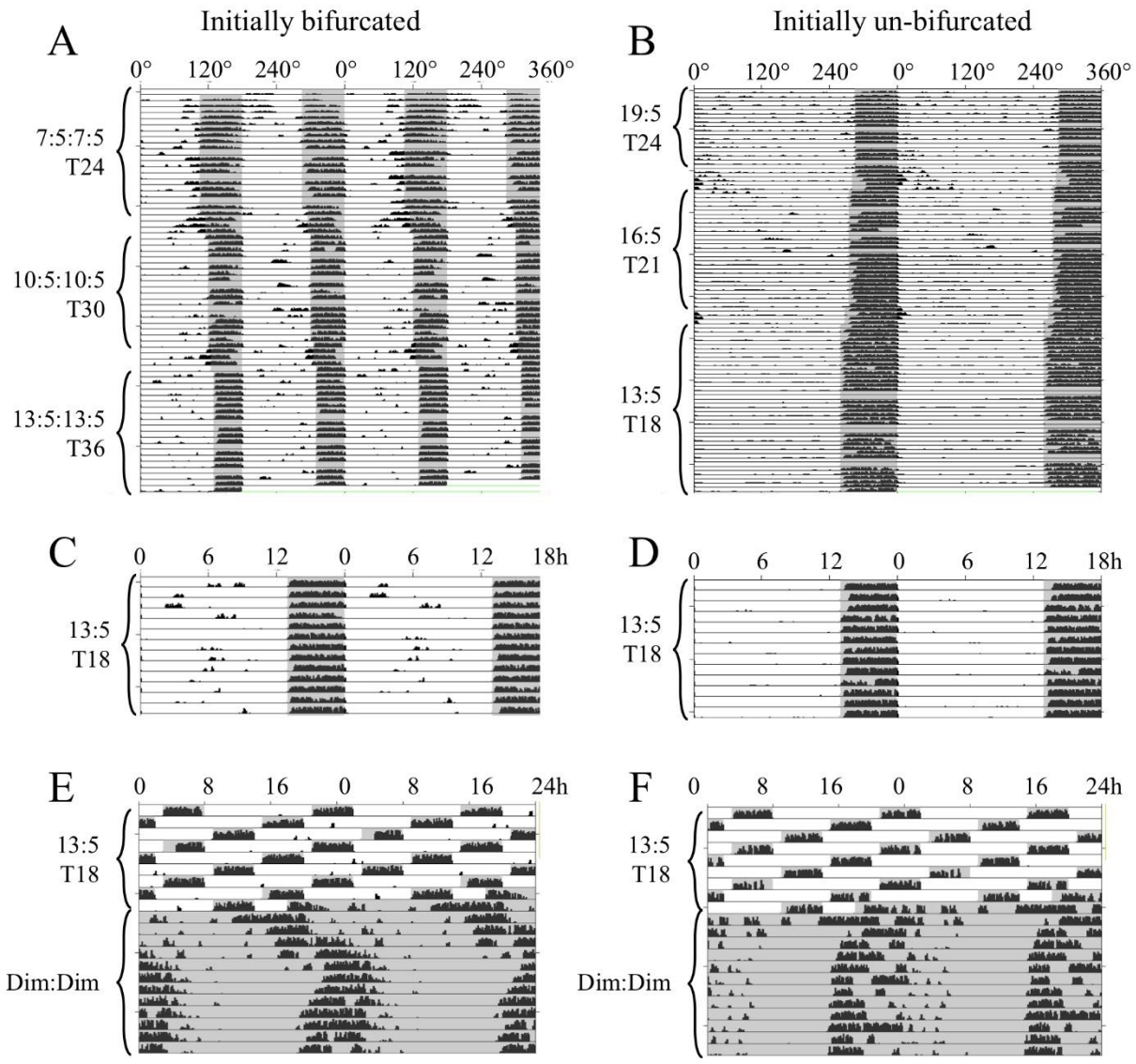

Figure 2.1. *Representative double-plotted wheel-running actograms from Experiment 1 of individual mice that were initially bifurcated (A) and not bifurcated (B) in LDimLDim 7:5:7:5 and LDim 19:5 respectively, and gradually transitioned to LDim 13:5. Data are plotted modulo 360 angular degrees. Gray shading indicates times of relative darkness. Gaps in data represent lack of wheel running activity while the wheels were blocked. Transitional photoperiods are not labeled. From the same animals, C and D replot, modulo 18h, the same 14 uninterrupted cycles from the final experimental phase. E and F show the transition from LDim13:5 to Dim:Dim, from the same animals. Y axes are scaled from 0 to 200 half revolutions per minute.*

Activity profiles averaged across subjects are depicted in Figure 2.2 demonstrating that, in each experimental phase including the initial bifurcation, activity was almost exclusively restricted to hours of darkness. EQ-values approached 1 and activity onsets shortly followed light offsets (Table 2.2, Figure S4). Under identical lighting conditions of T18 (LDim 13:5), quantitative analyses revealed no differences between Bif and nBif in LDim 13:5 in EQWheel (Figure S4), total amount of running wheel activity in day or night, or in phase angles between light offsets and activity onsets (Table 2.2, Figure 2.2). To distinguish between a 12h and 24h rhythm in Phase 1 (LDLD7:5:7:5), phase angles of wheel-running activity were compared between alternate scotophases. In 60% of animals there was a significant difference in phase angle in the odd nights compared to the even nights, indicative of a 24h basis to the entrainment. Under LDLD10:5:10:5 and LDLD13:5:13:5, however, no animals showed systematic differences between alternating nights, indicating 15/18h rather than 30/36h bases, respectively, of their rhythmicity (Figure 2.2). Therefore we averaged activity data from all scotophases to report a single phase angle under non-24h conditions.

Table 2.2 *Entrainment measures in LD_{im}13:5 from Experiment 1*

		Bif	**nBif**	**Statistic**	**p-value**
% RW activity in light		10 ± 3%	7 ± 2%	t(14.5) = 0.93	NS
Phase angle of entrainment		-21.7 ± 5.9 min	-10.6 ± 2.1 min	t (12.4) = 1.79	NS
Periodogram power (RW)	18h	559 ± 61	575 ± 56	t(16.7) = 0.20	NS
	22-26h	43 ± 13	18 ± 4	t(9.47) = 1.82	NS
Periodogram power (Tb)	18h	173 ± 53	179 ± 53	t(16.9) = 0.09	NS
	22-26h	94 ± 47	74 ± 28	t(13.2) = 0.36	NS
EQ (logit - transformed)	RW	2.79 ± 0.4	3.57 ± 0.3	Group: F(1,15) = 1.09 Measure: F(1,15) = 70.31 Interaction: F(1,15) = 0.85	NS < 0.001 NS
	Tb	0.85 ± 0.5	1.15 ± 0.5		

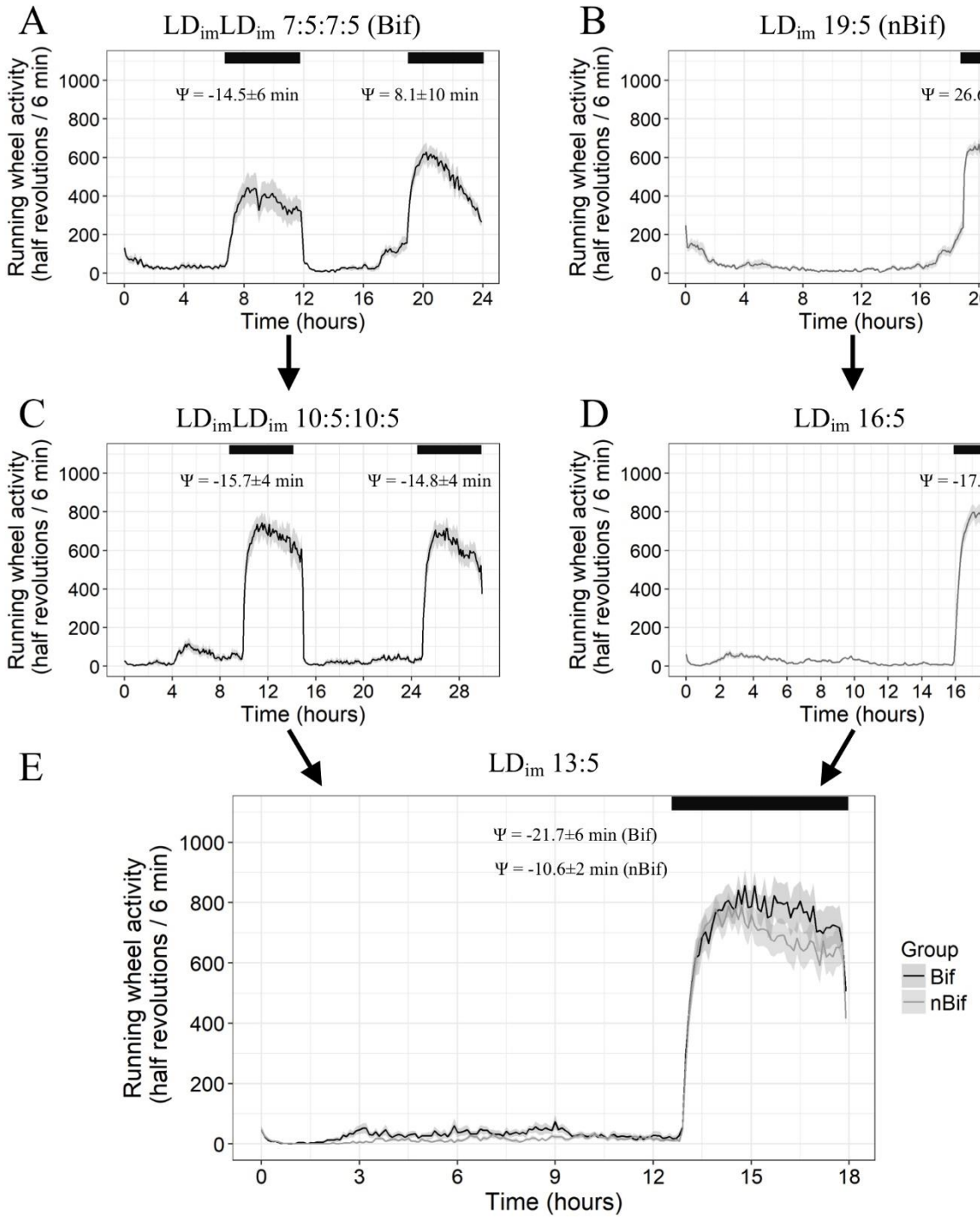

Figure 2.2. *Averaged time series of running wheel activity (half wheel revolution) in $LD_{im}LD_{im}$7:5:7:5 (A), $LD_{im}LD_{im}$10:5:10:5 (C), and LD_{im}13:5 (E) for initially bifurcated animals and LD_{im}19:5 (B), LD_{im}16:5 (D) and LD_{im}13:5 (E) for un-bifurcated animals from Experiment 1 across all days with wheel. Shading represents between-subject SE of the activity profiles. Black bars represent dark phases. Phase angles are shown mean ± sem (min) with positive values indicating that activity phase-leads onset of darkness. Y axes represent half revolutions per 6 min bin.*

In all animals the EQ_{Tb} was lower than the EQ_{Wheel} ($F(1,15) = 70.31$, $p < 0.001$, Figure S4), independent of Bif/nBif (Interaction: $F(1,15) = 0.9$, $p > 0.05$). Lower EQ_{Tb} was reflected not only by lower T18-power but also statistically greater power in the free-running, circadian (22-26h) range (Figure S5). Entrainment Quotients were significantly lower in males than in females ($F(1,15) = 4.88$, $p < 0.05$). A more comprehensive analysis of sex differences in this and other studies will be published separately.

Bifurcated Body Temperature in LDLD 7:5:7:5

Wheel blockage altered the Tb rhythm, as evident in the generally lower body temperatures during dark periods when wheels were blocked (Figure S6A&C). Moreover, Tb closely tracked GLA suggestive of additional acute effects of non-running activity on Tb (Figure 2.3A&C). As would be expected, nBif mice showed generally unimodal Tb with elevation during the night. Rhythms of bifurcated mice exhibited bimodality with elevations of Tb in both nights (Figure 2.3A&C). Similar to Tb, Tb' was elevated during the dim lit nights in both Bif and nBif. That is, when controlling for levels of GLA, Tb' is still bimodal in Bif (Figure S7). In N1 (shared night) Tb' trended lower in Bif than in nBif ($t(12.5)= 2.1$, $p = 0.056$; See Figure 2.3), yielding a lower amplitude rhythm. Averaged across the 5h dim nights, Tb' during N2 (additional night) was significantly higher in Bif-mice compared to nBif-mice ($t(16.0)= 4.91$, $p < 0.001$). There was no difference between N1 and N2 in Bif animals ($t(10.6) = 1.30$, $p > 0.05$), while for nBif mice, Tb' was significantly higher in the night than at anti-phase ($t(17.7) = 9.0$, $p < 0.001$).

Body Temperature in T18 LD13:5

Also in T18 (LD 13:5), Tb closely tracked activity (Figure S6B&D), with higher levels of activity and body temperature during the night ($F(1,16) = 88$, $p < 0.0001$; Figure 2.3B&D). However, lower EQ_{Tb} (i.e. higher power in circadian range) did not allow for similar methods used in T24 to control for levels GLA.

Release into constant conditions

When released into $D_{im}D_{im}$, animals reverted to free-run close to 24h within a few cycles (Figure 2.1E and F). The projected phases of activity onset were significantly clustered with a mean phase angle of -0.09h ($R(19) = 0.43$; $p < 0.05$, Figure S2) relative to dark onset. Individually, neither release group was significantly clustered ($R(9) = 0.49$; $p < 0.09$ and $R(9) = 0.37$; $p < 0.25$ for 0 and +9 respectively) but small sample sizes compromised the statistical power of these tests. When the time of release into $D_{im}D_{im}$ was delayed by 9h, the projected onsets were delayed 8.5h compared to those mice beginning $D_{im}D_{im}$ at the beginning of a previously scheduled scotophase ($F(1,18) = 14.3$; $p = 0.001$).

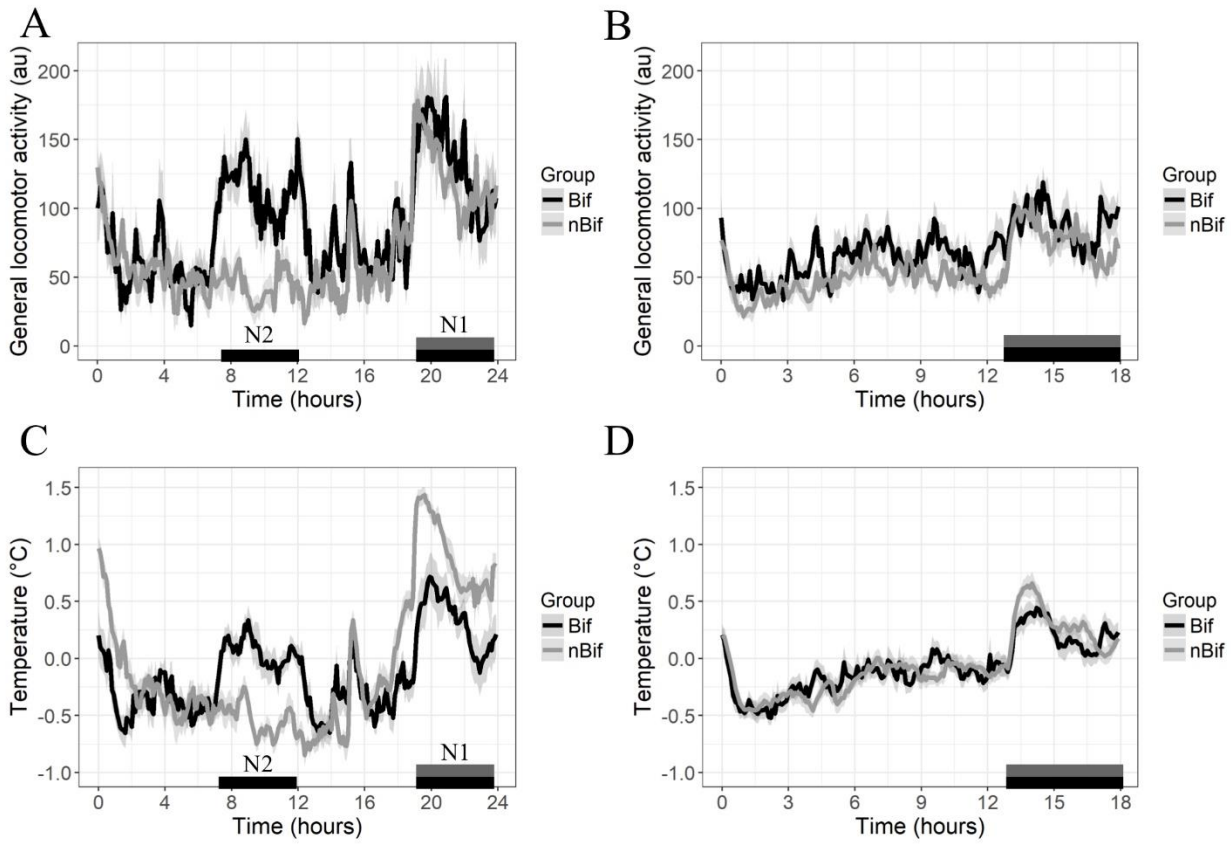

Figure 2.3. *(A, B) GLA and (C, D) Tb averaged across 72 hours of wheel blocking in Experiment 1 during 24h conditions and 18h conditions respectively. Temperature is plotted in degrees above/below the daily mean. Bars on bottom of the panels represent the light:dark cycle, with black/gray being dim and white being light. Black and gray represent Bif and nBif groups, respectively. Shading represents SE. N1 = 19-24h and N2 = 7-12h.*

Experiment 2: Cues for induction and maintenance of T18 entrainment

Induction

As in Experiment 1, representative mice that were bifurcated in $LD_{im}LD_{im}$7:5:7:5 entrained readily, as evident from alignment of activity with shaded dark periods, when transferred directly to LD_{im}13:5 (Figure 2.4A, C and E). The same was true for mice bifurcated in $LD_{im}LD_{im}$7:5:7:5 and transferred to LD_{ark}13:5 and mice transferred from LD_{im}14:10 to LD_{im}13:5 (actograms not shown). In contrast, mice transferred from LD_{ark}14:10 to LD_{ark}13:5 showed unambiguous evidence of free-running activity, recognized as a lack of alignment in with a clear free running period > 24h (Figure 2.4B, D and F).

Considering all subjects, entrainment failed only in the group without exposure to dim light or a history of bifurcation (nBif-Dark) as reflected in multiple measures (Table 2.3; Figure S8). Periodogram power at 18h was significantly different across groups ($F(3,24) = 5.931$, $p < 0.01$) and lowest in nBif-Dark compared to all other groups (Tukey HSD-adjusted p-values < 0.05). Power in the circadian range differed significantly between groups ($F(3,24) = 3.661$, $p < 0.05$). Both Dim groups showed significant lower power than nBif-Dark (Tukey HSD-adjusted p-values < 0.05). The EQ_{Wheel} was lowest in the nBif-Dark group, and significantly different from all other groups ($F(3,24) = 9.18$, $p < 0.001$; all Tukey HSD-adjusted p-values < 0.05).

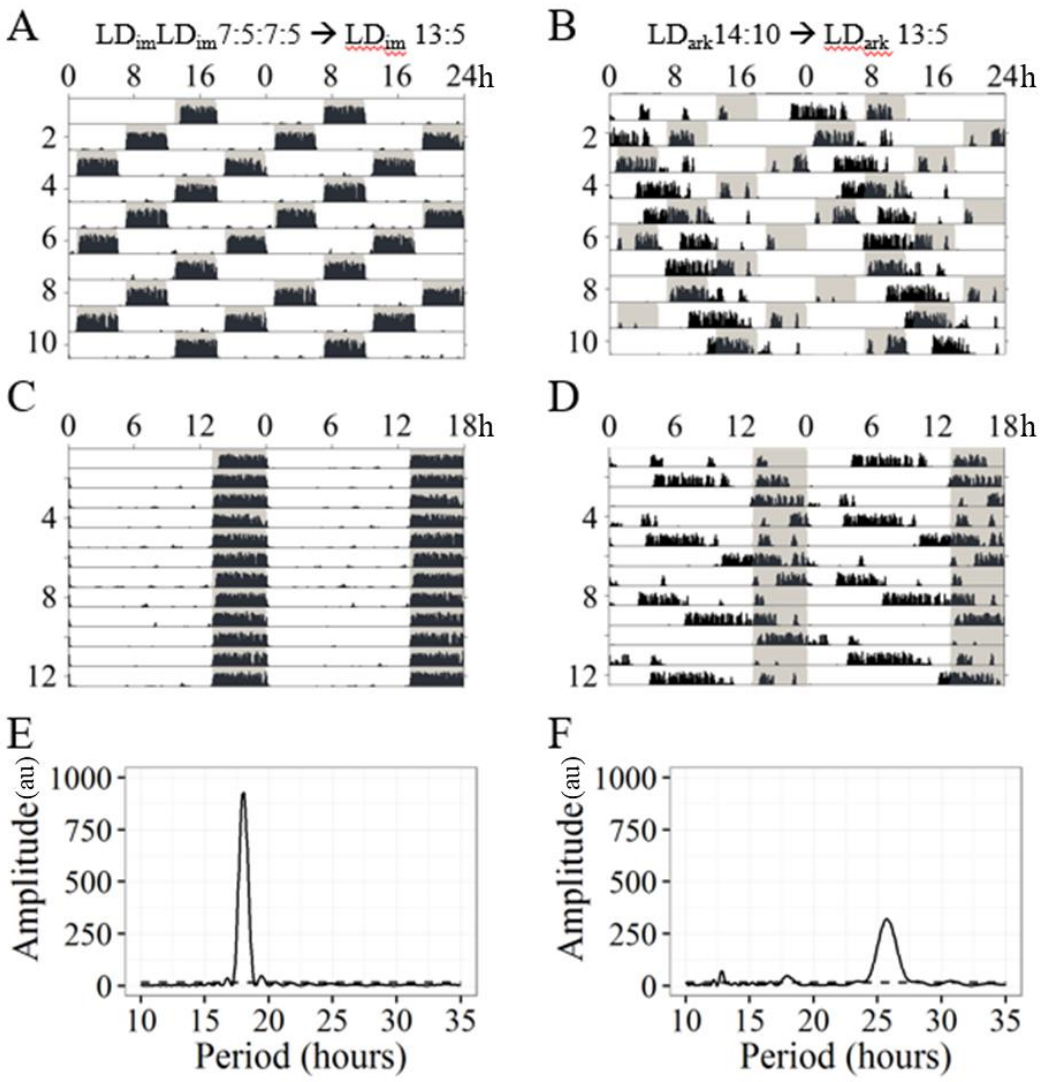

Figure 2.4. *Representative wheel running data from Experiment 2 with respective periodogram for a successfully entrained animal from Bif-Dim (A,C,E) and a free-running animal from nBif-Dark (B,D,F). A and B show the same data as C and D in modulo 24h and 18h respectively. Conventions as in Figure 2.1.*

Maintenance.

As the light to dark ratio in T18 was progressively reduced by shortening photophases over the course of the experiment, all animals eventually lost entrainment and started to free-run in or before reaching LD 1:17 (Figure 2.5A&B). Values of EQ_{Wheel} significantly decreased over the different phases within the experiment (Figure 2.5C; $F(1,23) = 728.48$, $p < 0.001$), and eventually approached zero (i.e. periodogram power exclusively in the circadian range). The pattern of entrainment loss differed by group as indicated by repeated measures ANOVA (Group: $F(3,23) = 5.51$, $p < 0.01$; Interaction with phase:($F(3,23) = 5.67$, $p < 0.01$). Group differences based on one-way ANOVA within each phase are indicated in Figure 2.5C. Phase angles did not change between photoperiods (b < -0.002, SE < 0.001, t = 1.80; 95% CI = -0.004; 0.0003).

Table 2.3 *Entrainment measures in LD_{im}13:5 from Experiment 2*

		nBif → Dark	Bif → Dark	nBif → Dim	Bif → Dim	Statistic	p-value
% RW activity in light		31 ± 6% a	14 ± 3% b	9 ± 2% b	7 ± 2% b	F(3,24) = 8.91 -> post hoc groups	<0.001
Phase angle of entrainment		Not determined	-13 ± 5 min	-19 ± 3 min	-16 ± 2 min	F(2,16) = 0.97	NS
Periodogram power (RW)	18h	363 ± 92 a	670 ± 92 b	678 ± 42 b	769 ± 60 b	F(3,24) = 5.931 -> post hoc groups	<0.01
	22-26h	107 ± 33 a	50 ± 28 ab	21 ± 6 b	14 ± 2 b	F(3,24) = 3.661 -> post hoc groups	<0.05
EQ (logit - transformed)	RW	0.92 ± .7 a	3.11 ± .5 b	3.65 ± .3 b	4.01 ± .2 b	F(3,24) = 9.18 -> post hoc groups	< 0.001

Groups not sharing a letter (a/b) are statistically different according to post hoc analyses

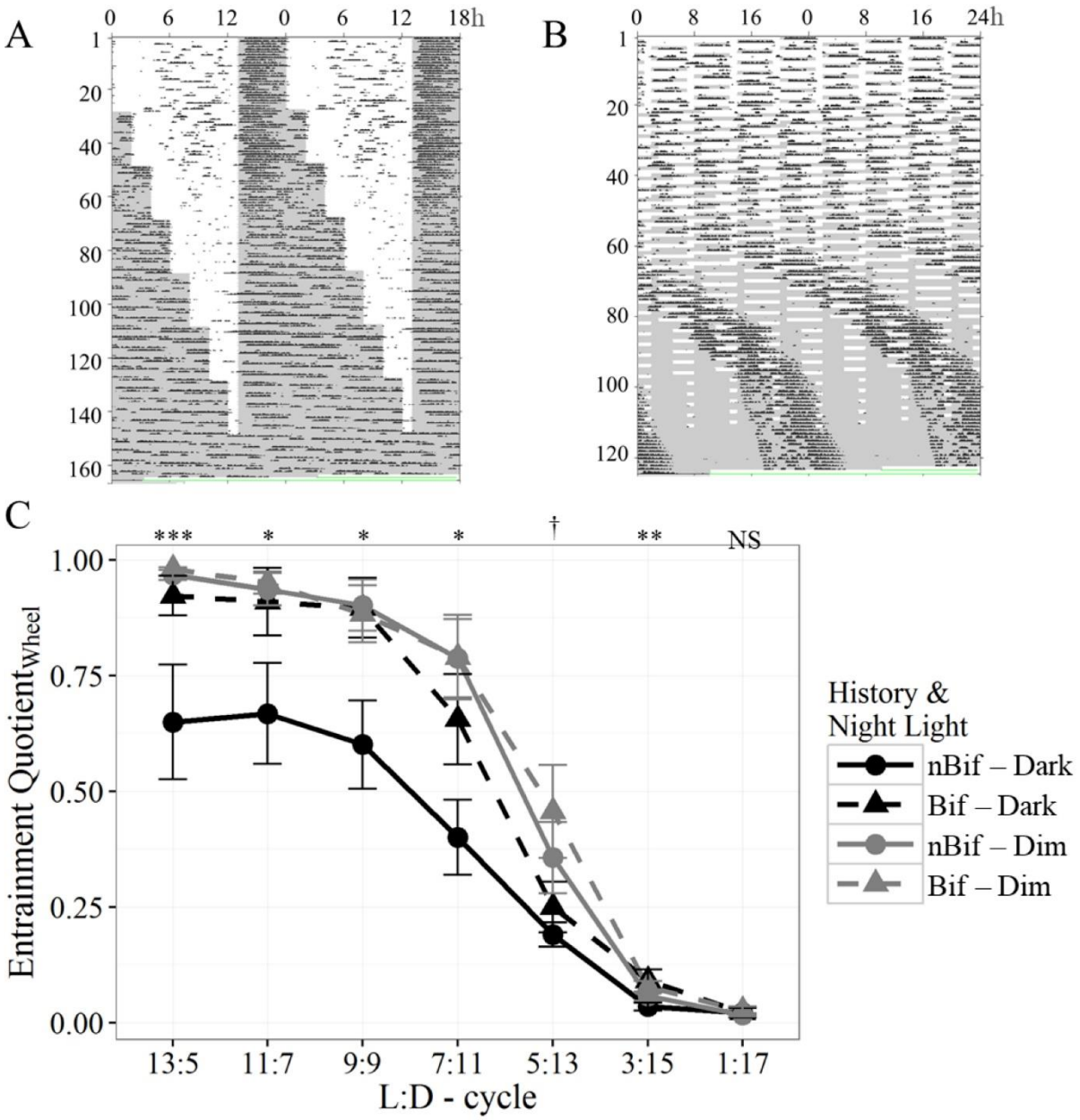

Figure 2.5. *Representative actograms from Experiment 2 plotted modulo 18h (A) and modulo 24h (B) of one animal across all different T18 conditions. (C) EQ_{Wheel} in all T18 conditions represented as mean ± SE. Significance codes show one-way ANOVA results for group differences in each phase: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < NS < 1*

Period of free-running animals.

Tau significantly decreased as the light to dark ratio decreased (*b* = -0.014, SE = 0.001, t = -12.80), such that for every 1% decrement in light duration, tau was reduced by 0.014 hour (95% CI = -0.016; -0.012). Tau was not significantly different between groups (*b* = -0.076, SE = 0.082, t = -0.93 and *b* = -0.099, SE = 0.082, t = -1.19 for history and dim light respectively).

Body weight

Relative weight gain over the full duration of the experiment negatively correlated with the ratio of entrainment in both Experiment 1 (*b* = -0.05, SE = 0.01, t = -5.28) and Experiment 2 (*b* = -0.07, SE = 0.02, t = -3.57) (Figure S9).

Discussion

The present study demonstrates that under permissive conditions, neurologically intact wild-type mice are capable of robust behavioral entrainment to 18h LD cycles; that exposure to dim light at night, an immediate history of rhythm bifurcation, and scotophases ≤ 9h each facilitate or permit this behavioral entrainment; that body temperature rhythms adapt also to 18h days but in some animals exhibit larger non-entrained rhythmic components than are apparent in wheel-running data; and that demasking of body temperature rhythms for effects of activity establish a bifurcated, but reduced amplitude rhythm of endogenous body temperature.

In Experiment 1, the most unanticipated result was that, regardless of the T-cycle (LD 7:5:7:5, 10:5:10:5, 13:5, 16:5, and 19:5) mice almost uniformly adopted activity rhythms with activity closely aligned with the scotophases, minimal activity in the photophases and rare or absent evidence of free-running rhythmicity. The strong apparent behavioral entrainment to T18 stands in marked contrast to previous reports from C57 mice or other rodents. As examples, 17% of mice failed to entrain wheel-running activity to T21 (Molyneux et al. 2008), and lacking a wheel, 80% failed to entrain to T21 (Casiraghi et al. 2012). Housed in groups of five without wheels, no mice entrained to T20 (Karatsoreos et al. 2011). In a paradigm that shortened T by

5 min daily, wheel-running mice maintained apparent entrainment as far as 20h, although mice were never exposed to more than one cycle of T20 (Pittendrigh and Daan 1976a; Aton et al. 2004). Finally, using various durations of light pulses, the maximum phase advance shown in C57 mice never exceeded 2-3 hours (Schwartz and Zimmerman 1990; Comas et al. 2006), leading to the prediction that 22h would be the lower limit of the entrainment range. Combined, the summarized experiments conclude that entrainment to T18, which requires a daily shaving of nearly 6h from the free-running period, even when housed in apparent optimal conditions (i.e, singly housed with wheel (Cambras et al. 2000; Chiesa et al. 2005)) is unprecedented.

In previous studies, a history of rhythm bifurcation in T24 LDLD was shown to confer extraordinary flexibility of entrainment and re-entrainment in rodents. Specifically, bifurcated hamsters exhibited nearly instantaneous entrainment to LD16:8 photoperiods of any phase, whereas unbifurcated hamsters maintained under conventional LD16:8 photocycles entrained more slowly and in proportion to the size of the required phase shift. Parenthetically, animals with a history of short daylength (LD8:16) exposure re-entrained to these same phase shifts at intermediate rates (Harrison and Gorman 2015). Additionally, bifurcated mice exhibited robust behavioral entrainment to a LDLD10:5:10:5 (T15/T30) schedule (Harrison et al. 2016). Knowing already that bifurcation allows entrainment to Phase 2 (T15/T30) conditions of Experiment 1, we predicted that further extension of the photophase to LD13:5 (T18/T36) would continue to permit behavioral entrainment. In contrast, after entrainment to long daylengths such as LD19:5 (T24) that preclude bifurcation, we expected failed entrainment upon reduction of the photophase to LD13:5 (T18). Contrary to expectation, mice adapted well and quantitatively equivalently to LD13:5 after these entrainment histories. Thus with respect to multiple indices of wheel-running behavior and Tb, we see no evidence for a bifurcation after-effect in the present study, although we cannot exclude the possibility that underlying clock mechanisms differ following these histories. Moreover, as such extreme entrainment outside of a bifurcation context was

unprecedented, we hypothesized *post hoc* that dim light alone may be sufficient to significantly extend the range of entrainment in the same manner as does rhythm bifurcation. Notably, dim light was previously shown to extend the limit of entrainment in hamsters (Boulos et al. 2002; Chiesa et al. 2005; Gorman et al. 2005) but was without a substantial effect in enhancing entrainment of mice to a different T30 photocycle (LD20:10, Harrison et al. 2016).

Since the T18 cycle represents an extreme with respect to expected limits of entrainment, it raises the possibility that the apparently entrained behavioral rhythms reflect masking rather than true entrainment. Experiment 2 excludes the possibility of acute *positive* masking effects of dim light by demonstrating that, under identical lighting schedules with dark nights, wheel-running rhythms match the T cycle or free-run depending only on their *prior* entrainment history. Thus, concurrent dim light cannot explain behavioral entrainment. Rather, prior bifurcation, enabled by dim light, is sufficient to permit entrainment to T18. Prior bifurcation, moreover, is not necessary for entrainment to T18 as dim light throughout the experiment yields the same strong entrainment in the absence of bifurcation. The uniformly robust entrainment in Experiment 1, thus, may be interpreted as resulting from the facilitative effects of dim light in the initially unbifurcated group. Likewise, positive masking was shown to be an inadequate explanation for apparent entrainment to T15/T30, as it too persisted following bifurcation without continuation of dim light (Harrison et al. 2016).

Strong *negative* masking by bright light could additionally contribute to a misleading impression of entrainment of rhythms that were, in fact, free-running. Such an interpretation is discounted by the EQ values obtained as the photophases were progressively shortened in T18. Despite the reduced opportunity for negative masking by bright light, EQs were unchanged in each group as the fraction of light in the cycle decreased from 72% to 50%. In rats, rhythmicity in the circadian range has been shown to increase with increasing scotophase length in T22 and T23 LD cycles (Cambras et al. 2004). Only when scotophases were lengthened to 11h did

our mice begin to show stronger free-running components and lower EQ_{wheel}. As the zeitgeber was further weakened, all mice eventually showed visually obvious free-running rhythms and correspondingly low EQ scores.

Additionally, a masking interpretation is discounted by demonstration of *phase control*, another important entrainment criterion, in $D_{im}D_{im}$. In Experiment 1, the phase of activity onsets in constant dim was significantly clustered around and controlled by onsets of constant conditions, and the mean phase difference between the two groups was close to the difference in time of release. This phase control is evident despite the fact that free-running rhythms quickly go back to species typical periods near 24h. If apparent entrainment in LD13:5 had been a masked free-running activity pattern, random phases with respect to onset of $D_{im}D_{im}$ would be expected, as phase would be predicted by the phase of the masked free-running rhythm (Harrison et al. 2016). Instead, circadian free-running rhythms with a phase controlled by time of release are indicative of a complete reset at time of release, discounting the possible explanation of a masked free-running rhythm for apparent entrainment in T18. These findings corroborate similar findings on phase of activity onsets after release in DD following entrainment in T15/T30 (Harrison et al. 2016).

Thus, multiple criteria indicate that behavioral entrainment to T18 is genuine and stable but needs a sufficiently strong zeitgeber to be maintained. Notably, however, T18 entrainment did not show the changes in activity onset phase angle as predicted by classical non-parametric entrainment theory (Daan and Aschoff 2001; Granada et al. 2013; Schmal et al. 2015) or reported in rodents under less extreme values of T (Molyneux et al. 2008; Schwartz et al. 2011). Rather, activity onset consistently occurred close to the time of lights off. This same pattern was previous observed in mice entrained to T30 LDLD cycles (Harrison et al. 2016). Thus, entrainment under these conditions likely does not derive from non-parametric resetting mechanisms. Indeed, even with single light pulses as long as 9h, C57 mice show only minimal

(e.g., 1-2h) phase advances (Comas et al. 2006) that fall far short of the ~6h advances required for non-parametric entrainment. Perhaps importantly, these long-pulse PRCs were collected against a background of complete darkness instead of dim illumination. Mechanistically, we speculate that this flexible behavioral entrainment is facilitated by altered SCN function although whether this involves changes in phase relationships and/or amplitudes of component oscillators awaits time series analysis of SCN clock gene expression.

Because wheel-running increases body temperature, we measured rhythms over three separate cycles when access to wheels was blocked. Tb rhythms, as well as GLA, during intervals of no wheel access were clearly bifurcated in T24 and showed nighttime elevations in T18 (Figure 2.3). In both cases, however, the amplitude was markedly reduced compared to mice in LD19:5. This is the first report of Tb in rodents entrained to T18. Body temperature is an important cue for synchronizing peripheral organs (Brown et al. 2002), as temperature cycles with 1.5°C amplitude have been shown sufficient to entrain isolated lung tissue to non-24h T-cycles (Abraham et al. 2010). Therefore, these findings are potentially important for functional rhythmicity of peripheral organs such as liver or kidney in extreme T- cycles.

While behavioral entrainment to T18 was extremely robust as reflected in EQ_{wheel} scores approaching 1, periodogram analysis of Tb data yielded discernable rhythmicity in the circadian range, thereby reducing EQ_{Tb} values relative to EQ_{Wheel} (Figure S4). Simultaneous expression of multiple periods -- some entrained and others free-running -- has been noted previously. Activity rhythms dissociate in mice in T21 (Casiraghi et al. 2012), and both activity and body temperature show multiple rhythmic components in rats in T22 and T23 (Anglès-Pujolràs et al. 2007). Additionally, in rats two motor activity components can be forced to decouple in T22 and are associated with subdivisions of the SCN (de la Iglesia et al. 2004). Decoupling of behavioral and Tb rhythms could rely on either reorganization of SCN subunits and/or decoupling of SCN

and extra-SCN oscillators that differentially contribute to regulation of activity and body temperature.

Even in the absence of a wheel, however, measures of Tb reflect the joint influences of any endogenous Tb rhythm and activity-induced temperature. Through correlational analyses, we further estimated general activity-independent rhythms in body temperature. The additive effect of activity on temperature has a clear ceiling effect: above a certain level of activity, more activity does not increase temperature any further. Therefore our non-linear model to estimate circadian body temperature may more accurately characterize endogenous Tb rhythms than would prior linear models (Weinert and Waterhouse 1998). Bimodality of body temperature (linearly de-masked with the Weinert and Waterhouse-model) in Siberian hamsters during bifurcation has been previously established (Rosenthal et al. 2005). However, in mice, it is novel that not only running wheel behavior, but also activity-independent body temperature, is bifurcated in LDLD 7:5:7:5.

Both human shift-work and multiple experimental models of circadian disruption in animal models are associated with weight gain and/or markers of metabolic disease (De Bacquer et al. 2009; Barclay et al. 2012). The critical dimensions of these complex exposure scenarios for metabolic risk are not yet fully defined. Although our experiments were not designed with a focus on body weight regulation, consistent negative correlations between weight gain and EQ_{Wheel} in LD 13:5 suggest that poor entrainment leads to greater weight gain than does stable entrainment to non 24h days. This association was obtained despite the fact that interval of weight gain included many different T-cycles (Experiment 1) and different photoperiods in T18 (Experiment 2).

In summary, rhythm bifurcation and/or very dim light at light permits robust running-wheel entrainment to an 18 hour light-cycle that, to our knowledge, exceeds the shortest demonstrated limit of entrainment in mice. As body temperature exhibited greater periodicity

~24h, adaptation to T18 is not complete and may reflect decoupling of multiple circadian oscillators as suggested in other experimental contexts (de la Iglesia et al. 2004; Anglès-Pujolràs et al. 2007; Casiraghi et al. 2012). These findings extend previous work establishing hyperplastic control of rest/activity cycles that, regardless of their mechanistic basis, may have translational utility for behavioral adaptation of humans to challenging shift-work or jet-lag exposures.

Acknowledgment

The authors would like to thank Elizabeth Harrison for comments on an early draft of the manuscript and Antonio Mora and Gil Sanchez for their excellent animal care. This work was supported by the Office of Naval Research N000141310285.

Chapter 2, in full, is a reprint of the material as it appears in *Walbeek, Thijs J., and Gorman, Michael R. 2017. "Simple Lighting Manipulations Facilitate Behavioral Entrainment of Mice to 18-h Days." Journal of Biological Rhythms 32 (4): 309–22. https://doi.org/10.1177/0748730417718347.* The dissertation author was the primary investigator and author of this paper.

Chapter 3. Enhanced circadian entrainment in mice and its utility under human shiftwork schedules

Abstract

The circadian system is generally considered to be incapable of adjusting to rapid changes in sleep/work demands. In shiftworkers this leads to chronic circadian disruption and sleep loss, which together predict underperformance at work and negative health consequences. Two distinct experimental protocols have been proposed to increase circadian flexibility in rodents using dim light at night: rhythm bifurcation and T-cycle (i.e. day length) entrainment. Successful translation of such protocols to human shiftworkers could facilitate alignment of internal time with external demands. To assess entrainment flexibility following bifurcation and exposure to T-cycles, mice in Study 1 were repeatedly phase shifted. Mice from experimental conditions rapidly phase-shifted their activity, while control mice showed expected transient misalignment. In Study 2 and 3, mice followed a several weeks-long intervention designed to model a modified DuPont or Continental shiftwork schedule, respectively. For both schedules, bifurcation and nocturnal dim lighting reduced circadian misalignment. Together, these studies demonstrate proof of concept that mammalian circadian systems can be rendered sufficiently flexible to adapt to multiple, rapidly changing shiftwork schedules. Flexible adaptation to exotic light-dark cycles likely relies on entrainment mechanisms that are distinct from traditional entrainment.

Keywords: Mouse; Shiftwork; Phase shift; Bifurcation; T-Cycle; Flexible Entrainment; Dim light.

General introduction

Many occupational settings, including hospitals, emergency response and transportation, require staff to work at any hour during the day. Working night shifts has been associated with both acute and chronic negative consequences (Moreno et al. 2019). Acute effects, including increased error rates, are likely a result of both work during the biological night and partial sleep deprivation. With chronic exposure to shiftwork, risks of attrition and development of physiological and mental health problems increase (Evans and Davidson 2013). The World Health Organization has labeled shiftwork as a probable carcinogen (Straif et al. 2007), and the American College of Emergency Physicians, to cite just one example, has published guidelines for best practices to mitigate some of its adverse effects (Benzoni 2017). Despite increased awareness of these issues, few effective treatments exist to alleviate these negative consequences. Many attempts to adjust work-schedules to optimize circadian health appear unsuccessful, in part due to competing priorities from individuals as well as organizations (Smith and Eastman 2012). As an alternative, a more flexible circadian system could provide a solution to adapt to rapidly changing work and sleep cycles. Enhanced clock resetting has been observed with pharmacological treatments (Kessler et al. 2008; An et al. 2013), or following exposure to constant light (Kaur et al. 2009) or short photoperiods (Glickman et al. 2012; Glickman et al. 2014; Ramkisoensing et al. 2014). While theoretically important, how these findings may be translated for human benefit is unclear.

Accumulating rodent studies have shown that non-invasive environmental manipulations can markedly increase circadian flexibility. First, compared to complete darkness, the mere addition of dim light at night (< 0.1 lux) a) cut in half the time required to re-entrain to acute phase shifts in the light-dark cycle (Evans et al. 2009); b) facilitated induction of stable bimodal activity patterns in 24h light:dark:light:dark cycles (i.e. rhythm bifurcation in LDLD) (Gorman and Elliott 2003); and c) expanded the range of behavioral entrainment far beyond conventionally

understood limits, including 18 and 30h cycles (Gorman et al. 2005; Walbeek and Gorman 2017). Second, following rhythm bifurcation under dimly lit nights, hamsters almost immediately adjusted to light:dark (LD) shifts to any phase (Harrison and Gorman 2015). In hamsters and mice, the range of entrainment to LDLD cycles was greatly expanded to include 18h–30h zeitgeber periods (Harrison et al. 2016; Walbeek and Gorman 2017; Gorman and Elliott 2019). In at least some cases, this enhanced entrainability after bifurcation persisted without the continuation of dim night-time lighting (Harrison et al. 2016).

In 2012, our group proposed consideration of such flexible entrainment as a potential strategy for shiftworkers to navigate complex conflicts between work schedules, social obligations, and circadian rhythmicity, with a focus on accommodation of permanent night-shiftwork (Harrison and Gorman 2012). In light of the enhanced resetting and T-cycle discoveries since 2012 and described above, we see potential for translation to the more common non-permanent shiftwork, where work times may occur at any hour. Thus, here we present three experiments to further characterize rhythm bifurcation and T-cycle entrainment, their underlying mechanisms, and their ability to facilitate adaptation to on-demand schedules. In Study 1 (“Jitter”), we investigated entrainment mechanisms of flexible behavior by probing animals entrained to exotic lighting paradigms with repeated, systematic phase perturbations. In Study 2 (“DuPont”), we used bifurcation to schedule activity according to a common DuPont work routine that alternates blocks of 12h shifts. Lastly, in Study 3 (“Continental”), we tested whether mice could adapt to a rapidly delaying shift schedule (modified Continental) during weekdays and still be well-entrained to normal conditions on weekends.

Results

Study 1/Jitter - Nature of entrainment to bifurcated and non-24h cycles

Previous studies have demonstrated flexible entrainment in lighting regimes generally considered to be far outside the normal range of entrainment, including exposure to both LD

(e.g. 13h of light, 5h dark; LD13:5 or T18LD) and LDLD (e.g. LDLD5:4:5:4 or T18LDLD) cycles (see methods for explanation of nomenclature) (Harrison et al. 2016; Walbeek and Gorman 2017; Gorman and Elliott 2019). Under those cycles that have been carefully characterized, behavioral adaptation reflects bona fide entrainment. Notably, an explanation of simple masking for entrained behavior has been rejected. For example, following both LDLD and extreme T-cycles there is phase control in DD (Harrison et al. 2016; Walbeek and Gorman 2017; Sun et al. 2019) and reorganized rhythms of core body temperature (Rosenthal et al. 2005; Walbeek and Gorman 2017). In LDLD conditions alone, further evidence includes persistence of biphasic behavior in skeleton cycles (Gorman and Elliott 2003), enhanced phase resetting (Harrison and Gorman 2015; Noguchi et al. 2018), entrainment after-effects (Sun et al. 2019), biphasic melatonin secretion (Raiewski et al. 2012), and altered c-Fos and clock gene expression in the Suprachiasmatic nucleus (SCN) (Gorman et al. 2001; Yan et al. 2010; Noguchi et al. 2018)

On the other hand, entrainment to extreme T-cycles lacks the characteristic large phase angle modulation (Gorman and Elliott 2019) and period after-effects predicted by classical non-parametric entrainment theory (Pittendrigh and Daan 1976a; Granada et al. 2013). This suggests that the enhanced circadian plasticity described above may differ mechanistically from classical entrainment. Study 1/Jitter, therefore, aimed to find evidence of oscillator-driven behavior in T24LDLD, T30LDLD, and T36LDLD. Transient misalignment following a rapid change in the phase of a zeitgeber is indicative of a slow-shifting oscillator. Hence, mice in Study 1/Jitter were exposed to repeated phase shifts, while the prevalence and magnitude of transients in onsets and offsets of behavior were scored. Any diminution of transients would be interpreted as behavior being controlled by light directly or an extremely rapidly shifting oscillator, rather than by the conventional strong oscillator believed to be the core of mammalian circadian rhythmicity.

Study 1/Jitter - Results

Male and female mice were exposed to a standard laboratory photoperiod (14h light, 10h dark; LD14:10) as a control or one of three experimental light-dark cycles. All three experimental conditions were first bifurcated in a 24h light:dark:light:dark (LDLD) cycle. One group remained in that condition (T24LDLD) and the other two groups were subsequently exposed to 30 or 36h LDLD conditions (T30LDLD and T36LDLD, respectively) as illustrated for representative animals in Figure 3.1. Each of these animals remained very well-adapted to the light-dark cycles throughout the experiment, as evident from little activity in the light, and no visible free-running components. A measure of equality of division of activity between alternate scotophases in LDLD, the Bifurcation Symmetry Index (BSI; (Harrison et al. 2016)), indicated that mice were well-bifurcated during the first phase of the experiment (0.71±0.08 and 0.56±0.11 for females and males respectively; $t(10.0) = 1.14$; $p = 0.28$). A complementary measure that quantifies behavioral adaptation in T-cycles, Entrainment Quotient (EQ; (Harrison et al. 2016)), showed that mice were likewise well-adapted in T30 baseline (0.96±0.02 and 0.83±0.06 for females and males) and T36 baseline (0.97±0.01 and 0.91±0.05 females and males). Considering data from the two non-24h conditions, EQ-values were significantly higher in females than males ($F(1,28) = 5.93$; $p < 0.05$), but did not differ between T30 and T36 ($F(1,28) = 1.32$; $p = 0.26$), and there was no interaction between T-cycle and sex ($F(1,28) = 0.77$; $p = 0.39$).

After stable entrainment in baseline, mice were exposed to alternating phase delays and phase advances of 2 and 4h each. In the three LDLD conditions, only every second scotophase was phase-shifted. Figure 3.2 represents average activity onsets and offsets following delaying and advancing phase shifts for each of the 4 lighting paradigms. Following a 2h phase delay in LD (Fig. 3.2A), onsets were delayed on the first shift, showed no transients over three days, and appeared to be negatively masked by lighting. On the other hand, activity offsets after the first

shift were delayed by approximately 1h, but also showed reliable transients, such that they were delayed by an additional 22 minutes on subsequent days. The opposite pattern was observed following a 2h phase advance, where onsets showed a first-day advance of 1.25h and additional transients of 15 minutes per day, while activity offsets coincided with the light transition. During the 4h phase shifts, a comparable pattern of re-entrainment was observed, but delaying and advancing transients were 35 and 25 minutes per day, respectively. In contrast, in all other conditions transients phase shifts in onsets and offsets were largely complete on the first day and transients were absent or minimal (7 and 8 min changes, respectively after 4h advance in T24LDLD (Fig. 3.2B) and 4h delay in T30LDLD (Fig. 3.2C)). No significant transients were observed in T36LDLD (Fig. 3.2D). If, as theorized elsewhere (Gorman and Elliott 2003; Gorman and Steele 2006), behavior in the LDLD conditions was controlled by strongly-coupled dual oscillators under mediating alternate bouts of activity, we anticipated that phase shifting one scotophase (N1) may exert spillover effects on the other (N2). Activity onsets in N2, however, did not display any differences in phase angles regardless of phase of N1, except a small relative delay in activity onsets (Δ9±3 min compared to baseline) after 4h delay in T24LDLD (Fig. 3.2).

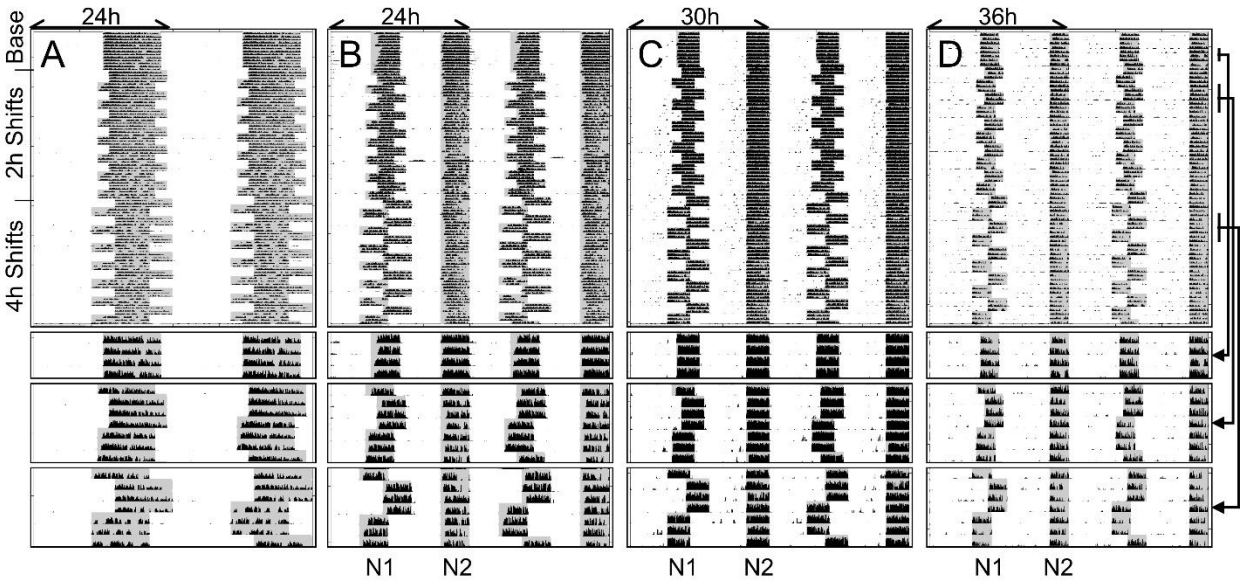

Figure 3.1*. Representative double-plotted actograms of mice in Study 1/Jitter that were exposed to T24LD (14:10; A), T24LDLD (7:5:7:5; B), T30LDLD (10:5:10:5; C), or T36LDLD (13:5:13:5; D), all with dimly illuminated scotophases (<0.1 lux). The second, third and fourth rows contain blow ups of the baseline, and repeated 2h and 4h phase shifts respectively. Wheel running activity is shown in black and scotophases are indicated by gray shading. Each line is scaled from 0–100 revolutions per minute.*

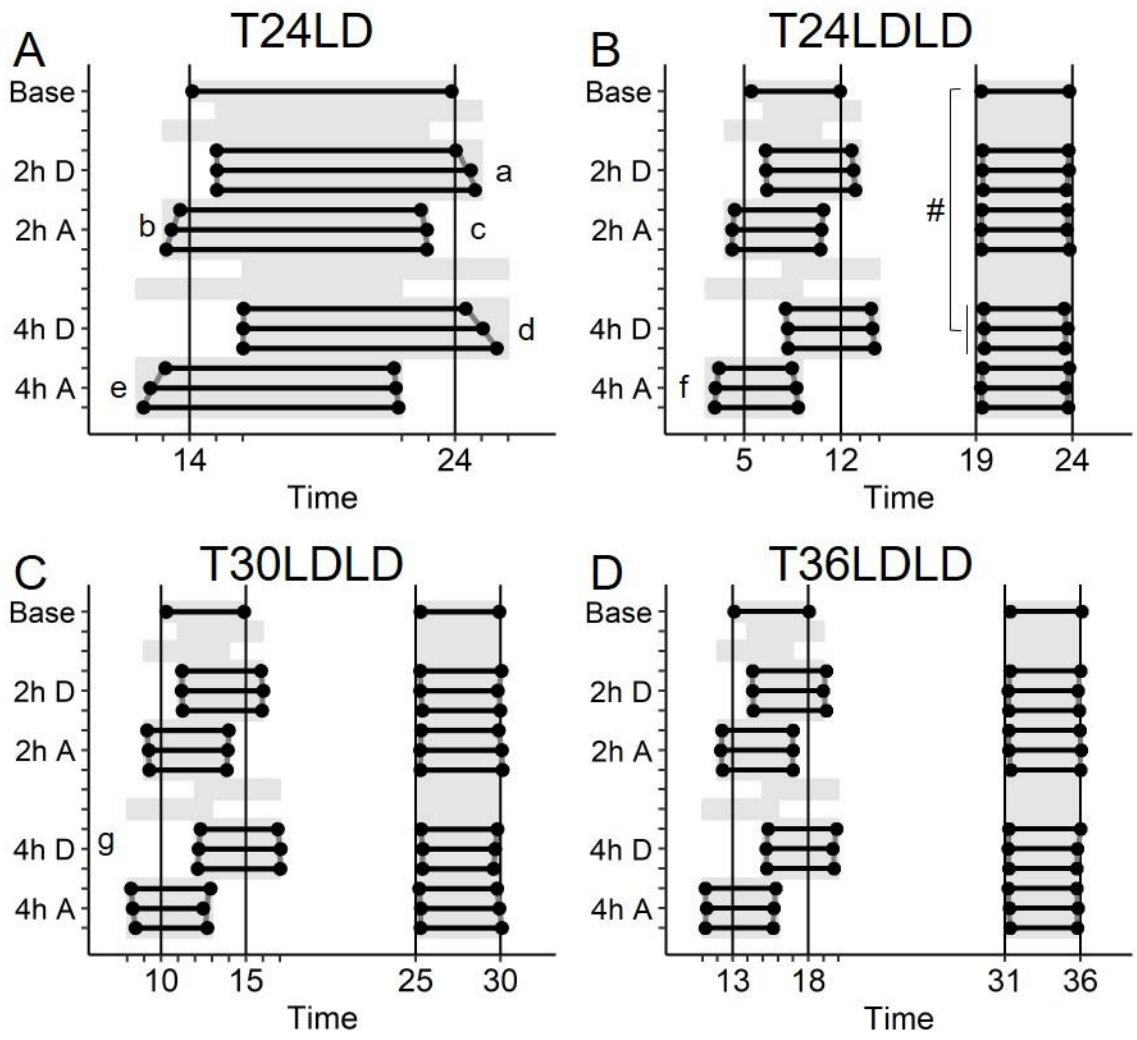

Figure 3.2. *Average activity onsets and offsets in Study 1/Jitter for animals entrained to T24LD (A), T24LDLD (B), T30LDLD (C), and T36LDLD (D). Onsets and offsets were averaged across the last 10 days of baseline, and across 5–7 repeats of 2h and 4h phase shifts each. Error bars representing SE for onsets and offsets are obscured by data points (all < 20 min). Scotophases are indicated by gray background. Significant slopes (min±SE/day) (i.e. "transients") are indicated by a lowercase letter: a:22±4, b: -15±2, c: 7±2, d: 35±6, e: -25±3, f: -7±3, g: 8 ±3. Positive slopes indicate onsets/offsets occurring later. Significant phase angle differences (min±SE) compared to baseline are indicated with special characters: #: 9±3. Positive phase angles mean later onsets than baseline. Note the large transients in T24LD (a,b,d,e), but the lack thereof in all other experimental conditions.*

Study 2/DuPont - DuPont work schedule with bifurcation

A DuPont Schedule is a common working schedule in U.S. manufacturing and involves alternating blocks of 3–4 12h day and night shifts, with non-working recovery days between different shift types. Under normal circumstances, the circadian system simply cannot adapt to the phase adjustments required to work such a schedule (Smith and Eastman 2012), but bifurcation in rodents has the potential to greatly enhance readjustments to large phase shifts (Harrison and Gorman 2015). In addition, Study 1/Jitter showed that with bifurcated behavior mice can rapidly adapt to repeated phase shifts. Therefore, in Study 2/DuPont we tested whether experimental entrainment conditions with bifurcation can facilitate adjustment to a simulated DuPont work schedule. Under LDLD conditions, this necessarily includes adjustments in the phase angle between the twice-daily scotophases, because a symmetric T24LDLD cycle with short photophases (such as LDLD8:4:8:4) does not allow for a long, 12h work shift. Thus, mice in LD were compared to mice in three alternative scheduling strategies with bifurcation, each approaching the rotation between simulated day and night work-shifts differently. All 12h "work" blocks were scheduled during the photophase, as they would be for diurnal humans. In nocturnal mice, that means that "work" is analogous to scheduling inactivity expected for the subject day. Additionally, successful adaptation to a shiftwork schedule in humans includes efficient sleeping during changing dark intervals. Thus, one goal of this study in a nocturnal rodent model was to produce an entrainment pattern where locomotor activity (a marker of subjective night) was absent from Work Blocks 1–4 (red boxes in Fig. 3.3), but instead occurred robustly in scheduled scotophases (shaded lightly in Fig. 3.3). Combining these two concepts, Percent Activity in the Light can be used as a good indication of overall adaptation (Brown et al. 2019).

Study 2/DuPont - Results

Prior to evaluating adjustment to a simulated DuPont schedule, mice were entrained conventionally to LD16:8 (control) or bifurcated in LDLD8:4:8:4 and assigned to one of three experimental conditions (n = 6/group). Figure 3.3 depicts a representative actogram from each schedule employed in Study 2/DuPont. The conventionally entrained LD16:8 mice demonstrated predictable shortcomings in adjusting to the changing LD cycle: During the first set of simulated night work shifts (Work Block 1), the representative animal showed significant persistence of subjective night behavior that diminishes over four cycles. The dark periods following these simulated night shifts included robust locomotor activity, but activity stopped well before the end of darkness on the first two nights. There was poor adaptation, moreover, in the adjustment of activity in the first cycle after return to conventional hours, though rest/activity rhythms were largely well-adapted for Work Block 2, which simulates day-work. In Work Block 3 (night-shifts), there was greater intrusion of nocturnal behavior than in Work Block 1. Dark periods after these night shifts had diminished and sporadically-timed activity, indicative of poor adaptation. In Work Block 4, there was good adaptation, as with the previous simulated day-work.

The three experimental groups that incorporated rhythm bifurcation tested alternative approaches to accommodate the work schedule. On “days off”, the first bifurcation group was designed to keep the two 4h-scotophases in antiphase (Anti), previously hypothesized to be the most stable form of bifurcated entrainment (Gorman and Steele 2006). The second group aimed to keep the phase relationship between the scotophases at LDLD12:4:4:4 or LDLD 4:4:12:4 (Phase), to limit the total number of required phase adjustments. For the third group, large phase shifts were replaced by incremental 2h-steps (Step), as gradual rather than abrupt shifts have been recommended for human rotating shiftworkers (Eastman 1990). The representative mice in Anti, Phase, and Step (Fig. 3.3B, 3.3C and 3.3D, respectively) all appear comparatively

well-adapted throughout the experiment and show high alignment of activity with the imposed light-dark cycle during baseline, on simulated work days, and on days off. On average, baseline BSI-values were high for the three LDLD groups and did not differ from each other (0.69±0.07, 0.83±0.02, 0.82±0.04 for Anti, Phase and Step respectively ($F(2,15) = 2.53$; $p = 0.11$).

Because groups were different and continuously changing, simple metrics were used to quantitatively compare the successful adaptation to the DuPont schedule. First, during the 28-day simulated work shift protocol, activity in the light was not different between groups, including LD (0.05±0.02, 0.04±0.01, 0.07±0.02 and 0.12±0.05 for LD, Anti, Phase and Step respectively, ($F(3,20) = 1.3$; $p = 0.30$). Additionally, no differences between all groups in average deviations on days off were found (Fig. 3.4, $F(3,20) = 2.50$; $p = 0.08$). On work-days, however, LD displayed a large mismatch, with activity starting 4.0±0.2h away from lights-off (Fig. 3.4), while LDLD groups all showed an average mismatch of less than 1.5h (1.4±0.2, 1.0±0.1, 1.4±0.2h for Anti Phase and Step respectively; $F(3,20) = 49.4$; $p < 0.0001$; post hoc group comparisons depicted in figure).

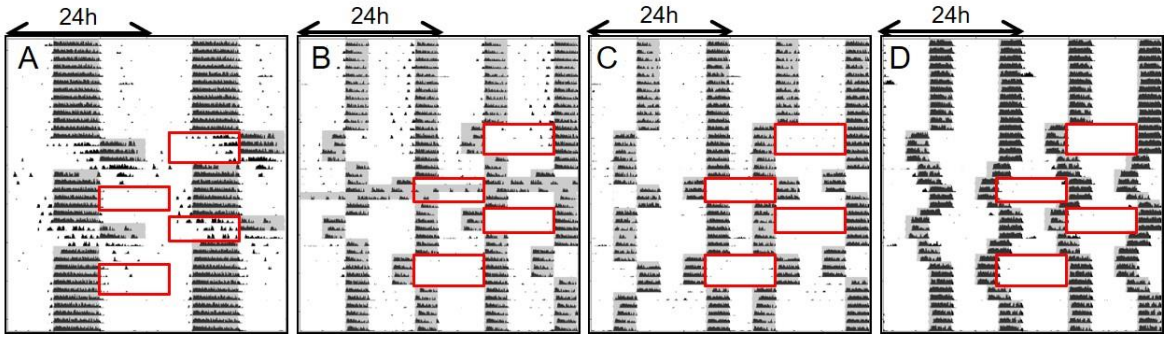

Figure 3.3*. Representative actograms from mice in LD (16:8; A), and LDLD (8:4:8:4) schedules designed to maintain antiphase scotophases (Anti; B), stable phase angles between scotophases (Phase; C), or gradual transitions for phase shifts (Step; D). All scotophases were dimly illuminated (<0.1 lux). For more details on lighting schedules see methods. Superimposed red boxes, single-plotted, represented required work times (Work Blocks 1–4) in a DuPont schedule. In the middle of the experiment a technical failure caused the lights to be off for 48 consecutive hours for group B. Those days were excluded from all analyses, and this did not appear to disrupt behavior after the issue was resolved. Notably, activity in the three LDLD groups appeared to adjust rapidly to the frequent changes in the light schedule.*

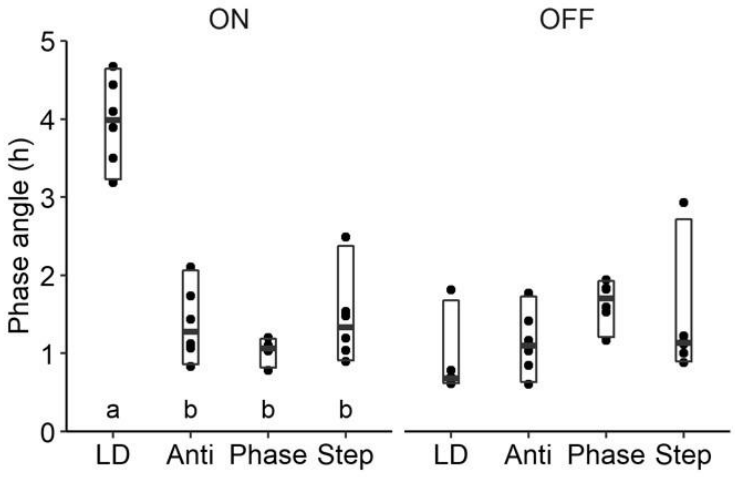

Figure 3.4*. Average absolute phase angles of activity onsets to lights off for mice in Study 2/DuPont on "work days" (left) and "days off" (right). Post hoc group comparisons are indicated with lower-case letters under the groups. Groups that share the same letter were not significantly different from each other. On work days, LDLD groups significantly differed from LD.*

Study 3/Continental - Rotating work hours using T-cycles.

Study 2/DuPont demonstrated that in bifurcation N1 and N2 need neither a stable phase nor phase relationship to maintain entrainment, and that bifurcation facilitated adjustment of wheel activity according to a pre-determined "work schedule". Study 3/Continental extended these findings by testing if a strategy with T-cycle entrainment can also be used to schedule wheel activity according to human shiftwork paradigms. A T30 (LDLD10:5:10:5) entrainment paradigm allows mice to start activity 6h later each consecutive day, which could allow a shiftworker to work different shift types on successive days. Therefore, this strategy would be particularly suited for a work schedule involving quickly rotating work times. Here, we used an adapted version of a "Continental" rotating shift schedule, in which real workers rotate between 8h day, swing, night shifts with days off. Specifically, five 24h work days (120h) were replaced with four 30h cycles (120h), to allow efficient rotation between different shift types throughout the week. Each 30h cycle was an LDLD cycle, which allowed two 10h subjective days (one for work and one personal) and two 5h subjective nights for sleep. These work-days were

alternated with two 24h weekend days, which allow diurnal days off for the theoretical shiftworker (a social requirement for many). Because we hypothesized that bifurcation was needed to maintain entrainment, in the first phase of the study, weekend days were LDLD7:5:7:5, while in the second phase this hypothesis was tested by substituting the LDLD weekends with LD14:10. Mice were exposed to these hybrid T-cycle paradigms with either dimly lit nights following a history of bifurcation — conditions designed to facilitate behavioral adaptation — or with dark nights without the bifurcation-history. The latter condition is typical for laboratory rodents in chronobiological studies. Adaptation was quantified using wavelets and activity in the night. Again, as mice are nocturnal, evidence of good adaptation was absence of activity in the light and presence of activity in the dark.

Study 3/Continental - Results

Figure 3.5 shows an example actogram from one animal from each group, with all wheel data plotted modulo 24h (Fig. 3.5A/5D) and modulo 30h (Fig. 3.5B/5E). As anticipated, dim light at night during the stable T-cycles in baseline (Phase 0: top lines in actogram) predicts entrainment, while animals with dark nights mainly fail to entrain. Corroborating the represented individuals, average EQ-values were 0.92±0.03 and 0.67±0.10 for dim and dark, respectively ($F(1,20) = 21.2$; $p < 0.001$), with females displaying higher EQ-values (0.93±0.02) than males (0.70±0.09; $F(1,20) = 17.6$; $p < 0.001$).

In Phase 1, all mice were exposed to alternating T30LDLD work weeks and a T24LDLD weekend. The representative dim light animal in figure 3.5D/E showed adaptation to both weekend schedules (best visible in modulo 24h; Fig. 3.5D) and work-week (best visible in modulo 30h; Fig. 3.5E) as evident from low activity levels in the light and dominant wavelet-periods alternating between 12 and 15h (Fig. 3.5F). The dark-night animal, on the other hand, predominantly showed free-running rhythms (best visible in modulo 24h; Fig. 3.5A, Fig. 3.5C). Adaptation in Phase 1 of the experiment was highly predictive of behavioral adaptation in Phase

2, where weekend days were un-bifurcated, meaning that free-running animals — a group largely consisting of animals from the Dark condition — continued to free-run, while animals that were behaviorally adapted — mainly from the Dim condition — maintained adaptation.

Wavelet analysis can evaluate periodicity at any point along a time series, as is shown for the illustrative animals in Figure 3.5C/F. Wavelets were used to extract the dominant rhythms in wheel running for each 2–4 day block of the schedule by taking a point estimate from the middle of the window. Period estimates generally fell categorically into entrained (i.e. matching the zeitgeber period) and free-running (i.e. > 24h period) values with very few estimates in between (Fig. 3.6). The mice in Dark conditions predominantly display free running rhythms, while mice with Dim nights mainly show rhythms matching the light-cycle (generalized linear mixed effect model; all $p < 0.05$). The only exception is the 24h LD weekends in Phase 2, where entrained and free-running animals produce overlapping distributions, but the period for animals with dark nights was significantly longer than for animals with dim nights (25.7±0.3 vs 23.5±0.2h; $F(1,20) = 38.56$; $p < 0.0001$).

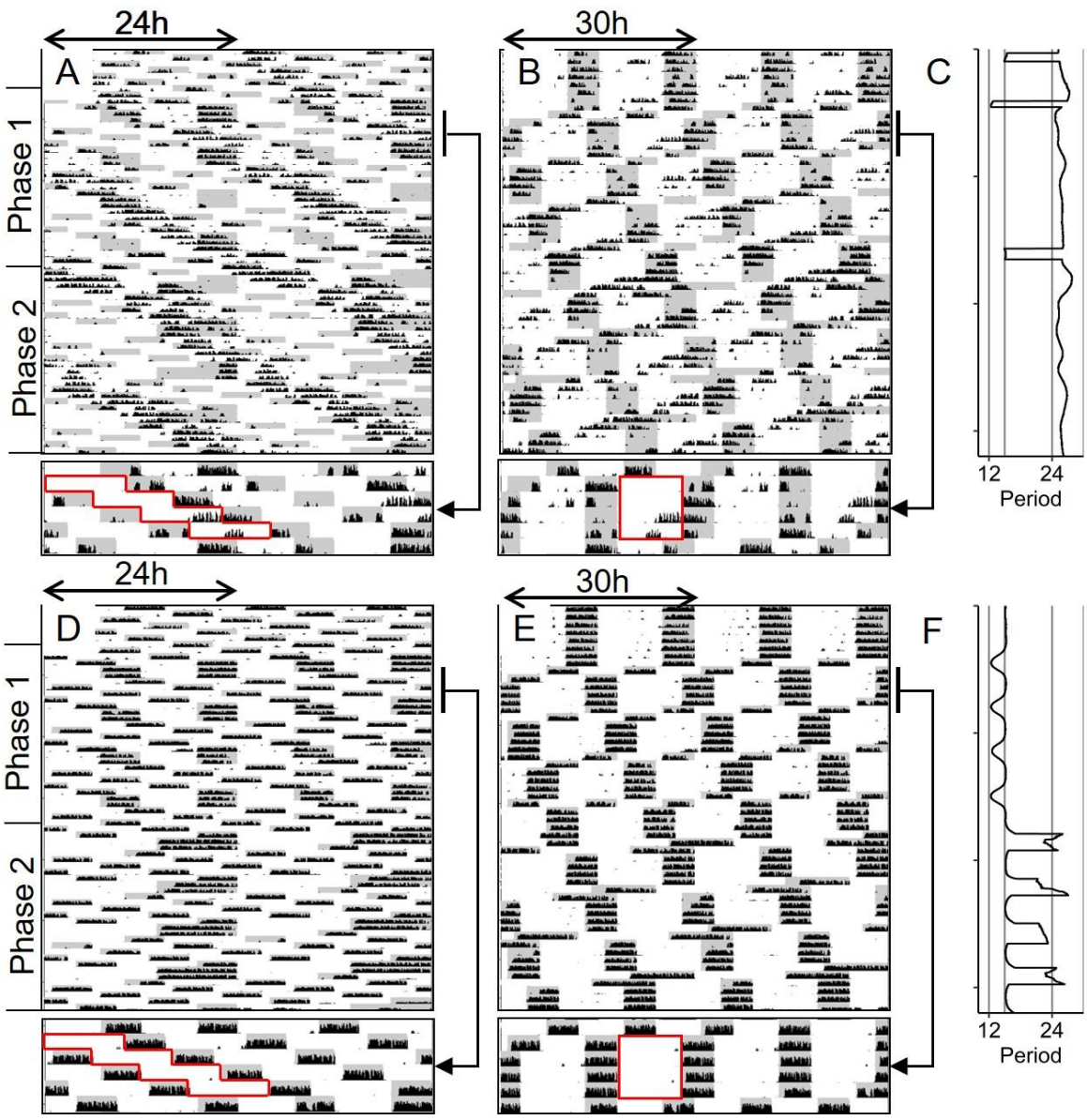

Figure 3.5. *Illustrative double-plotted actograms and wavelet ridge-plots for 2 animals from Study 3/Continental. Mice were exposed to alternating T24LDLD (7:5:7:5) and T30LDLD (10:5:10:5) cycles in phase 1 and T24LD (14:10) and T30LDLD (10:5:10:5) in phase 2, see methods. Data for an animal with dark nights is plotted modulo 24h(A) and modulo 30h (B), and data for an animal in the dim night condition (<0.1 lux) are plotted in (D) and (E) for modulo 24 and 30h respectively. Blow-ups at the bottom of each panel represent the first T30 work-week. Red boxes represent a time where 4 delaying 10h work shifts could be scheduled. Wavelet ridges for the animals with dark nights and dim nights are plotted in (C) and (F) respectively. Vertical gray lines are at 12, 15, 24 and 30h. Values in the ridge plots for animals in F alternated rapidly and almost completely between the values matching the changing zeitgebers (12 and 15 in Phase 1 and 15 and 24 in Phase 2), indicating maintenance of entrainment throughout the protocol. Values in C on the other hand did not follow the period of the light cycle.*

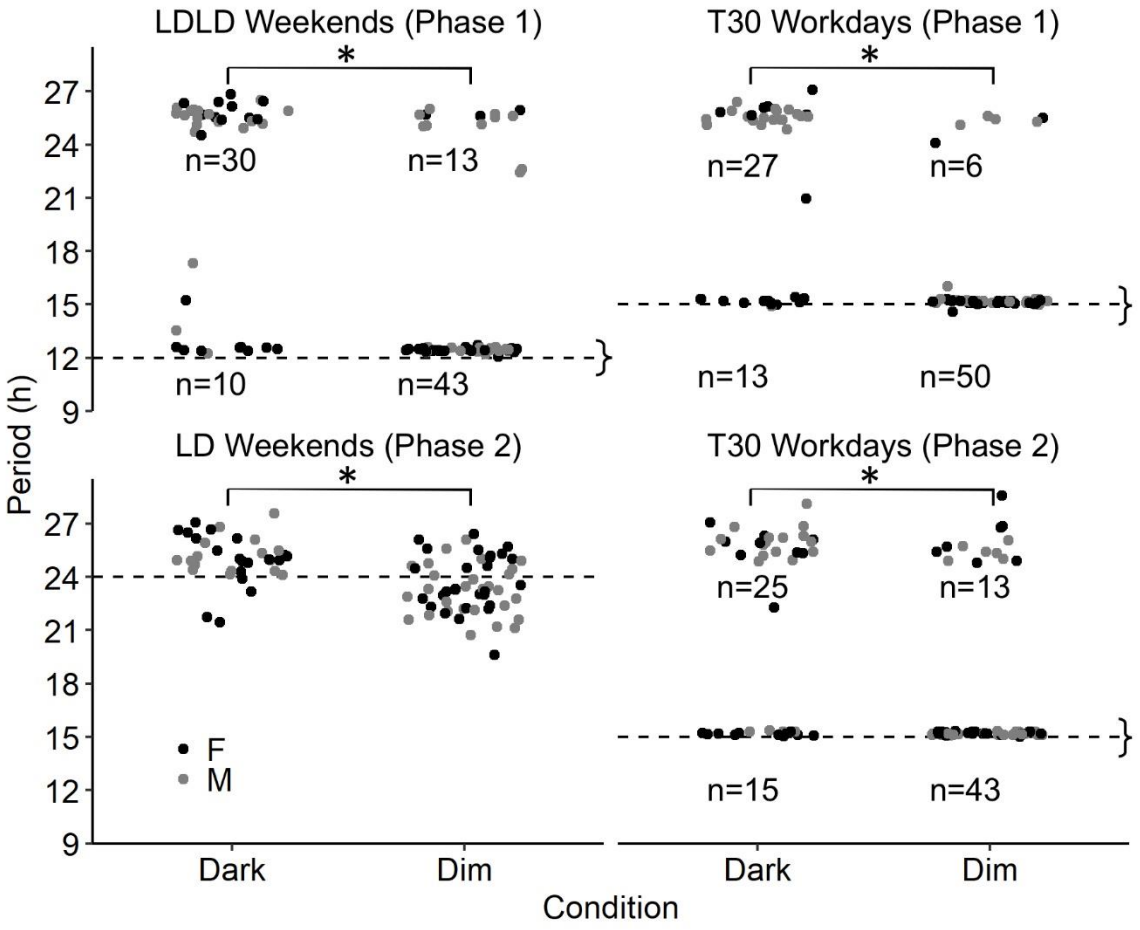

Figure 3.6*. Dominant wheel running periodicity based on wavelet analyses. For each animal, the main period is extracted for each week/weekend separately, therefore each animal is represented in each panel 4 times. Horizontal dashed line indicates the period of the light-dark cycle, and "}" indicate data points consistent with entrainment. Points deviating from the dotted lines are likely reflective of non-entrained animals, but this is not decisive because some animals could have entrained by frequency demultiplication. Number inside the graph indicates the number of data points in each cluster. In T24LDLD weekends and T30 workdays animals with Dim spent more time entrained that animals in Dark. Significant group differences are indicated by *.*

In addition to the wavelet rhythmicity in activity, timing of activity in relation to light-cycles was scored. During T24Baseline, activity in the light did not differ by group or sex (Fig. 3.7E), but during this phase mice with dark nights were still in LD14:10, while mice with dim nights were in LDLD7:5:7:5. In all other phases, activity in the light was greater in Dark than Dim (all $p < 0.01$), and did not differ depending on sex, but sex and condition did interact such that males with dark nights had more activity in the light (all $p < 0.05$).

As in Study 2/DuPont, activity in the light was measured; however, it is sensitive to negative masking. Therefore, we also measured activity in the dark. If a mouse is well-adapted, the full scotophase would be expected to be filled with activity. More importantly, each week- or weekend-day would be expected to have the same respective activity profile. A mouse that is free-running, but masked by the LD cycles, would be expected to have different activity profiles in subsequent weekends depending on the relative phase between internal free-running rhythms and the LD-cycle. Figure 3.7 represents activity profiles of T24LDLD weekends (Fig. 3.7B), T24LD weekends (Fig. 3.7C) and of all T30 weeks in between (Fig. 3.7D). Animals are sorted based on subjective entrainment. Animals in Dim showed large agreement between weekends, as apparent by near-identical patterns across repeats, while Dark animals showed more of a “checkerboard pattern”, indicative of irregular weekend behavior.

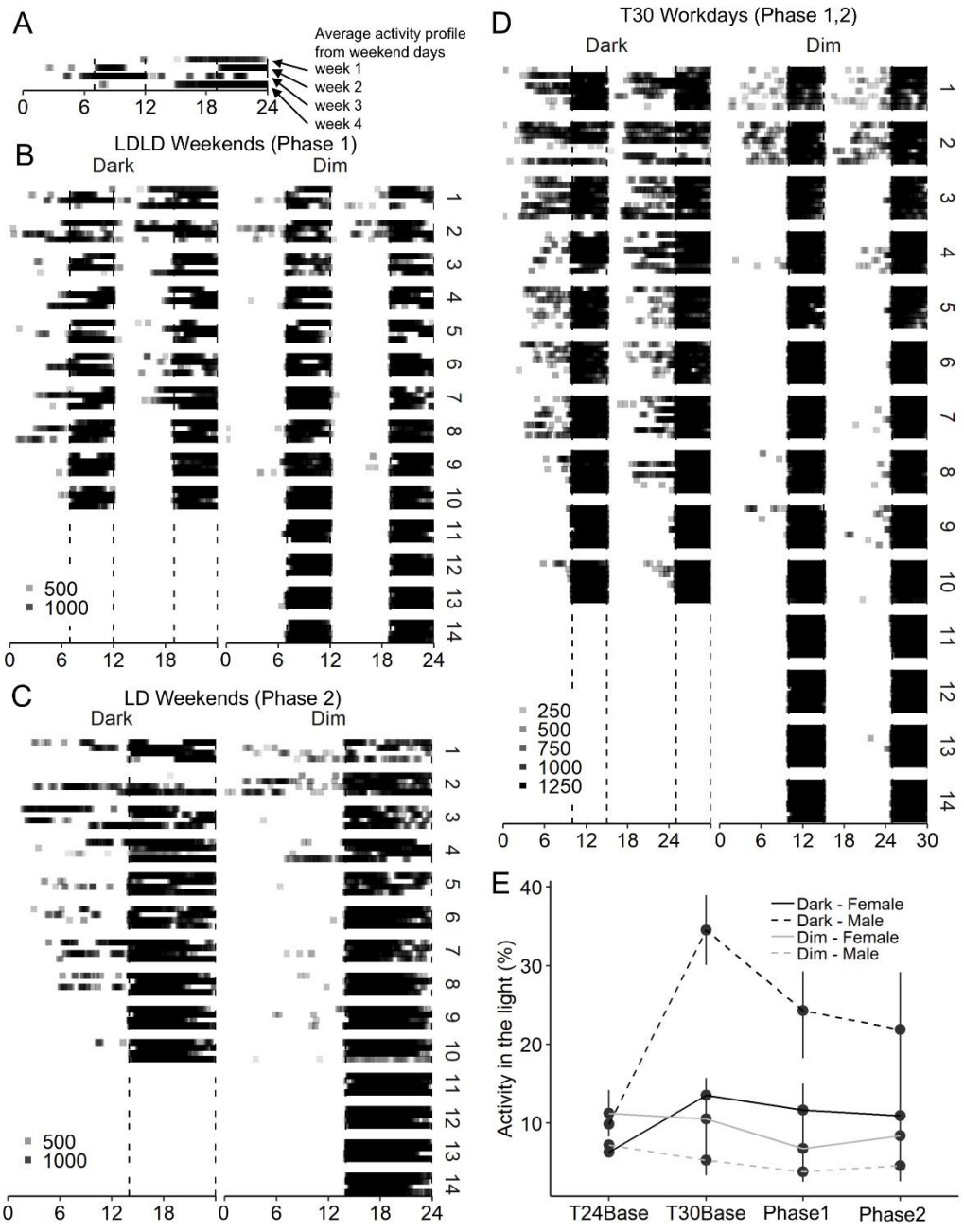

Figure 3.7*. Wheel running activity profiles for each individual in Study 3/Continental from LDLD weekend (**B**), LD weekend (**C**) and T30 weekdays (**D**). A blow-up of the first animals in LDLD weekends/Dark is shown in (A). Each block represents 4 (A/B/C) or 8 (D) weeks of data from a different animal. Animals were ranked subjectively from worst to best adapted (1-14, listed on the right side of the panels). Within each block, each line represents the averaged activity profiles for 2 T24 weekend (A/B/C) or 4 T30 week (D) days. Only activity above the daily average is plotted. Intensity of the color represents number of half-wheel revolutions per 6 min bin. (**E**) Average percent activity in the light for each condition represented as mean±SE through the experiment. Activity profiles of animals in Dim were more consistent from week to week and showed less activity in the light compared to animals with Dark nights.*

Discussion

Summary

Baseline BSI and EQ values corroborate earlier reports that mice are capable of behavioral adaptation to extreme light dark cycles (Study 1–3), that dim light at night facilitates flexible entrainment (Study 3) and that females, on average, adapt to extreme T-cycles better than males (Study 1,3). Furthermore, in Study 1/Jitter, mice in 24hLDLD, T30LDLD, and T36LDLD adjusted wheel running activity to a phase shifted light-dark cycle on the first day, while mice in T24LD show expected transient misalignment in activity onsets and offsets following phase advances and delays respectively. In Study 2/DuPont, bifurcated mice in T24LDLD adjusted their behavior to schedules designed to simulate a DuPont working paradigm better than un-bifurcated animals. Lastly, in Study 3/Continental mice provided dim light at night were capable of adjusting behavior to rapid and frequent changes in T-cycles and this could be used to simulate rotating shiftwork with diurnal days off, while mice with dark nights mainly failed to adjust. Combined, these studies expand the characterization of flexible entrainment associated with bifurcation and T-cycles and strengthen the support for translation to aid shiftworkers with their schedules.

Building rodent shiftwork models

Rodent models of human shiftwork typically assess consequences of circadian disruption similar to that observed in human shiftwork, applying a variety of approaches including forced work during the normal rest phase (e.g. Saderi et al. 2019), chronic jetlag (e.g. Oike et al. 2015) and non-entraining T-cycles (e.g. Karatsoreos et al. 2011). Few studies compare alternate schedules to minimize circadian disruption (McGowan and Coogan 2013), and we are aware of no study testing different strategies to achieve a common simulated work schedule in animal models. For Study 2/DuPont and 3/Continental, we therefore developed a strategy where we aimed to evaluate and compare light-dark scheduling practices designed to

avoid misalignment. We deployed protocols where adaptation to a predetermined simulated "work-schedule" on both days-on and days-off was quantified. This novel approach allows for efficient comparison of alternative scheduling paradigms that are otherwise hard to compare because of the dynamic nature of such experiments.

Using this method in Study 2/DuPont, three bifurcation-based schedules were compared to an LD cycle for adaptation. Because the LD group had the most consistent schedule, with only two adjustments in activity for night-time work-blocks and no further phase shifts on days off, mice in the control group tended to better alignment on days off than LDLD animals. Despite having more phase shifts (as many as 15 in Step Group) and changing photophase duration and phase angles between scotophases, bifurcated mice not only maintained entrainment throughout the experiment, but outperformed un-bifurcated controls on work-days. Because no differences were observed between the LDLD groups, invariant phase angles or minimization of the number and magnitude of phase shift appear to be unimportant considerations for achieving successful adaptation. Human shiftworkers often break up sleep to accommodate for family and other activity (Akerstedt 2003). Given the variety of bifurcation-based schedules that support adaptation to shiftwork schedules, rhythm bifurcation might grant freedom to schedule sleep as desired without sacrificing quality.

While Study 2/DuPont improved alignment to a DuPont schedule, other schedule types are perhaps more common. Study 3/Continental used T-cycles to test adaptation to a delaying rotating shift schedule (modified Continental) with normal weekend days. Indicators of successful adaptation revealed that mice with nocturnal dim light can adjust behavior to match frequently changing periods. Because bifurcation on weekends is not necessary to maintain adaptation on work days, hypothetical shiftworkers could remain flexible in how to structure their days off. Together, these experiments show that both bifurcation and T-cycles can be used to schedule rest/activity around a variety of very different simulated real-world shiftwork schedules,

without loss of alignment of activity with the light-dark cycle. The scheduling of activities around a given work schedule, despite being one of the few areas of control for the worker, has received little attention historically (Gamble et al. 2011; Petrov et al. 2014). Proactive scheduling strategies such as those reported here, might serve as an alternative or even complementary approach. Future studies could directly compare bifurcation and T-cycles and examine the extent to which they might augment one another. Additionally, adaptation may be further improved using timed feeding (Patton and Mistlberger 2013), exercise (Edgar and Dement 1991), or other known zeitgebers in addition to light. Furthermore, studies could investigate the consequences for adaptation of extended intermittent periods of normal T24LD entrainment (e.g. long weekends, vacations). Additionally, a Continental shiftwork schedule calls for 8h delays between subsequent shifts, while T30 only allows for 6h delays daily. Because no qualitative differences between T30LDLD and T36LDLD were found in Study 1/Jitter, we anticipate these findings could be easily extended to a T32 schedule (8h delays daily), but this should be empirically tested.

The current studies served as a proof of concept that behavioral misalignment is not an inevitable consequence of shiftwork schedules. Behavioral adaptation does not require minimally changing and near 24h conditions. With bifurcation and dim light, the overall level of adaptation to challenging schedules was greatly increased, even if some animals under such conditions were not ideally adapted. There is little reason to assume that our lighting conditions and schedules were optimal for enhancing circadian flexibility. We have only just begun to evaluate parameters of the lighting environment on entrainment flexibility primarily in T24 LDLD, but see Sun et al. 2019. Factors that predict entrainment to LDLD or T-cycle paradigms include sex, age of the animal and light parameters like duration and intensity (Evans, Elliott, et al. 2012; Harrison et al. 2016; Walbeek and Gorman 2017; Sun et al. 2019; Walbeek et al. 2019). The superior entrainment flexibility in females observed here (in Studies 1 and 3) corroborates

previous findings from our lab (Walbeek and Gorman 2017; Walbeek et al. 2019), but is seemingly opposite that reported in humans where males may have higher tolerance than females as reflected in self-reported level of complaints related to sleep or health (Saksvik et al. 2011), but see (Nachreiner 1998). Mechanisms underlying sex differences in entrainment flexibility in our studies have not yet been investigated. Other individual differences in humans that correlate with shiftwork tolerance include chronotype, age, family structure, and certain personality traits (Ritonja et al. 2019). Awareness of these individual differences, however, has not yet resulted in individualized or actionable solutions to aid shiftworkers. Mouse studies with well-defined shiftwork adaptation endpoints may help distinguish biological factors from social covariates to inform such solutions.

The current report identifies interventions to reduce behavioral misalignment, but the long term health consequences in any of these extreme lighting paradigms are yet to be investigated. Few formal studies have been published yet, but preliminary observations are encouraging. For example, mice that are successfully entrained in a variety of non-24h conditions in our lab do not seem to develop an obesity phenotype (Walbeek and Gorman 2017; Walbeek et al. 2019), and do not have impaired reproductive function ((Evans and Gorman 2002) and unpublished data) as seen in circadian disruption models (Evans and Davidson 2013). Lastly, unlike mice in a simulated jetlag protocol, bifurcated mice do not have deficits in cued memory retrieval in Pavlovian fear conditioning (Harrison et al. 2017). Even if long term health were not improved, flexible adaptation might alleviate acute effects of shiftwork by improving alertness on the job or the ability to get quality sleep between shifts.

Mechanisms of behavioral adaptation

The flexible entrainment to extreme lighting conditions observed here is exceptional and far beyond traditional limits of entrainment (Daan and Aschoff 2001). *By what mechanism are mice able to adapt their behavior to these highly artificial schedules?* A variety of studies with

different light cycles and species show converging evidence that an explanation of simple masking can be rejected in favor of a true reorganization of the circadian system (Gorman et al. 2001; Gorman and Elliott 2003; Rosenthal et al. 2005; Watanabe et al. 2007; Yan et al. 2010; Raiewski et al. 2012; Harrison and Gorman 2015; Evans and Gorman 2016; Harrison et al. 2016; Gorman et al. 2017; Walbeek and Gorman 2017; Noguchi et al. 2018; Sun et al. 2019). Whereas Study 2/DuPont and 3/Continental took a phenomenological approach to explore overall entrainment under complex photoperiod regimes, Study 1/Jitter was designed to test specific entrainment hypotheses by evaluating oscillator characteristics in bifurcation and T-cycle entrainment in two ways. First, *does bifurcation make the system more responsive to light changes?* In traditional entrainment, phase shifts induce transient misalignments between behavior and zeitgeber. These transients are a characteristic of oscillator-driven behavior, and an observable manifestation of the slow rate of adjustment of the central pacemaker after a schedule change. If behavioral adaptation in LDLD relies on similar mechanisms as classical entrainment, transients would be expected in wheel running activity in N1. Because in none of the three LDLD conditions did we find transients comparable to those in T24LD, LDLD entrainment renders the system more resettable. The phase shifts in activity onsets on the first day in response to a phase advance may be a reflection of a resetting action of dark onset and/or incomplete adaptation in this rapidly cycling pattern of advancing and delaying shifts. With respect to transients, changing the T-cycle from 24 to 30 or 36h did not qualitatively change regulation of behavior. These results complement findings of enhanced phase-resetting in hamsters to simulated time-zone travel (Harrison and Gorman 2015) and extend the observation to T-cycle entrainment.

Second, *is entrainment in LDLD affected by strong oscillator interactions?* If activity in alternating scotophases in non-24h LDLD were controlled by antiphasic coupled oscillators, as has been proposed in T24LDLD (Gorman and Elliott 2003; Gorman and Steele 2006), phase

shifts in N1 would be expected to also alter activity onsets in N2. The overall independence of activity in N2 from changes in N1 suggests a lack of strong functional interactions between multiple oscillators in these paradigms. The small delay in activity onsets in N2 following a 4h delay in N1 in T24LDLD is the only spillover effect observed. Because in earlier reports with asymmetric 24h LDLD cycles the dependence of N1 and N2 was in the opposite direction (Gorman and Steele 2006), we can not reject a more parsimonious interpretation that these spillover effects reflect homeostatic mechanisms: with the 4h N1 delay, there are only 5h of rest between N1 and N2, which may contribute to the small delay in activity onset in N2. Overall, there is no compelling evidence of interacting oscillators in LDLD nor in T30 and T36.

Combining the observations from all studies, activity rhythms during or following bifurcation and extreme T-cycles appear to be more directly controlled by light than driven by a strong underlying circadian oscillator. At the same time, an explanation of only positive and negative masking of a strong oscillator driving behavior in these conditions has also been rejected by prior evidence (Gorman et al. 2001; Gorman and Elliott 2003; Rosenthal et al. 2005; Watanabe et al. 2007; Yan et al. 2010; Raiewski et al. 2012; Harrison and Gorman 2015; Evans and Gorman 2016; Harrison et al. 2016; Gorman et al. 2017; Walbeek and Gorman 2017; Noguchi et al. 2018; Sun et al. 2019) as well as by the lack of 24h rhythmicity in Study 3/Continental (Fig. 3.6). Therefore, we propose an alternative explanation that remains to be empirically tested. While in T24LDLD behavior might be driven by two dissociated circadian oscillators, potentially in subregions of the SCN (Evans and Gorman 2016), under extreme T-cycle entrainment, behavior may be uncoupled, wholly or partially, from its normal circadian regulators. Decoupling or partial entrainment of multiple circadian oscillators had been demonstrated rodents in T-cycles (de la Iglesia et al. 2004; Anglès-Pujolràs et al. 2007; Casiraghi et al. 2012; Walbeek and Gorman 2017). To date, among the best-entrained animals in our studies with dim light and T-cycles, there is no evidence of a freerunning circadian

oscillator that continues to keep track of 24h time while behavior is adapted to extreme light cycles. Moreover, upon release in DD, animals rapidly reverted to typical free-running periods with a phase determined by the time of release, rather than at random phases as would be expected from a free-running circadian oscillator or at a specific time which would be predicted by an entrained 24h-oscillator (Harrison et al. 2016; Walbeek and Gorman 2017; Sun et al. 2019). Although our neurobiological investigations have not yet included T-cycles, in bifurcation, clock gene expression in the SCN shows dampened rhythmicity and strong resetting (Noguchi et al. 2018). Recently, another group reported similar enhanced resetting of SCN PER2::LUC rhythms in mice exposed to T-cycles without successful entrainment (Leise et al. 2018). Neurobiological studies in progress will test alternative mechanistic hypotheses. Light manipulations, in other context, have also led to increased circadian resetting, for example photoperiod manipulations (Glickman et al. 2012; Glickman et al. 2014; Ramkisoensing et al. 2014) or short exposure to constant light (Kaur et al. 2009). Furthermore, both VIP treatment (An et al. 2013) and a serotonin receptor antagonist, NAN-190, (Kessler et al. 2008) have been shown to increase the speed of re-entrainment following a phase shift. Whether dim light-induced enhanced circadian plasticity relies on overlapping mechanisms with any of these remains to be determined.

Conclusion

Together, the present studies demonstrate that entrainment in these exotic interventions is regulated by mechanisms that are distinct from classical entrainment. Furthermore, adaptation is compatible with multiple large perturbations as often occurs in real-world shiftwork settings. Therefore, bifurcation and T-cycle paradigms may have translational potential to aid shiftworkers in coping with complex schedules.

Methods

Nomenclature

In non-traditional light-dark paradigms like the ones studied here, standard circadian nomenclature is ill-suited to characterize the complexity of the schedules. In this report, zeitgeber period is noted with a T (e.g. T24 for a 24h cycle). Because prior characterization of bifurcation in T24LDLD demonstrated a 24h organization (Gorman and Steele 2006; Yan et al. 2010), the LDLD notation is preferred over the logically equivalent T12LD. In T30LDLD and T36LDLD, which are formally equivalent to T15 and T18, on the other hand, no evidence of 30 or 36h oscillations has yet been found both in current and prior studies (Harrison et al. 2016; Walbeek and Gorman 2017). Regardless, because of the asymmetric design in phase shifts between N1 and N2 in Study 1/Jitter, the LDLD notation is used there. For consistency, the same notation is used in Study 3/Continental.

Housing and lighting

During all experiments, mice (C57BL/6) were singly housed in shoebox cages (28×18×17 cm) and provided with a running wheel (13 cm diameter). Cages were placed in light-tight, ventilated chambers that fit up to 16 cages each. Chambers were equipped with fluorescent lamps that provided 327 ± 162 lux at the cage level during photophases, and dim green LEDs (555±23 nm) that could provide illuminance no greater than 0.1 lux (irradiance of 3.90 × 10-5 W m^{-2}) in the brightest parts of the cage during the scotophase. Use of dim lights is indicated in experimental details and denoted as LD_{ark} or LD_{im}. Room temperature was continuously monitored and regulated at 22±2 °C. Food (Mouse Diet 5015; Purina, St. Louis, MO) and water were provided ad libitum and wheel-running activity was recorded continuously (VitalView Version 4.2, Mini-mitter, Bend OR) as the number of half wheel revolutions per 6 min bins. Before the experiments, mice were co-housed with same-sex siblings (3–4 per cage) in the colony in LD_{ark}14:10 without a wheel. At the end of the experiments animals were humanely

euthanized. All experiments received approval of the Institutional Animal Care and Use Committee, University of California, San Diego before being conducted and followed all university guidelines.

Quantitative assessments of entrainment and analyses

To objectively quantify behavioral adaptation to extreme light-dark cycles, two metrics were deployed. First, for evaluating bifurcation in 24h LDLD cycles, the Bifurcation Symmetry Index (BSI) quantifies symmetry in the distribution of activity between alternate scotophases. For each 24h-cycle, the scotophase (N1 and N2) with the lesser number of activity counts is determined. The sum of all activity counts in that dark interval is then divided by the total activity across 24h (Total24) and multiplied by 2 (Daily score = 2 * min(N1,N2) /Total24). Daily symmetry values are then averaged across 10 days to yield a BSI score. This provides an objective measure ranging from 0 (all activity consolidated in one of the two nights) to 1 (completely symmetrical activity). Any light-time activity — which in large amount indicates poor entrainment — also lowers BSI.

Similarly, for non-24h light-dark cycles, an Entrainment Quotient (EQ) was calculated to evaluate behavioral adaptation to the zeitgeber period. Using 10 24h-days of wheel-running data, Lomb Scargle periodograms were created to evaluate rhythmicity at any period. Peak periodogram power (PPP) at the period of the LD cycle (e.g. 18h in T18) is an indicator of entrained behavior ($PPP_{entrained}$), while peak periodogram power in the circadian range (i.e. 23–26h) is an indicator of non-entrained, or free-running components ($PPP_{circadian}$). EQ is calculated by dividing $PPP_{entrained}$ by the sum of both: ($EQ = PPP_{entrained} / (PPP_{entrained} + PPP_{circadian})$. EQ gives an objective evaluation of fully entrained (EQ approaches 1) or free-running (EQ approaches 0) behavior. Free-running rhythms combined with negative and positive masking (induced by light and dark, respectively) will likely result in intermediate EQ values.

Study 3/Continental used fast-changing T-cycles, which do not allow for sufficiently long stable behavior needed for EQ determinations. Therefore wavelet analyses were used to evaluate the period of the dominant frequency at each point in time. Clocklab was used to perform wavelet analyses using the default settings (Start: 2h; End: 30h; Step: 0.1h; Cycles: 8; Sigma; 2, Bin: 6min). Ridge data — consisting of the highest power period at each point in time — were extracted from the full frequency spectrum. For each mouse, the dominant period for each 120h work-week and 48h weekend block was extracted by taking the average ridge value from the 6h surrounding the midpoint of the window. Entrainment was defined as dominant period within T-cycle period ±1h..

Activity onsets and offsets were eye-fitted in ClockLab. Activity was recorded using VitalView. Periodogram-analyses and the creation of actograms were performed using ClockLab version 2.72, while wavelet analyses were done in ClockLab version 6.0.26. All other statistical analyses and plotting were done using R.

Study 1/Jitter

4.4.1. Stable entrainment

Group-housed male and female mice from the colony (5–11 weeks of age) were moved to single housing with running wheels during the first 2h of the light-phase, and divided into four groups. The first group (LD; n = 10, half female) was exposed to 14h of bright light and 10h of dimly illuminated relative darkness (LD_{im}14:10). The other three groups were exposed to $LD_{im}LD_{im}$7:5:7:5 (Table 1). For the transition to LDLD, animals were placed in darkness (with dim light) for 5h (N1) at the end of the 2h transition window then 7h of photophase, followed by another dark phase (N2) that started at time of original lights off, but was truncated. After 2 weeks of LDLD, the three groups were separated: one group (Bifurcation, n = 14, half female) remained on $LD_{im}LD_{im}$7:5:7:5; a second group (T30, n = 16, half female) was exposed to $LD_{im}LD_{im}$10:5:10:5; and a third group (T36, n = 16, half female) was exposed to

$LD_{im}LD_{im}$13:5:13:5. Both light-schedule transitions were implemented by extending the photophases while always keeping every scotophase 5h.

Repeated phase shifts

After 2 weeks, mice were stably entrained. Animals in group LD were then exposed to a 1h phase delay for three days. This shift was not analyzed but was performed to render future advances and delays symmetric with respect to the initial entraining conditions. From here, mice were exposed to repeating cycles of 2h phase shift every 3 days alternating between phase advances and phase delays. After 7 repeats, there was 1 transitional 3h phase delay, followed by another 7 repeats of alternating 4h phase advances and 4h phase delays, all occurring every 3 days.

The groups with LDLD light cycles (Bifurcation, T30 and T36) were exposed to a similar pattern of repeating 2h, then 4h, phase shifts. Importantly, in these groups only N1 was phase shifted, while N2 maintained a stable phase, causing the two scotophases to not be antiphasic anymore but rather asymmetrical. Because each cycle is longer than 24h, we have fewer iterations of these shifts so that they were performed over the same 12 week interval as the shifts in LD.

Exclusion of non-entrained animals

For each of the three LDLD conditions, only the 8 most well-entrained — defined by BSI and EQ values from the last 10 days of baseline — were included in the analyses. Results and conclusions did not depend on decisions for exclusion: although not reported, qualitative results did not change with post-hoc analyses using different criteria.

Table 3.1. *Lighting schedule for Study 1/Jitter.*

	Phase 0: Stable entrainment		**Phase 1: 2h - Phase shifts**	**Phase 2: 4h - Phase shifts**
LD (n = 10)	28 cycles: LD_{im} 14:10		7 x 6 cycles: LD_{im} 14:10	7 x 6 cycles: LD_{im} 14:10
Bifurcation (n = 14)	28 cycles: $LD_{im}LD_{im}$ 7:5:7:5		7 x 6 cycles: $LD_{im}LD_{im}$ 8:5:6:5 / 6:5:8:5	7 x 6 cycles: $LD_{im}LD_{im}$ 9:5:5:5 / 5:5:9:5
T30 (n = 16)	14 cycles: $LD_{im}LD_{im}$ 7:5:7:5	11 cycles: $LD_{im}LD_{im}$ 10:5:10:5	6 x 6 cycles: $LD_{im}LD_{im}$ 11:5:9:5 / 9:5:11:5	6 x 6 cycles: $LD_{im}LD_{im}$ 12:5:8:5 / 8:5:12:5
T36 (n = 16)	14 cycles: $LD_{im}LD_{im}$ 7:5:7:5	10 cycles: $LD_{im}LD_{im}$ 13:5:13:5	5 x 6 cycles: $LD_{im}LD_{im}$ 14:5:12:5 / 12:5:14:5	5 x 6 cycles: $LD_{im}LD_{im}$ 15:5:11:5 / 11:5:15:5

Onset and Offsets / Activity in the light

For every day in the experiment, activity onsets and offsets were determined for every scotophase and expressed in relation to the light transitions to calculate phase angles. In the LDLD cycles this was done for N1 and N2 separately. Using mixed effect linear regression, prevalence and magnitude of transients — non-zero slopes of phase angles across 3 days — were determined in N1. Averaged phase angles for the four experimental phases in N2 were compared to baseline. Any significant slopes or changes in phase angle smaller than the temporal resolution of the measurement (6 min recording bins) were not reported. Total activity during the light was calculated for each 24, 30, or 36h cycles and averaged across days for each phase.

Study 2/DuPont

In Study 2/DuPont, mice in both standard and bifurcated conditions were exposed to a variant of a Dupont work schedule, requiring four, large-magnitude phase adjustments across the course of four weeks.

Stable entrainment

Group-housed male mice aged 4–5 weeks were divided into 4 groups (n = 6/group). For two weeks, the first group was exposed to $LD_{im}16:8$, and the other three were exposed to $LD_{im}20:4$. Animals were then moved to individual cages equipped with running wheels. For the $LD_{im}16:8$ group, this occurred shortly before lights off. For the other three groups, wheels were introduced at the beginning of the new, second scotophase in an $LD_{im}LD_{im}8:4:8:4$ cycle to facilitate uniform bifurcation across animals. Animals then remained in baseline conditions ($LD_{im}16:8$ or $LD_{im}LD_{im}8:4:8:4$) for four weeks to allow for stable entrainment before the experimental phase commenced.

Experimental phase

After 4 weeks, all groups were exposed to a simulated DuPont shift-schedule. A DuPont schedule is a commonly used shiftwork schedule in U.S. manufacturing and consists of alternating blocks of 3–4 12h day (e.g., 8am–8pm) and night (8pm–8am) shifts with days off in between. To accommodate night shifts, the LD group was phase delayed by 8h prior to the first night shift and phase advanced back to the original phase afterwards. The LD group's schedule was based upon self-reported behavior of typical shiftworkers (e.g. staying up all day to work a first night shift, and then sleeping after) (Akerstedt 1998). The three bifurcated groups each had their own scheduling strategy to adjust their activity-rest schedule to work shifts. The first group maintained scotophases in antiphase wherever possible and only changed the phase angle when needed to accommodate 12h work-shifts (Anti). The second group maintained a stable phase relationship whenever possible, even on days off (i.e. LDLD12:4:4:4; Phase). The third group minimized the magnitude of individual phase shifts by adjusting gradually in 2h shifts rather than 4h as in the other groups (Step).

Quantification of adaptation

To quantify adaptation, activity in the light and activity onsets were determined. Onsets were separately calculated for work days (defined as the first onset after a work schedule until the last onset after the last day of each work-block) and days-off. Activity in the light was calculated over the entire 28 day protocol starting with the day of Work-Block 1.

Study 3/Continental

Table 3.2. *Lighting schedule for Study 3/Continental.*

	Phase 0: Stable entrainment		**Phase 1: Bifurcated weekends**	**Phase 2: non-Bifurcated weekend**
Dark (n = 10)	14 cycles: LD_{ark} 14:10	6 cycles: $LD_{ark}LD_{ark}$ 10:5:10:5	4 repeats of: 4x $LD_{ark}LD_{ark}$ 10:5:10:5 2x $LD_{ark}LD_{ark}$ 7:5:7:5	4 repeats of: 4x $LD_{ark}LD_{ark}$ 10:5:10:5 2x LD_{ark} 14:10
Dim (n = 14)	14 cycles: $LD_{im}LD_{im}$ 7:5:7:5	6cycles: $LD_{im}LD_{im}$ 10:5:10:5	4 repeats of: 4x $LD_{im}LD_{im}$ 10:5:10:5 2x $LD_{im}LD_{im}$ 7:5:7:5	4 repeats of: 4x $LD_{im}LD_{im}$ 10:5:10:5 2x LD_{im} 14:10

A separate cohort of male and female mice (7–11 weeks old) was assigned to one of two groups upon transition from the colony to singly housing with wheels. The first group (Dark, n = 10, half male) was maintained on 14 more days of LD_{ark}, while the second group (Dim, n = 14, half male) was exposed to 14 days of $LD_{im}LD_{im}$ to facilitate flexible entrainment. From here on, both groups were exposed to identical bright-light schedules. The dim light illumination, however, remained as it was before in each respective group. Following the 24h cycles, both groups were exposed to six cycles of LDLD 10:5:10:5 (Phase 0), followed by four repeats of four 30h “work days” (equivalent in length to 5 24h days), and two bifurcated 24h “weekend days” (Phase 1). The six T30LDLD cycles of baseline, combined with the first workweek provided 10 full cycles of stable T30 to quantify entrainment using EQ-values. In Phase 2, four additional

weeks of T30 work-weeks with T24 weekends followed. The weekends, however, were now unimodal LD cycles rather than LDLD (Table 2).

Author Contributions

Conceptualization, Thijs J Walbeek (Study 1,3), Elizabeth M Harrison (Study 2), Robert R Soler (Study 3) and Michael R Gorman (Study 1–3); Data curation, Thijs J Walbeek (Study 1), Elizabeth M Harrison (Study 2) and Robert R Soler (Study 3); Formal analysis, Thijs J Walbeek (Study 1–3), Elizabeth M Harrison (Study 2) and Robert R Soler (Study 3); Funding acquisition, Michael R Gorman; Methodology, Thijs J Walbeek (Study 1,3), Elizabeth M Harrison (Study 2), Robert R Soler (Study 3) and Michael R Gorman (Study 1–3); Resources, Michael R Gorman; Supervision, Michael R Gorman; Visualization, Thijs J Walbeek; Writing – original draft, Thijs J Walbeek; Writing – review & editing, Elizabeth M Harrison and Michael R Gorman.

Funding: This research was funded by Office of Naval Research, grant number N00014-13-1-0285

Conflicts of Interest: The authors declare no conflict of interest

Acknowledgments: The authors would like to thank the staff from UCSD's Animal Care Program for their help with animal husbandry. Additional thanks go to Jonathan Sun for assistance with data collection.

Chapter 3, in full has been submitted for publication of the material as it may appear in *Walbeek, Thijs J., Elizabeth M. Harrison, Robert R. Soler, and Michael R. Gorman. "Enhanced Circadian Entrainment in Mice and Its Utility under Human Shiftwork Schedules." Manuscript under Review.* The dissertation author was the primary investigator of Study 1 in the paper and author of this paper.

Chapter 4. Clock gene expression rhythms in the SCN and peripheral organs in mice entrained to 18h days.

Abstract

In mammals, endogenous circadian rhythms are generated at the cellular level via a transcriptional-translational feedback loop. In the core loop, a handful of clock genes (e.g., *Bmal1*, *Clock*, *Per* and *Cry*) interact to produce near 24h rhythms and additionally induce daily expression patterns in many clock-controlled genes. On the organismal level, the circadian system is orchestrated by the suprachiasmatic nucleus (SCN) in the hypothalamus, which through its network properties generates very precise and robust rhythms. These rhythms are synchronized to the environment through light input from the eye.

Recent studies have discovered simple environmental manipulations that induce unexpected exceptional plasticity in the circadian adaptation of behavior. With the addition of dim light at night no brighter than 0.1 lux, mice can adapt to short, 18 hour days that are far outside the conventional limits of entrainment. So far, the molecular bases, and involvement of canonical circadian oscillators in the regulation of behavior in these exotic conditions have not been characterized. In the present study, RNA rhythms of common clock genes in the SCN, liver and kidney of mice entrained to T18 were characterized using in situ hybridization and qPCR.

In both the SCN and peripheral tissues, clock gene rhythms seem to predominantly follow 24h rhythms, rather than 18 hours. This rejects the hypothesis that dim light facilitated extreme plasticity in the SCN, but rather suggests that control of behavior in T18 is uncoupled from the SCN. While behavior is controlled at 18 hours, the SCN and peripheral tissues are not. In summary, this work expands on the understanding of the mechanistic basis of under-explored circadian plasticity.

Introduction

Many species have evolved a circadian timekeeping system to anticipate predictable daily changes in the environment that are a result of earth's relative position and orientation in the solar system. In mammals, the molecular basis of the circadian system is a transcription-translation feedback loop (TTFL) that is found in almost every cell (Mohawk and Takahashi 2011). The main loop of the TTFL consists of two positive elements -- transcription factors *Bmal1* (brain and muscle ARNT-like 1) and *Clock* (circadian locomotor output cycles kaput) -- that together regulate transcription of negative elements -- *Per1* and *2* (Period), and *Cry1* and *2* (Cryptochrome) -- which downregulate BMAL1 and CLOCK and thereby suppress their own transcription. The TTFL, which is regulated by at least a dozen other known genes, including nuclear receptor *Rev-erbα*, takes about 24h to complete one cycle and controls rhythmic regulation of thousands of other genes (Panda et al. 2002). At the level of the tissue, these cellular rhythms control rhythmic organ function. In the liver, for example, glucose and lipid metabolism are controlled by the circadian clock (Zarrinpar et al. 2016).

In the anterior hypothalamus, the suprachiasmatic nucleus (SCN) is the core pacemaker of the mammalian circadian system. Its endogenous and self-sustained circadian rhythms are synchronized to the outside environment through direct light information from the retina (Welsh et al. 2010). The SCN keeps peripheral rhythms synchronized through various direct and indirect communication signals including changes in body temperature, melatonin secretion or activity and feeding rhythms (Mohawk et al. 2012). Proper coordination and synchronization among all parts of the hierarchical circadian system are important for acute and chronic physiological and mental health. Disruption of the circadian system, either through environmental or genetic manipulations, is associated with a wide range of disease (Evans and Davidson 2013).

Entrainment (i.e. synchronization) of the mammalian circadian system has been well characterized for several decades. One of these properties is the limited range of environmental cycles (T-cycles) to which the circadian system can entrain. Non-parametric entrainment of a system with an internal rhythm of 24h ($\tau = 24$) to an environmental rhythm of 22h ($T = 22$), requires a daily 2h phase advance. The magnitude of the phase shift that can be induced by light is limited — 1-2h by 9h light pulse (Comas et al. 2006) — consequently, the range of entrainment is limited. Recently, simple lighting manipulations that seem to render the rodent circadian clock markedly more flexible were discovered. With the addition of ecological levels of nocturnal dim light no brighter than 0.1 lux, mice and hamsters displayed behavioral entrainment to T-cycles as short at 18h and as long as 30h (Harrison et al. 2016; Walbeek and Gorman 2017; Gorman and Elliott 2019). Non-parametric entrainment to T18 would require daily near 6h phase advances, which are unprecedented in any mammalian system. Additionally, short T-cycle entrainment lacks large phase angles (Walbeek and Gorman 2017; Gorman and Elliott 2019) and transient misalignment following a phase shift (Walbeek et al.) predicted by classical entrainment theory (Daan and Aschoff 2001; Granada et al. 2013; Schmal et al. 2015). Thus, enhanced plasticity of behavioral entrainment in these artificial conditions likely does not derive from nonparametric resetting mechanisms. On the other hand, an explanation of simple masking of a free-running circadian oscillator has been rejected based on a predictable phase of activity after release in constant dark (Harrison et al. 2016; Walbeek and Gorman 2017) and 18h rhythms in activity-independent body temperature (Walbeek and Gorman 2017). In summary, the mechanism of control of behavior in extreme T-cycle entrainment, as well as involvement of traditional circadian regulators, remains unknown.

In the present study, we aimed to measure central and peripheral clock gene rhythms in mice entrained to T18 (LD13:5). The characterization of rhythmicity in the SCN could distinguish between two alternative hypotheses explaining flexible entrainment. If the addition of dim

scotopic illumination increases flexibility of the SCN beyond its conventional limits, 18h clock gene rhythms might be expected. If, on the other hand, dim light uncouples control of behavior from the SCN, near 24h rhythms might be expected despite behavioral adaptation to the 18h LD cycle. Furthermore, measuring peripheral molecular rhythms could distinguish a complete reorganization of physiological rhythms to match behavior vs a mainly free-running physiology uncoupled from wheel running behavior. Molecular rhythms in SCN and liver and kidney were measured using *in situ* hybridization (ISH) and reverse transcription quantitative polymerase chain reaction (RT-qPCR), respectively, in mice entrained to T18 or T24 control conditions.

Methods

Animals, Housing

Female mice (C57BL/6, born at UCSD from Jackson Labs Stock) were housed in plastic cages (28x18x15cm) inside environmental chambers. Each cage was provided with a running wheel (13cm diameter) and ad libitum food (Mouse Diet 5015; Purina, St. Louis, MO) and water. The well-ventilated chambers that hold up to 16 cages each were equipped with fluorescent lamps (327±162 lux) and additional dim green LEDs (555±23 nm, <0.1 lux, irradiance of 3.90 × 10-5 W m-2), and temperature (22±2°C) was continuously monitored and regulated. Wheel-running activity was recorded from each cage and stored as the number of half wheel revolutions per 6 min bins (VitalView Version 4.2, Mini-mitter, Bend OR). Mice were born from breeding pairs housed in either LD14:10, LD13:5 or LD14:10 with weekly 6-hour phase advances. All animals were placed in the colony at LD14:10 at weaning. No statistical differences were found between birth conditions. All experimental procedures were pre-approved and followed the guidelines and policies of UCSD's Institutional Animals Care and Use Program.

Entrainment, Groups, Timepoints, Dissections, tissue preservation

All animals were transferred from group-housed colony conditions (LD_{ark}14:10) to single housing and were provided with a wheel and nocturnal lighting (LD_{im}14:10). After three weeks of acclimating to the new chambers and wheels, each mouse was assigned to one of two groups. The first group remained entrained to LD_{im}14:10 (n= 36), while the other group was entrained to LD_{im}13:5. (initial n = 64, only 52 best-entrained animals were selected; EQ values were 0.97±0.003 and 0.75±0.05 for included and excluded animals respectively). On the tenth day of experimental entrainment, mice from T24 were split between 9 three-hourly time points (ZT0-ZT24, n = 4 / TP), while mice from T18 were split between 13 time points (ZT0- ZT36, n = 4 / TP), with ZT0 defined as “lights on”. Mice were euthanized within 30 minutes of their predetermined time-point using 4% isoflurane followed by decapitation. If occurring during the scotophase, mice were euthanized and enucleated in dim red light (<1 lux). Brains, as well as tissue from the liver and kidney, were collected and immediately frozen on dry ice then stored at -80°C until processed.

***In Situ* Hybridization (ISH)**

Using a cryostat, brains were sliced coronally at 20um and divided into 5 series. Brain sections were thaw-mounted onto Superfrost-plus slides and stored at -80°C until the day of ISH. *Bmal1* (Genbank AB015203; bp 864-1362) and *Per2* (Genbank bp AF035830; 9–489) probes were previously established (Shearman et al. 1997; Shearman et al. 2000) and were generously provided by Dr. David Weaver (UMass Medical School). Radiolabeled ISH was performed as previously described (Stephens et al. 2018). In short, for each probe, one full set of brain sections containing the SCN was fixed in 4% paraformaldehyde, treated with acetic anhydride, rinsed in saline-sodium citrate (SSC), and dehydrated with ethanol, before being treated with chloroform, again dehydrated and air dried for 90 min. Radiolabeled *Bmal1* (35S) or *Per2* (33P) antisense riboprobe (0.04 pmol/mL) were combined with tRNA, heat denatured, and

added to hybridization buffer to create the probe mixture. Each probe mixture (*Bmal1* or *Per2*) was then applied to the tissue (100 ul/slide). Cover-slipped slides were then placed in a humidity chamber at 55°C to hybridize overnight. The next day, slides were washed in SSC, treated with ribonuclease A, washed again in SSC, dehydrated in ethanols and air dried for 90 min. In a dark room, slides were then dipped in Kodak nitroblue tetrazolium salt emulsion, air dried and stored at 4°C for 14 and 13 days for *Bmal1* and *Per2* respectively before being developed and coverslipped.

ISH quantification

ISH slides were scored by an investigator blind to condition or time point using automated imaging software called Grains (Dr. Don Clifton, University of Washington). For each slide containing SCN, each hemisphere of the SCN area was outlined manually. The number of silver grains, signifying *Bmal1* or *Per2* mRNA, in the selected areas was counted using the Grains software and averaged between hemispheres. For each brain, the three SCN sections with the highest silver grains counts were averaged.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Liver and kidney tissue was homogenized before using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) to purify RNA. Using the Super Script III (Thermo Fisher Scientific, Waltham, MA, USA), cDNA was synthesized before qPCR was performed on a CFX384 Real-Time Detection System with SYBR Green (Bio- Rad, Hercules, CA, USA) according to the manufacturer's instructions. A relative fluorescence unit threshold of 500 was used to obtain quantification cycle (Cq). Cq values were averaged across duplicate qPCR reactions, and used to calculate gene expression levels relative to the average of *Actβ* and *ApoE* using the ΔΔCT method (Livak and Schmittgen 2001; Pfaffl 2001) with approximate qPCR efficiency of 2. If the difference between duplicate reactions of the same sample exceeded 1 cycle, the value closer to the mean of the time point was used (12 determination). Four liver samples were excluded

because of low DNA yield, and five samples with expression levels > 3 S.D on three or more genes were additionally excluded from analyses. Primers for Actβ, *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per2* were all provided by Dr. Tsuyoshi Hirota (Nagoya University), *ApoE* primers were found in the PrimerBank (ID: PrimerBank ID 6753102a1) and *Rev-erbA* had been described before (Lau 2004).

Table 4.1: *qPCR Primers*

Gene	Forward	Reverse
Actβ	ggctgtattcccctccatcg	ccagttggtaacaatgccatgt
ApoE	tgacaggatgcctagccg	cgcaggtaatcccagaagc
Bmal1	agataaactcaccgtgctaaggatg	gttggcttgtagtttgcttctgtg
Clock	atggtgtttaccgtaagctgtag	ctcgcgttaccaggaagcat
Cry1	actgaggcacttacacgtttgg	gcagggagtttgcattcattc
Cry2	tgcactggttccgcaaag	accacgggtcgaggatgtag
Per1	ggcaatggcaaggactcag	ggaggctgtaggcaatggag
Per2	tgttccgacatgcttgcg	gaaacagcttcctctgctccag
Rev-erbA	ccacaccgctgggagagt	gccctggcgtagaccattc

Analyses, Statistics and Software

Periodogram analyses were used to calculated entrainment quotients (Harrison et al. 2016), which are a measure to quantify entrainment in T-cycles ranging from 0 (free-running) to 1 (entrained). For both ISH and qPCR data, MetaCycle (Wu et al. 2016) was used to assess statistically significant rhythmicity and estimate period, phase, and amplitude with minimum and maximum period set to 12 and 36h respectively. Additionally, clock gene data were fit using cosinor analyses with both an 18 and 24h period. The ratio of R^2 was used to determine which is the better fit. Peak phase was expressed both relative to the LD cycle (ZT, 18h) and to clock time (i.e. 24h). Actograms and periodograms were created used ClockLab (version 2.72); all

other analyses and creating graphs were performed in R (Vienna, Austria, version 3.5.1; Rstudio, Boston, MA, v. 1.1.456; tidyverse v. 1.2.1; ggplot2 v. 3.1.1; MetaCycle v. 1.2.0; cosinor v. 1.1).

Results

Behavior

All animals that were used for tissue collections were well-entrained to light-dark cycles. Figure 4.1 shows example actograms from an animal in T24 (Fig. 4.1A), and a well-entrained animal in T18 (Fig. 4.1B/D) as well as an animal that did not adapt to T18 and was therefore excluded (Fig. 4.1C/E). As expected, the animals in T24, displayed robust wheel-running activity throughout the dark phase (gray shading) with little activity in the light. Behavior of the entrained animal in T18 (EQ: 0.98) is highly aligned with the light-dark schedule (best visible in modulo 18h; Fig. 4.1D) and does not display any free-running components (best visible modulo 24h; Fig. 4.1B). In contrast, the non-entrained animal in T18 (EQ: 0.26), which represents only a minority of the animals shows less consistent wheel-running activity in the dark (Fig. 4.1E) and has clearly visible free-running patterns (25.3h) in behavior (which is also reflected in lower EQ-value; Fig. 4.1C). All animals included in the tissue analyses had EQ-values larger than 0.9 (mean±SE: 0.97±0.003)

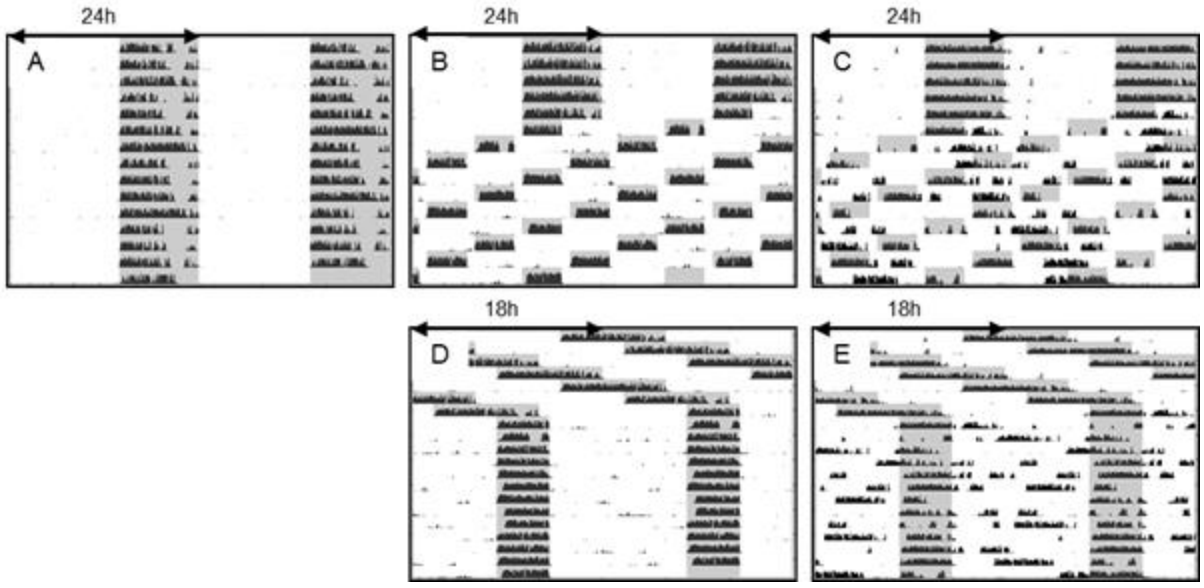

Figure 4.1. *Double plotted actograms of an animal in T24 (A), a well-entrained, included, animal in T18 (B/D) and a non-entrained, excluded, animal in T18 (C/E). D and E show the same data (modulo 18h) as B and C (modulo 24h), respectively. The last day of data is the day of dissection. Wheel running activity is represented in black and scaled to 100 counts / minute. Scotophases are indicated with background gray shading.*

ISH

In T24, *Bmal1* and *Per2* expression in the SCN (Fig. 4.2) was rhythmic with an estimated period of 23 and 24.8h, respectively (both $p<0.0001$; Fig. 4.3). *Bmal1* expression was highest 2.5h after lights off (acrophase = ZT 16.5), while *Per2* expression was highest 3 hours before lights off (acrophase = ZT 11.0). Likewise, in T18, *Bmal1* and *Per2* expression were rhythmic with estimated period of 22.8 and 23.0h, respectively ($p<0.0001$). The relative amplitudes were ~15% and ~11% lower in T18 than in T24 for *Bmal1* and *Per 2,* respectively.

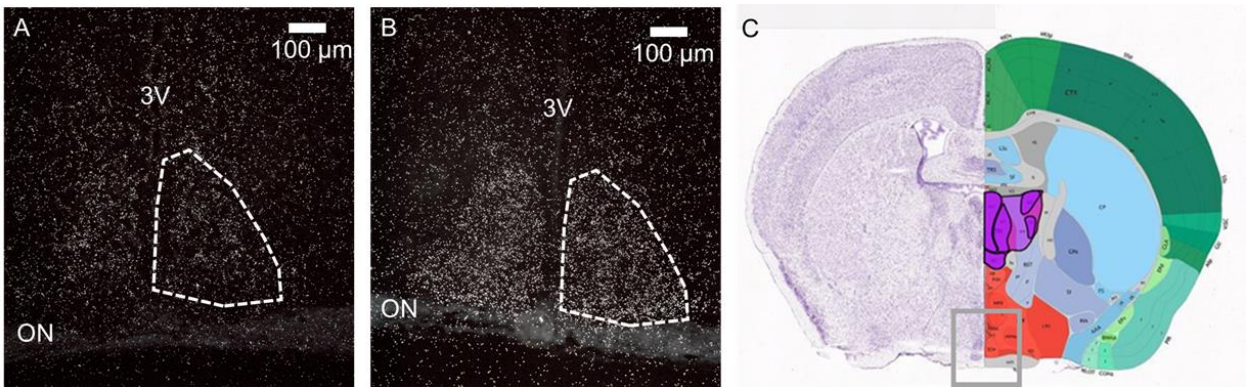

Figure 4.2. *Pictures of the SCN (~0.4mm posterior of bregma) from the T24 Bmal1 assay from ZT6 (A) and ZT15 (B) 3V: Third ventricle, ON: Optic Nerve.* Image 58/132 of The Allen Mouse Brain Atlas "P56, coronal" *(C), the gray box approximates the area of the photo in (A/B).*

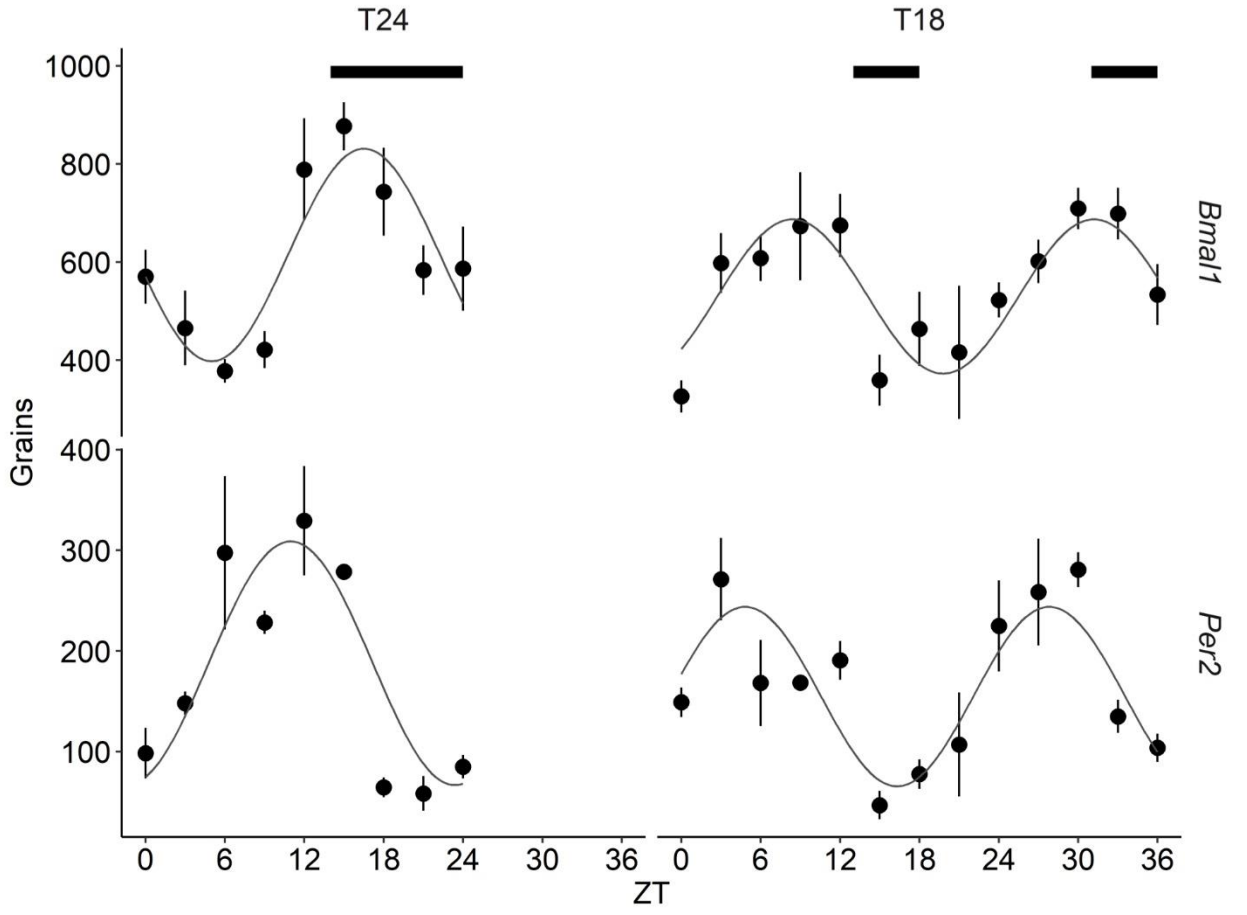

Figure 4.3*. Average number of grains per hemisphere Bmal1 and Per2 expression in the SCN. Spread indicates SE. Black bars at the top of each panel represent the scotophases. Although MetaCycle does not make assumptions about waveform, for graphing only a sinusoidal function was plotted based on period, acrophase and amplitude estimates from MetaCycle.*

qPCR

Expression levels were rhythmic for all clock genes in the liver for T24 and T18 (Figure 4.4). In the kidney, *Clock* (T24 and T18) and *Per1* (T18) were not significantly rhythmic, while all other genes were (Fig 4.4). The period estimates for gene expression in the liver and kidney in T24 were 24.2±0.76 and 24.4±0.73 (mean±SD), respectively (Fig. 4.5A). To make comparisons with T18 more conservatively, liver *Cry1* and *Cry2*, which have long period estimates > 27h, were excluded from standard deviation estimates. The periods of clock genes in T24 are expected to be 24 hours; indeed all rhythmic genes significantly fit a 24h cosinor ($p < 0.001$). Therefore the range of periods observed in genes from T24 can be considered as "noise" or inaccuracies in period estimation. For T18, all period estimates fall within 2 T24-based standard deviations of 24h, except liver *Per1* (33h) and *Cry2* (19.9h), and kidney *Per2* (22.3h) and *Cry1* (25.6h) (Fig 4.5A). Additionally, all rhythmic genes were fit to a 24h and 18h cosine. Based on R^2 values, all genes except liver *Cry2* and *Per1* are better explained by the 24h than an 18h model (Fig 4.5B).

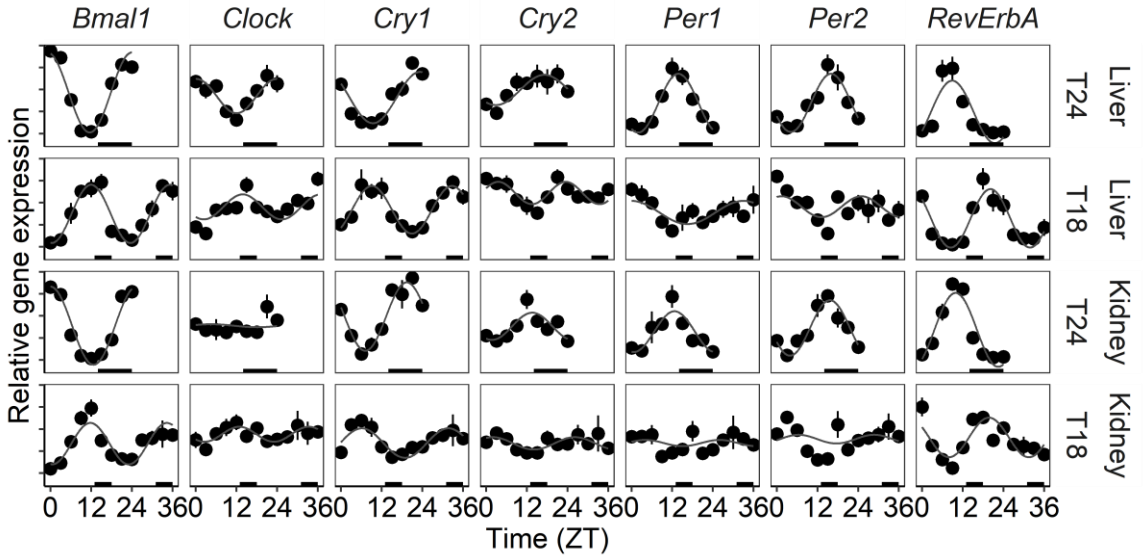

Figure 4.4*. Clock gene expression patterns in peripheral tissues from mice in T24 and T18. Data points and range indicate the mean and standard error for each time point. The line represents the sinusoidal fit based on mean, amplitude and phase estimates from the MetaCycle analyses. Black bars at the bottom of each panel indicated the scotophases. Each panel is normalized to the highest expression within each gene before averaging.*

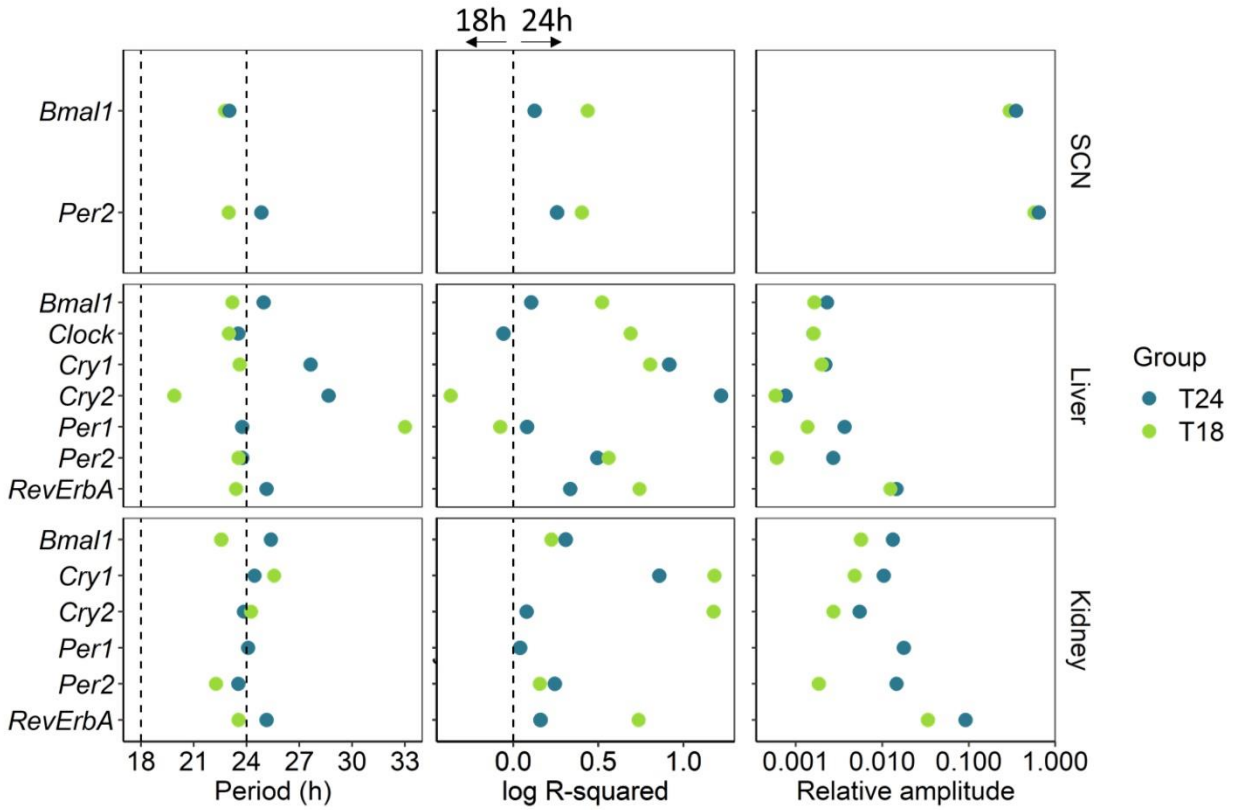

Figure 4.5. *Left panels: MetaCycle based period (dashed line at 18 and 24h). Middle panels: Log ratio R^2 calculated at log(R^2_{T24}/R^2_{T18}). Dashed line is ratio of 1 (i.e. equal fit) and positive values are better fit for T24 than T18. Right panels: log transformed relative amplitude calculated as ratio amplitude to baseline. Only values for rhythmic genes are plotted.*

Amplitude

Relative amplitude was lower in T18 in all genes in all tissues, with the exception of Clock in the Liver, where relative amplitudes were equal. Relative amplitude of the *Bmal1* and *Per2* rhythms in the SCN in T18 was 85% and 89% of that in T24, respectively. On average, the amplitude of clock gene expression in T18 was 69±10% and 38±7% of the amplitude in T24 for liver and kidney respectively (Figure 4.5C).

Phase

Because most genes in T18 across all three tissues appear to have a period closer to 24 than 18h, acrophase was expressed relative to clock time rather than ZT. While the relative phase between clock genes was highly preserved in both liver and kidney. the absolute phase in T18 was 13.1 hours later compared to T24 (Fig 4.6). The absolute phase angle between acrophases of *Bmal1* and *Per2* in the SCN was 5.5h in T24 and 3.6h in T18.

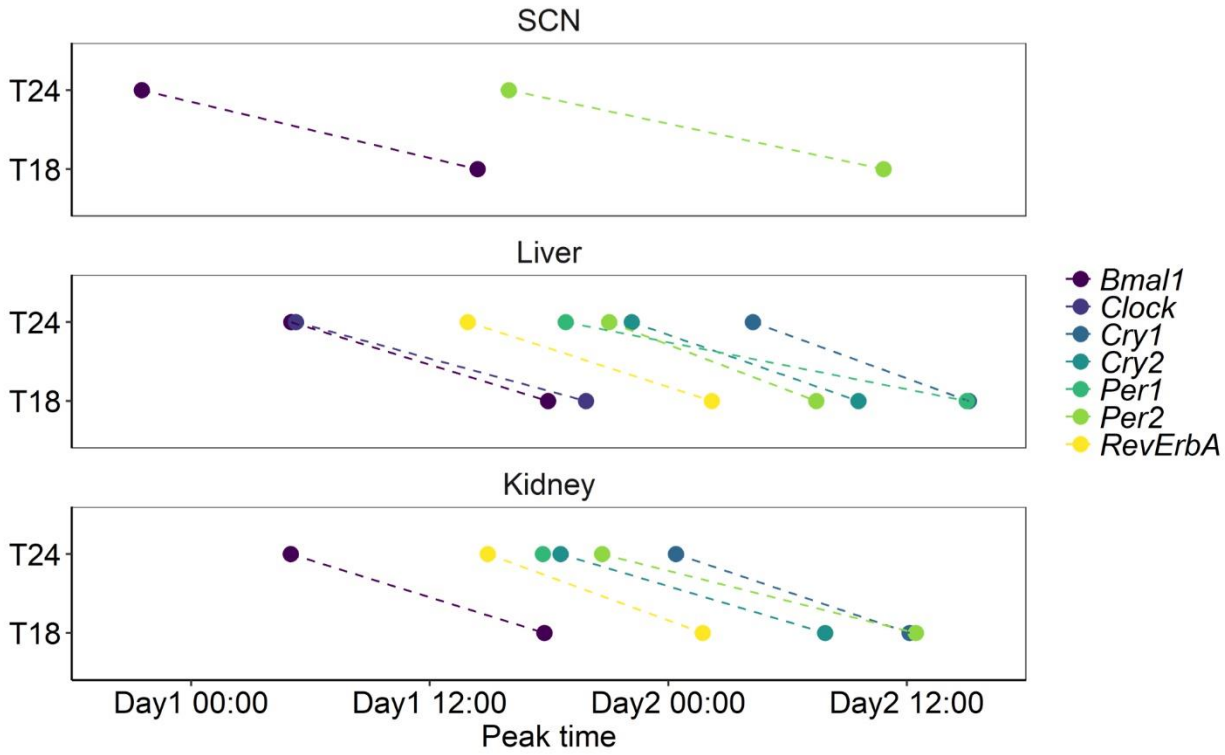

Figure 4.6. *Peak phase of each of the genes relative to clock time on the day of dissection. Some points were plotted 1 cycle later ("double plotted") to visualize the phase relationship between genes better.*

Discussion

The current project presents clock gene expression rhythms in SCN, liver and kidney in mice adapted to 18 hour rhythms. Behavioral entrainment to 18 hour light:dark cycles is unexpected from mammals and likely is not the result of classical non-parametric entrainment, yet is robust, repeatedly and reliably shown and is not a result of simple positive and negative masking. Overall, across 3 tissues and 7 different genes, clock gene rhythms appeared to be best described by 24 hours, and not 18 hour rhythms. Although not true in each individual gene, the average estimated period was much closer to 24h than 18.

Per1 and *Per2* expression patterns were altered in hamsters entrained to short (23.33h) and long (24.67h) T-cycles, indicating the reorganization in the SCN to facilitate entrainment in these lighting regimes (Schwartz et al. 2011). Furthermore, rats in a forced desychronization protocol (i.e. not entrained) in T22 showed SCN clock gene expression with two distinct periods, matching those observed in behavior (de la Iglesia et al. 2004). In our data, the main difference between T24 and T18 was a shift in phase and a reduction in amplitude, while period remained unchanged. Future studies on additional genes may allow for detection of dissociation as seen in earlier reports.

The dissociation between behavioral rhythms (18h) and SCN rhythms (24h) in 18h light:dark cycles suggests that the SCN is decoupled from the control of behavior. Control of behavior by non-canonical circadian clocks had been demonstrated before. For example, mice with *Period1/2/3* triple-mutations -- which are arrhythmic in constant conditions -- displayed evidence of a wheel-inducible circadian oscillator (Flôres et al. 2016). This novel oscillator can produce free-running locomotor rhythms with a 21h period. Whether control of behavior in T18 in our mice relies on the same wheel-inducible or any other extra-SCN oscillator (Pendergast and Yamazaki 2017) remains to be determined. Additionally, it remains unknown by what

mechanisms dim light at night uncoupled control of behavior from the SCN in genetically intact mice.

Overall, gene rhythms in T18 were best explained by a 24h rhythm with a shifted phase relative to T24. Importantly, the overall phase relationship between genes as well as between tissues was highly conserved in T18, while other circadian manipulations (e.g. photoperiod) alter clock gene expression rhythms in central and peripheral tissues differently (Sosniyenko et al. 2010). With the resolution in the data, it is impossible to distinguish between gene rhythms that are exactly 24 hours (entrained) vs near 24 hours (free-run). Therefore, we cannot without further experiments separate several possible hypotheses. The first, and likely most intuitive, hypothesis is that of a free-running SCN and physiology, while wheel running behavior is uncoupled and adapted to the light:dark cycle. The period of the free-running component in the poorly-entrained animal represented in Fig. 4.1C/E was 25.3 hours, which is a typical FRP for non-entrained animals in these conditions (average period of free-running component of all animals with EQ < 0.75 is 25.3±0.6; mean±SD). A FRP of 25.3h causes a daily 1.3h drift relative to clock time. Since animals were exposed to T18 for 10 days, a total 13h drift would be expected at the day of dissection. Because the phase difference in peak gene expression between T24 and T18 was 13h, this phase difference might result from a free-running circadian oscillation that matches the period of free running wheel running behavior. Alternatively, it is possible that the transition from LD14:10 to LD13:5 caused an initial phase shift to the SCN, which subsequently entrained to a harmonic of the 18h LD-cycle (e.g. 4x18 = 3x24 = 72h). This would predict a 24h rhythm in the SCN, which then entrains the liver and kidney, for example through corticosteroids (Pezük et al. 2012). One way to distinguish these non-exhaustive alternative explanations would be to collect more tissue 20 days into T18 conditions. If organs were entrained to a multiple of 18h, phases would be expected to be identical between 10 vs 20 days, while free-running tissues would be expected to continue to drift. Without these additional

time points we can not distinguish between these hypotheses. The data provide strong evidence, however, to reject the hypothesis that the entire circadian system is entrained to T18. Additionally, because gene expression rhythms were observed between animals, we can excluded that tissues in individuals under T18 entrainment were either non-rhythmic or cycle with a random phase. Furthermore, all three tissues had comparable phase differences between T24 and T18. We cannot, however, distinguish between independently free-running tissues with similar FRP and the possibility that peripheral tissues were entrained to the SCN.

In addition to their circadian regulation, some clock genes are known to be inducible by exogenous factors. For example, in the SCN, both *Per1* and *Per2* levels increased in response to an acute light pulse during subjective night, when expression is naturally low (Shearman et al. 1997). This inducibility plays an important role in the SCN's ability to be entrained by light; reviewed in (Welsh et al. 2010). Likewise, clock gene expression of some genes in the liver are inducible by zeitgeber input such as body temperature (Brown et al. 2002) or metabolic signals (Oike et al. 2011). In addition to acute responses, clock gene expression in the liver can be entrained to metabolic zeitgebers. In mice fed only during the day, clock gene expression in the liver will phase shift to follow feeding rhythms rather than the un-shifted SCN (Damiola et al. 2000; Stokkan et al. 2001). In our experiment, despite 18h rhythms in behavior and body temperature (Walbeek and Gorman 2017) peripheral organs did not follow 18h rhythms, but instead matched the (near) 24hour rhythms found in the SCN. In vitro, mouse liver failed to entrain to a 20 hour temperature cycle (35-37.5°C) while entrainment was stable at T24 and T28 (data unpublished). This might indicate that the liver is incapable of entraining to 18 hour cycles, and therefore, unlike the restricted feeding experiments, entrains to the SCN (or free-runs with comparable FRP) rather than following physiological rhythms. Characterization of feeding rhythms in T18 will be important in understanding these results. Importantly, rhythm amplitudes in T18 were lower than in T24 and in addition to period and phase, amplitude is an important

circadian parameter (Abraham et al. 2010). If the tissue clocks in fact run with a (near) 24h period, the periodic and harmonic influence of light, activity-induced increases in body temperature, and potentially feeding events might induce changes in gene expression independent of the circadian regulation. This likely induces “noise” that could explain digression from 24h in the estimated periods or a lower amplitude in some genes. The combination of a reduced amplitude and slightly altered phase-relationship between *Bmal1* and *Per2* in the SCN suggests that while wheel-running behavior in T18 is not controlled by the SCN, the SCN might still be sensitive from non-photic feedback such as from behavior (Hughes and Piggins 2012).

Together, these data present a model in which control of wheel running behavior appears to be uncoupled from the molecular rhythms in the SCN. Surprisingly, peripheral tissues follow the SCN, and not behavior or core body temperature rhythms. Future studies should investigate how dim light, mechanistically, induces this uncoupling.

Funding

This work was supported by discretionary departmental funds to MRG and NIH R01 HD090161 and P50 HD012303 to ASK

Acknowledgments

The authors would like to thank David Weaver for generously providing *Bmal1* and *Per2* plasmid for ISH, and Tsuyoshi Hirota for providing qPCR primer sequences for *Bmal1*, *Clock*, *Per1/2*, *Cry1/2* and *mActβ*. Additionally, we thank Adriana Esparza for assisting with ISH and Andrew Farkas, Alexandra Mendez, Andrew Zhao and Konstancia Ziegert for with help with tissue collection. Furthermore, the authors would like to thank UCSD’s Animal Care Programs Staff for help with animal husbandry. Chapter 4, in full is currently being prepared for submission for publication of the material. *Walbeek, Thijs J., Shannon B.Z. Stephens, Jennifer A. Yang, Takako Noguchi, Michael R. Gorman, Alexander S. Kauffman.* The dissertation author was the primary investigator and author of this material.

Chapter 5. Effects of short T-cycle entrainment on rodent reproduction

Abstract

In rodents, the female reproductive system is tightly connected with circadian timing. Precisely timed signals from the suprachiasmatic nuclei (SCN) trigger a cascade of neural activation and endocrine processes that comprise the estrous cycle. Consequently, circadian disruption has been shown to lead to decreased fertility. Mice exposed to repeated 6 hour advances of the light cycle (chronic jet-lag; CJL), for example, have fewer successfully completed pregnancies than un-shifted controls.

We have demonstrated increased circadian flexibility that expands the range of entrainment and markedly reduces behavioral jet-lag after phase shifts. With the provision of dim light at night (<0.1 lux), as compared to complete darkness, mice rapidly and robustly entrain to 18h light:dark-cycles (T18; LD13:5). Here we test the hypothesis that increased entrainment flexibility induced by dim light will ameliorate subfertility in non-24h environments.

In Study 1, estrous cycle length and stability were assessed via vaginal cytology in female mice entrained to T24 and T18. Mice in both groups cycled reliably but cycles of T18 mice were ~24h longer than in T24 mice as a consequence of additional day/cycle spent in estrus. And whereas T24 mice exhibited a Luteinizing Hormone (LH)-surge near the end of the light phase on pro-estrous, T18 mice sampled at the same phase did not. The absence of the predicted LH surge under conditions in which mice are fertile suggests that LH may surge in an altered phase. In Study 2, breeding success over 90 days was compared between mouse pairs entrained to T18, T24 or undergoing CJL. In contrast to earlier studies, fertility was unaffected by entrainment condition or circadian disruption as measured by number of litters, number of pups per litter, pup weight etc. One possible explanation to reconcile the paradox of the lack of a properly timed LH surge, yet unimpaired fecundity in T18 is that in T18 male presence is required to induce ovulation. In Study 3, however, ovarian histology confirmed spontaneous

ovulation in T18 mice, and therefore rejects this hypothesis. Lastly, in Study 4, timing of mating behavior was scored in breeding pairs in T18. Timing of mating was consistent relative to LD-transition both within and between breeding pairs. This suggests that at least part of the reproductive axis adapts to 18h rhythms.

Together, these data identify conditions under which fertility is preserved under CJL; establish that fertility and estrous cycling persist under 18 h behavioral entrainment, and identify changes in the temporal patterning of neuroendocrine events.

Introduction

Many industries, including in health care, transportation, manufacturing, and emergency response, require staff to work at any time of the day. Working night shifts exposes a shift-worker to a combination of overall sleep loss and inappropriately timed exposure to food and light (Smith & Eastman, 2012). Chronic circadian disruption has been associated with increased risk for mental and physiological impairments across many biological domains (Evans and Davidson 2013; Moreno et al. 2019) including cognition (Cho et al. 2000), metabolism (De Bacquer et al. 2009), immune function (Mohren et al. 2002), and reproduction (Mahoney 2010). Shiftworkers are subject to increased risk of infertility, miscarriage, irregular menstrual cycles, and low birth weight or pre-term delivery (Mahoney 2010; Gamble et al. 2013). With over 15% of US adults doing shift-work, and an associated annual cost of worker under-performance and increased health care combined exceeding 200 billion USD (Kerin and Aguirre 2005), these negative consequences are a clear society level problem. Despite awareness of these consequences of working at night, few preventive solutions exist. Better understanding of mechanisms contributing to the development of increased health risks, as well as developing strategies for shift-workers to minimize these health effects of night shifts is important.

Many of the symptoms observed in human shift-workers, can be reproduced in rodent models (Evans and Davidson 2013). For example, mice that were genetically manipulated to

have a dysfunctional circadian clock displayed disrupted estrous cycling (i.e. rodent ovulatory cycle) and pregnancy success (Miller et al. 2004). Moreover, mice that are genetically intact, but in an environmentally induced state of circadian misalignment similar to that observed in human shift-workers (i.e. chronic jetlag) had low percentage of pregnancies carried to term (Summa et al. 2012). Additionally, reproductive efficacy was impaired by exposure to 22 or 26h days (Endo and Watanabe 1989). Although reproductive consequences of circadian disruption have been extensively quantified, less is known about the exact mechanisms. Working night shifts affects a whole host of environmental and physiological factors, including sleep loss, altered light exposure, overall stress, and disrupted melatonin rhythms. Which combination of these factors mediates the effects of chronic shift-work on health is largely unknown. Consequently, very few explicit and actionable suggestions exist to minimize consequences (Lowden et al. 2019). Rodent studies with reproduction endpoints (as an example from the long list of consequences above) that model human problems, can be used to identify mechanisms and test strategies for improving shift-worker's health.

The ovulatory (or estrous) cycle in a mouse is 4-5 days long and is divided in three different stages. Ovulation happens at the day of proestrus and is triggered by a cascade of neuroendocrine events in the hypothalamic–pituitary–gonadal axis (HPG). During diestrus, developing follicles in the ovaries produce estradiol (E2), leading to a buildup of serum E2-levels. When reaching sufficiently high E2 concentration, kisspeptin neurons stimulate gonadotropin-releasing hormone (GnRH) expressing neurons in the hypothalamus to release a bolus of GnRH. The bolus of GnRH triggers the pituitary to produce a surge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulates ovulation. This surge of endocrine events is dependent on a combination of E2 concentration and temporal "gate" (Everett and Sawyer 1950; Legan et al. 1975), which tightly connects the reproductive axis and the circadian system. Although the origin of the temporal gating system is not identified, the

functional existence has been well characterized. For example, lesioning the SCN (core of the mammalian circadian clock) eliminated LH surges (Gray et al. 1978; Samson and McCann 1979). Furthermore, an injection of barbiturates to temporarily block the CNS postpones the surges by 24 hours, suggesting that if the window is missed, a next opportunity arises the next day (Everett and Sawyer 1950; Siegel et al. 1976). Lastly, E2 treated rodents have daily surges, confirming the presence of a daily gating signal that together with high levels of E2 produces a surge and ovulation (Legan and Karsch 1975; Christian et al. 2005). Because of the tight connections between circadian rhythms and HPG-axis, perhaps the reproduction consequences of circadian disruption are not surprising.

One proposed strategy to minimize circadian misalignment in a rodent model of shift work is dim-light induced flexible entrainment. With the addition of nocturnal lighting no brighter than 0.1 lux, mice can be rendered markedly more adaptable, such that they can behaviorally adapt to lighting schedules far outside the traditional range of entrainment including 18h days (i.e 13 hours of light, 5 hours of dark; LD13:5; T18) (Walbeek et al.; Harrison et al. 2016; Walbeek and Gorman 2017). Behavioral entrainment in T18 does not require genetic or pharmacological intervention and can be rapidly induced. Additionally, because it facilitated adjustments to large phase shifts, T18 entrainment has been proposed as a strategy for shiftworkers to adapt to quickly rotating working-hours (Walbeek et al.). In addition to the potential effect of increased alertness on the job and improved sleep quality, behavioral alignment could mitigate long term health effects; however, this has not been formally tested yet. In human shift-work schedules, as well as in rodent shift-work models, subjects are exposed to non-24h paradigms, which they fail to adapt to. It is unclear whether non-24 *per se* is bad for health or that the non-adaptation is critical. Testing for reproductive health while behavior is adapted to a T-cycle, could help separate some of these effects. With T18, we have

a model that facilitated adaptation in non-24h conditions, and therefore allowed to test the hypothesis that adaptation is critical and not the non-24 nature of the schedules.

In the current studies, we aimed to characterize reproductive function in mice entrained to T18. First, using a three-month fecundity assay, we tested the hypothesis that T18 entrainment does not produce the same reproduction deficits seen in other non-24h context. Specifically, functional fertility of mice in T18 is quantified and compared to positive control animals in standard T24 lighting conditions and chronically jetlagged negative control animals. Second, estrous cycle length and regularity were measured under T18 and compared to T24 controls. Additionally, through ovarian histological examination, follicle development and spontaneous ovulation were characterized. Lastly, through video analysis, timing of mating behavior in breeding pairs entrained to T18 was scored. Combined, these studies test one aspect of health consequences of flexible entrainment, as well as deploy a research strategy that can develop a mechanistic understanding of health consequences of circadian disruption and interactions between circadian and endocrine systems.

Methods

General

In all experiments, mice (C57BL/6) were house in plastic cages (28x18x15cm) equipped with a running wheel (13cm diameter). Food (Mouse Diet 5015; Purina, St. Louis, MO) and water were provided ad libitum and refreshed regularly. Well-ventilated environmental chambers that accommodate up to 16 cages were used to regulate temperature (22±2 °C) and light exposure. Chambers were equipped with fluorescent lamps for bright light (327±162 lux) and additional dim green LEDs for dim light illuminance (555±23 nm, <0.1 lux, irradiance of 3.90 × 10-5 W m^{-2}). Light-dark cycles, and the use of dim lights are indicated for each experiment (i.e. LD_{ark} vs LD_{im}). Wheel-running was continuously recorded as the number of half wheel revolutions per 6 min bin (VitalView Version 4.2, Mini-mitter, Bend OR). At the end of the experiments, all

animals were euthanized humanely. All experiments and procedures were pre-approved by and followed the guidelines of UCSD's Institutional Animal Care and Use Program.

Across the presented experiments, two different lighting cycles each with different day lengths (i.e. T-cycles) were used. In the colony, as well as in control conditions, animals were housed in 14 hours of light and 10 hours of darkness (i.e. LD14:10). In experimental T18 conditions, light cycles were 13 hours of light and 5 hours of darkness (i.e. LD13:5). Lastly, in Study 2, a Chronic Jetlag paradigm is used as negative control. Here, animals were housed in LD14:10 but phase advanced by 6 hours every week, a form of environmental circadian disruption that has been shown to induce impaired reproductive function (Summa et al. 2012) as well as other negative health consequences; e.g. (Davidson et al. 2006; Adams et al. 2013).

To quantify entrainment in T18, Entrainment Quotients (EQ) were used. EQ values are based on Lomb-Scargle periodogram analysis, which evaluates rhythmicity in the data for any possible period. Periodogram power at 18h (peak value 17.8-18.2) and the circadian range (peak value 23-26h) were extracted and used to calculate EQ using the following formula: $EQ = power18h / (power18h + powerCirc)$. EQ-values therefore range from near 0 (all power in circadian range) for a free-running animal to near 1 (all power at 18h) for a completely adapted animal. Any free-running rhythms that are partially masked will result in intermediate EQ-values.

Study 1a - Estrous cycling

Female (born into our colony) mice were divided into either T24 or T18 (n=10/group). The 2 groups were split between two environmental chambers that additionally each housed 6 males. Males were not used for any part of the analyses, but to provide pheromonal cycling cues (McClintock 1978). All mice were initially entrained to LD_{im}14:10. After 3 weeks of habituation, mice in T18 were entrained to LD_{im}13:5, while mice in T24 remained on LD_{im}14:10 for the full duration of the experiment. After 10 days, stable entrainment was established and daily (every 24h) vaginal lavages were performed during the photophase. Lavages were

performed for 20 and 24 consecutive days in T24 and T18 respectively. Samples were spread out on microscopic glass in 3ml water, dried, and stained with Methyl Blue Staining. Estrous cycle stages were identified based on microscopic cytological evaluation (Byers et al. 2012; McLean et al. 2012). Each sample was scored by 2 independent researchers (T.J.W./A.M./A.Z./K.Z.). If needed, a 3rd observation would resolve any conflicts. To count cycle length, only completed cycles from proestrus to proestrus were counted.

Study 1b - Endogenous LH surge

After tracking the estrous cycle, serial blood blood samples (3µl) were collected from the tail vein for characterization of luteinizing hormone (LH) surges. T24 mice were sampled 1 hour before light-off on 4 consecutive days (i.e 24h between samples), while T18 mice were sampled on 2 series of 3 consecutive light-off transitions (i.e 18h between samples) with 1 day in between. During days of blood collection vaginal lavages were continued. Hormone determinations were done as described before (Tonsfeldt et al. 2019). In short, blood samples were kept in collection capillary at room temperature to clot for 90 minutes before centrifuging at 2000 x g for 15 minutes. Serum was separated and frozen at -20°C until further processing. For LH-measurement, samples were run in singlet on Milliplex analyzer (MPTMAG-49K; MilliporeSigma, Burlington, MA) using a Luminex Magpix (LH: lower detection limit, 4.8 pg/mL; intra-assay coefficient of variation, 15.2%; inter-assay coefficient of variation, 4.7%).

Study 2 - Fecundity

Male and female mice (N=52, 8-12 weeks old, ordered from Jackson labs or from Jackson stock) were individually housed with a running wheel on LD14:10. After 12 days of habituation, mice were split into three groups. The first group remained on LD14:10 (LD), the second group was transitioned into LD13:5 (T18), and the third group was chronically jet-lagged by exposure to weekly 6h phase advances (CJL). Only the T18 group received dim light at night to facilitate entrainment. After 12 days in experimental conditions, 1 male and 1 female were

excluded from T18 for poor entrainment (EQ < 0.9). The remainder male and female mice were paired by transferring the females into the cage of a male from the same condition, creating 8, 9 and 8 breeding pairs for LD, T18 and CJL respectively. Breeding pairs remained under the same housing and lighting conditions for the remaining of the experiment. Cages were changed weekly, except within the first week of a new litter being born.

Cages were monitored daily for new litters. For each litter pups were counted on the day they were found (day 0) and again on day 1, while trying to minimize disruption to the nest. Any dead pups were removed from the cages, but included in the Day 0 count. On day 21 pups were weaned, weighed, and sex of the mice was determined. Pups received identifying earmarks under 2% isoflurane anesthesia. The sex of the mice was confirmed 7 days post weaning and records updated retrospectively if needed. 70 days after pairing, males were removed from cages. Females and pups were left alone until last pups were weaned. One breeding pair in T18 did not complete the experiment because of dystocia during the second litter; this breeding pair was excluded from the pups/litter analyses, but the first litter was included in appropriate analyses.

Study 3 - Ovarian histology

A separate cohort of female mice was entrained to either LD_{im}14:10 (n=10) or LD_{im}13:5 (n=11) for 3 weeks. Following stable entrainment, mice were anesthetized using 4% isoflurane and ovaries were surgically removed. After dissection, ovaries were fixed in 60% EtOH, 30% 37% formaldehyde and 10% glacial acetic acid on a shaker at 4°C for 24 hours. After that, tissue was stored at 70% EtOH at 4°C until further processing. Fixed tissue was embedded in paraffin and sliced at 20µm using a microtome. Every fifth slide was stained using hematoxylin and eosin and follicles and corpora lutea were counted in each section using a microscope. Follicles were not staged. For each ovary, the slice with the highest count was used as the

number of follicles or corpora lutea for that animal. For each animal, only 1 of the ovaries was counted.

Study 4 - Mating behavior

Sixteen mice (10 females, 6 males; 8 weeks old from Jackson labs) were entrained to LD_{im}13:5 following 2 weeks of LD_{im}14:10. On the 5th day in T18, male mice were gonadectomized and received testosterone implantation as described before (Schoeller et al. 2016) to prevent pregnancy. In short, bilateral gonadectomy was performed under 2% isoflurane anesthesia. Silastic brand silicone capsules (Dow Corning Corp; internal diameter 1.02 mm; 6mm length) filled with testosterone (Sigma; T1500; 6mm) were implanted subcutaneously between the shoulder blades of the animal. At the end of the surgery animals received a single subcutaneous injection of 0.05mg/kg buprenorphine. Following 5 days of recovery, the 4 best-entrained males were paired with 4 well-entrained females. One week later, animals were separated for 48 hours before males were paired with a new set of well-entrained females for one week. Male cages were video recorded throughout the experiment. Video recordings were scored for successful mounting attempts.

Results

Study 1a - Estrous cycling

Wheel running behavior for two illustrative animals are presented in Figure 5.1. The mouse in T24 (Fig. 5.1A) is well entrained to the light dark cycle (gray shading). As in many individuals, a 4-5 day pattern in activity emerged that is a characteristic signature of the estrous cycle (Fahrbach et al. 1985; Kopp et al. 2006; Lightfoot 2008). On every 4-5 days, on days of proestrus, activity counts are higher than on surrounding days, especially during the second half of the scotophase where activity counts are more than twice as high compared to other day (red arrows in Fig. 5.1A). Data for the animals in T18 are represented twice: modulo 24h (Fig. 5.1B) and modulo 18h (Fig. 5.1C). Combined these actograms show high levels of behavioral

entrainment. Activity reliably lines up with the scotophases (best visible in Fig. 5.1C) and there is no free-running component with a near 24h cycle (best visible in Fig. 5.1B). There is no display of estrous cycling in this or any other animals in T18. All females in T18 were well entrained, as evident from high EQ-scores (0.99±0.0052). Mice for this study were born from dams entrained to either T24 or T18 and housed in those respective conditions until moved to the colony at weaning (Day 21). Pups were counterbalanced between groups and no evidence of statistical differences depending on history were found.

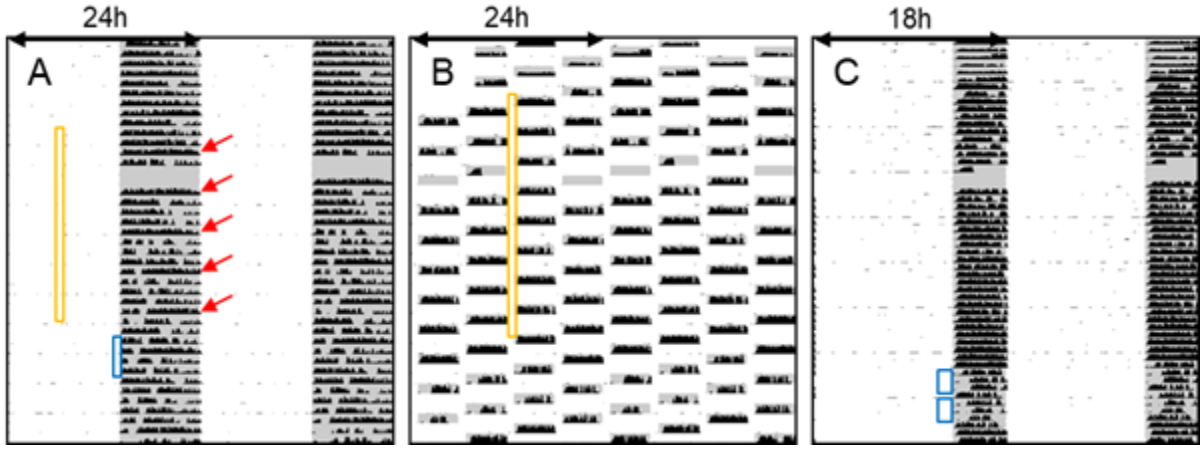

Figure 5.1. *Example double-plotted actograms from female mice from Study 1a that are exposed to T24 (A) or T18 (B/C). Data for animal in T18 are plotted in modulo 24h(B) and modulo 18h(C). Orange boxes indicate the 1h window during which smears were performed (every 24h in T18, therefore represented in B) and blue boxes indicate time of tail blood samples (every 18h in T18, therefore represented in C). Red arrows point at increased activity on days of proestrus, only indicated where confirmed by cytology. The gap in activity about one third into the experiment, indicates 48h of loss of wheel-running data. Only data were lost; animals or lighting were not affected.*

Estrous stages for four animals are represented in Figure 5.2. All illustrative animals progress through multiple complete estrous cycles (diestrus -- proestrus -- estrus) in the measured period (Fig 5.2A). Mice in T18 took, on average, 24h longer to complete a full cycle through all stages than did mice in T24 (4.3 ± 0.18 day vs 5.3 ± 0.32 day for T24 and T18 respectively, ($t(13.9) = 2.71$, $p < 0.05$. Fig. 5.2A/B). All of the difference is explained by extra time spent in estrus, no difference in time spent in diestrus or proestrous (Fig. 5.2C). Cycle length variability within individuals did not differ between groups ($t(8.4) = 0.74$; $p = 0.48$). In T24

animals, days of proestrus based on cytology did align with activity based “proestrus days” (red arrows in Fig. 5.1A). Also post hoc (after knowing the proestrus days), no estrus-related locomotor activity patterns could be detected in T18 animals.

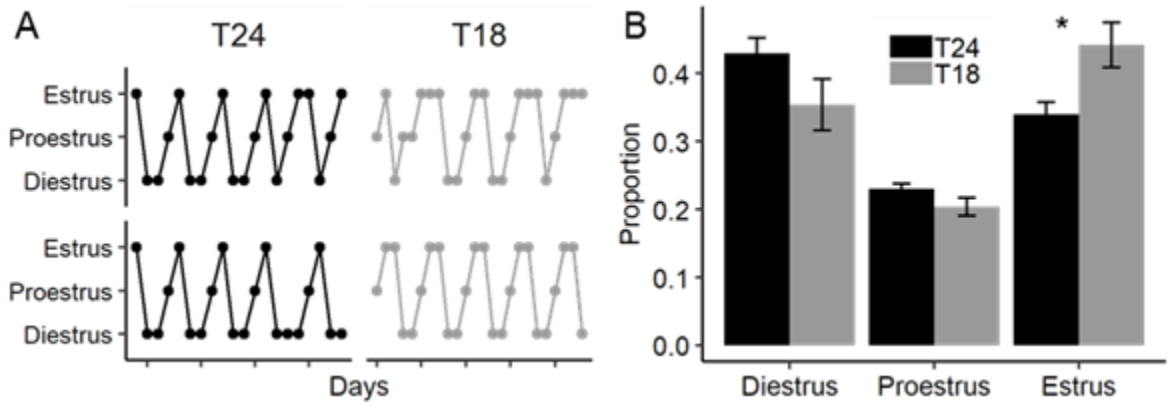

Figure 5.2. *Example cycling data for an animal in T24 and in T18 (A). The upper two animals in A are the same individuals represented in Figure 5.1 (Actograms). Staging is based on cell type composition in vaginal lavages performed every 24h for 20-24 days. Group averages of the proportion of days in each stages (B). * indicates significant group differences.*

Study 1b - Endogenous LH surge

As expected, all T24 animals showed significantly elevated LH concentrations on days of cytology-confirmed proestrus (Fig. 5.3). In T18, no elevated LH was observed in any of the days, despite 9/10 animals going through at least 1 day of proestrus (based on cytology) during the days of sampling (Fig. 5.3).

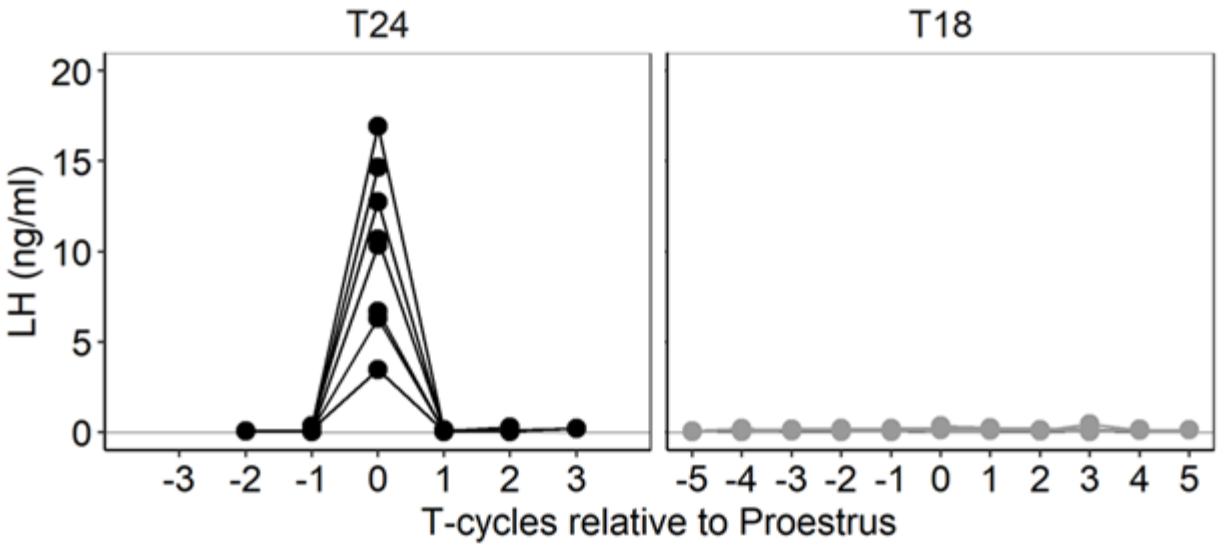

***Figure 5.3**. Serum Luteinizing Hormone concentrations from animals in Study 1b. Sampling days are aligned based on cytology-confirmed proestrus. Because proestrus was not on the same day in each animal, the total range of cycles ranges from -3 to +3 and -5 to +5 for T24 and T18 respectively. Each individual animal, however, only had 4 or 6 samples in each condition respectively.*

Study 2 - Fecundity

Three example actograms from the three-months long fecundity study are shown in Figure 5.4. Because the cages housed multiple animals, and the setup does not distinguish which individual is in the wheel, no observations about individual entrainment can be drawn. EQ-scores in T18 from individual animals before pairing were 0.97±0.003 and 0.94±0.01 for females and males respectively. In both T24 (Fig. 5.4A) and T18 (Fig. 5.4B) the majority of activity occurs during the scotophases, with little activity during the day. Daily activity profiles appear to change depending on pregnancy and litters in the cage, but the effects are likely to be less pronounced than might be expected because there are males in the cage. The mice in CJL (Fig. 5.4) appear to be less well adapted. After each 6h phase advance, activity onsets occur hours after lights off, and catch up in about a week, right when the next phase shifts occur, causing chronic misalignment (as intended).

For each of the breeding-pairs, reproductive output was scored (Table 5.1). Among none of the measures were any significant groups differences found. For both number of litters per

breeding pair and number of pups per litter on Day 0, groups trended to be different. None of the post hoc group comparisons are significant, but the trends were toward the experimental groups having more pups per litter, and more litters in CJL than T24 controls, against our expectations.

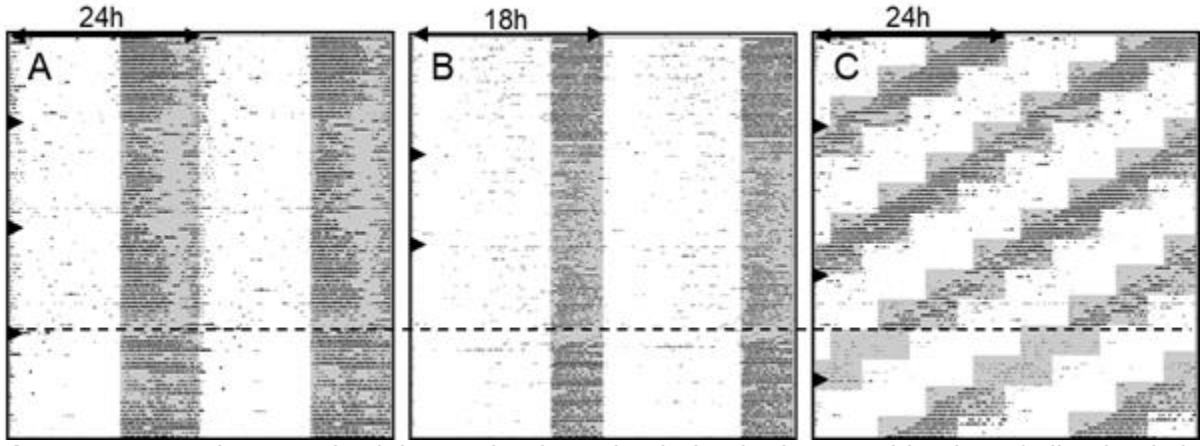

Figure 5.4. *110 days worth of data. Wheel running behavior is a combination of all animals in the cage, can't separate who's running. Horizontal line is male removal. Triangle are litters being born.*

Table 5.1: *Reproductive outcomes in Study 2.*

	T24 (n=8)	T18 (n=9)	CJL (n=8)	Statistics
Number of litters (count)	2.5±0.3	2.4±0.2	3.1±0.2	H(2) = 5.4; p = 0.07
Pups / litter at birth (count)	6.8±0.5	8.1±0.6	8.4±0.5	H(2) = 5.2; p=0.08
Pups / litter at wean (count)	4.9±0.8	6.5±0.7	5.4±0.8	H(2) = 1.7; p=0.42
Weight of the pups (grams)	8.2±0.4	8.5±0.3	7.8±0.3	$X^2(2) = 2.7$; p = 0.25
Time until first litter (days)	24±2	31±4	24±2	H(2) = 4.0; p = 0.13

Study 3 - Ovarian histology

Ovaries from animals exposed to T18 were indistinguishable from those collected from females in T24. The number of follicles (T24: 6.1±0.5; T18: 7.4±0.7; t(18.2) = -1.53; p = 0.14) and CL (T24: 4.2±0.4; T18: 4±0.8; t(14.7) = 0.22; p = 0.83) did not differ between groups.

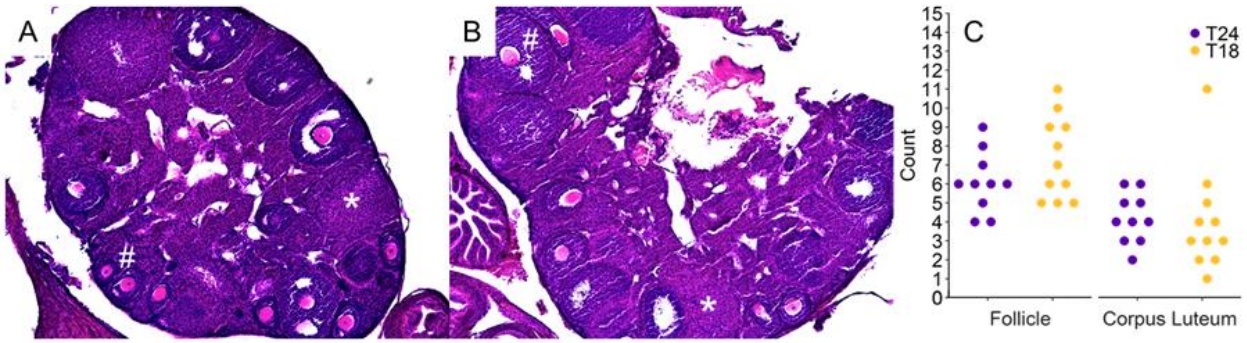

Figure 5.5. *Illustrative ovary images from and animals in T24 (A), and T18 (B). Example follicles are indicated with "#" and example corpus luteum with "*". Counts for all animals are shown in (C).*

Study 4 - Mating behavior

Mating behavior was observed in seven of eight breeding pairs, with one breeding pair (Breeding pair 2, see Fig. 5.6) showing two independent mating events. Each mating event contained multiple (3-20) mounting attempts over about an hour-long window. Time points for all mounting events were averaged. No synchronization was found between breeding pairs. Timing of the mating events relative to clock time and relative to zeitgeber time (i.e. LD-transitions) are plotted in Figure 5.6. All mating events occur between ZT7 and ZT12 ($R(7) = 0.90$; $p<0.001$), while there is no apparent consistency relative to clock time ($R(7) = 0.01$; $p<0.93$).

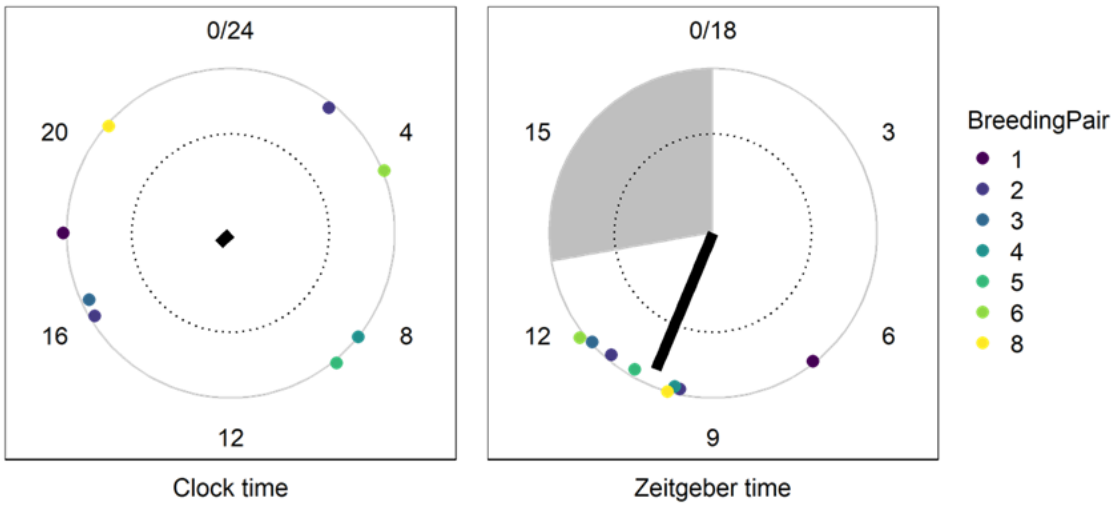

Figure 5.6. *Timing of each mating event relative to clock time (left) and zeitgeber time (right). Vector is indicated with thick black line, the dotted circle indicates the significance threshold. Light:dark cycle is indicated with gray shading.*

Discussion

In the present studies, reproductive function of female mice entrained to T18 was investigated. Mice in T18 had regular, but slightly lengthened estrous cycles compared to mice in T24. Unlike control animals, on days of proestrus, females in experimental conditions did not display altered activity profiles and lacked a surge in serum LH at the time of lights off transition. Despite the absence of an appropriately timed LH surge, breeding pairs in T18 did not display impaired fecundity compared to controls. Similarly, unlike other studies, mice exposed to chronic jet-lag also did not display fecundity deficits. Additionally, T18 females did display normal ovarian morphology and signs of spontaneous ovulation. All mating behavior occurred during the light phase, with seven out of eight events within a few hours before lights off. Together, these data identify conditions under which fertility is preserved under CJL; establish that fertility and estrous cycling persists under 18h behavioral entrainment and identify changes in the temporal patterning of neuroendocrine events.

Estrous cycles

Estrous cyclicity had been demonstrated to be distorted as a consequence of altered circadian rhythmicity. Therefore, the regular and stable estrous cycles in females mice in T18

are somewhat surprising. The precise mechanisms of the circadian gating system regulating ovulation is still unknown, but probably involves a network of hypothalamic nuclei. If, like behavior, the hypothalamus, which also included the SCN, entrained to 18h rhythms, a faster estrous cycle could be expected in T18 compared to T24. If, on the other hand, hypothalamic areas uncouple from behavioral control and continue to cycle on near 24h rhythms, equal estrous cycles might be expected. Surprisingly, estrous cycles in T18 were about a day slower than in T24. Although no mechanistic studies have been performed thus far, slower estrous cycles, despite a faster circadian system providing the timing of the gating, could be explained by a simple coincidence model: To trigger an ovulation-inducing LH surge, a combination of sufficiently high levels of E2 and a temporally defined gating signal are required (Wintermantel et al. 2006; Williams et al. 2011; Williams and Kriegsfeld 2012). Our histological ovarian analysis indicates normal follicle development, which suggests E2 build up, independent of the circadian system. One could expect the time it takes to reach threshold E2 levels to be equally long independent of circadian conditions. Then, ovulation happens in the first circadian window after threshold has been reached. If, for example, E2 build up takes 95 hours to reach threshold, T24 would have its next gate, and therefore ovulation at 96 hours (4x24), but T18 would have just “missed” the window at 90 hours (5x18) and now needs to wait until 108 (6x18) before ovulation occurs. Therefore, a simplistic mathematical coincidence model could explain slower estrous cycles in T18 than T24, even if critical areas in the hypothalamus run at 18 hours.

Surge & ovaries

The lack of LH surges at the end of the light phase suggest that the timing of the LH surge relative the the LD-cycles has been altered in T18. Alternatively, male-induced ovulation, as seen in other mammalian species (Carter et al. 1989), could explain the combined observations of the lack of an LH surge at lights off in isolated females, yet full fecundity in breeding pairs in T18. However, ovarian morphology and counts of follicles and corpora lutea in

T18 indicate that spontaneous ovulation persists in T18. Therefore, it remains to be determined if LH surges, and therefore ovulation, in T18 happen at different consistent time, or that surges are unpredictable under these conditions. Additionally, we cannot rule out the possibility that any LH surge in T18 has markedly lower amplitude and therefore might be undetectable, as seen in *Bmal1*$^{-/-}$ mice that showed ovulation but seemingly lack a detectable LH surge (Chu et al. 2013).

Fecundity

Mice that were exposed T22 or T26 had impaired pregnancy efficacy as long as exposed to T-cycles both before and during pregnancy (Endo and Watanabe 1989). Presumably, mice in these T-cycles were not entrained, but that is not explicitly commented on in the paper. Furthermore, four 6h phase advances during pregnancy had lower percent of pregnancies carried to term than did control mice (Summa et al. 2012). Because T18 entrainment requires daily instead of weekly phase advances from a near 24h FRP, and T18 is more extreme than T22, exposure to T18 could be expected to be worse for reproductive health than either CJL or T22/T26. On the other hand, mice in T18 do not show the misalignment of activity with the LD cycle that is common in CJL, but instead are behaviorally entrained. Comparing performance in T18 and CJL could distinguish between effects of non-24h cycles and lack of entrainment on health. In Study 2, surprisingly, neither T18 nor CJL breeding pairs showed impaired fecundity compared to T24 control animals. Although not significant, the experimental groups may even have had more litters per breeding pair and more pups per litter. These trends make it unlikely that negative effects of CJL were not observed due to insufficient statistical power in the study. The absence of an effect in T18 suggests that a lengthening of estrous cycles by 1 day (Study 1) did not affect fecundity and may suggest that T18 is not a form of circadian disruption. The lack of a negative control (in this case, CJL), however, does not allow for a strong case on the latter interpretation.

Notably, CJL did not affect fecundity in our study, while it did in the same strain of mice in earlier report (Summa et al. 2012). Potential methodological differences explaining these seemingly contradicting results include the phase shifting schedule. Whereas in our study animals were phase shifted every 7 days, starting before pairing and all the way through weaning, in the earlier report animals were phase shifted every 5-6 days and only during gestation. However, weekly phase shifts have been shown to be severe enough to induce deficits in other contexts; e.g. (Davidson et al. 2006; Adams et al. 2013). Additionally, mice in our study had access to a running wheel. Voluntary activity, or maybe even wheel running per se, has been shown to be protective against stress-induced depression (Solberg et al. 1999), and caused changes in overall physiology and circadian activity patterns; reviewed in (Novak et al. 2012). Although we are not aware of any direct studies on the mediation of running wheels in the effect on circadian disruption on reproductive health, it is theoretically possible that wheels are important. In addition to highlighting the potential importance of wheels or any other methodological details, the lack of replication of these findings might suggest that paradigms used to study circadian disruption might not be not very generalizable to other context in rodents, let alone human shiftworkers. Importantly, despite common objection, it has been argued that access to wheels represents a more ecologically relevant condition (Meijer and Robbers 2014).

Timing of mating behavior

In T24, the majority of mating occurs between three and six hours *after* lights off (Snell et al. 1940). In contrast, all mating events in T18 occurred during the light phase, with seven out of eight mating events occurring between 1 and 3.5 hours *before* lights off. Because males were castrated, we were able to observe multiple events within the same breeding pair. Only one pair however, did show a second mating event. The interval between the two mating events was six 18h cycles, corroborating estrous cycle lengths of ~5 24h days from Study 1a. In other

pairs females may have experienced pseudo pregnancy following the first penetration, delaying a potential second mating beyond the scope of the experiment. Overall, the timing of mating behavior relative to the 18h light:dark cycle is consistent between and within breeding pairs. This timing, however, is 6 hours earlier relative to lights off than reported mating time in T24. If the relative timing between LH surge, mating behavior, and ovulation remains consistent (Snell et al. 1940) in T18, this relative phase shift might explain the lack of LH surge around lights off (Study 1b).

In summary, we have demonstrated that the temporal organization of reproductive function in female mice entrained to T18 has been altered. This does not, however, lead to impaired reproductive function, unlike mice in non-24h conditions in earlier reports. These results expand on the characterization of physiology under enhanced circadian plasticity and provide a model to study temporal dynamics in the hypothalamus.

Acknowledgments

The authors would like to thank the staff from UCSD's Animal Care Program for their help with animal husbandry. Additional thanks go out to Hanne Hoffman for consulting on experimental design, and Alexander Pena for scoring videos on mating behavior.

Chapter 5, in full is currently being prepared for submission for publication of the material. *Walbeek, Thijs J., Karen J. Tonsfeldt, Alexandra Mendez, Andrew Zhao, Konstancja Ziegert, Michael R. Gorman.* The dissertation author was the primary investigator and author of this material.

Chapter 6. General discussion and conclusion

Summary

In this dissertation, I have demonstrated that in permissive conditions the circadian system of a mouse can be markedly more flexible than predicted by classical entrainment theory. I have demonstrated that entrainment in 18 hour days can be induced repeatedly, rapidly, and robustly. Enhanced behavioral plasticity showed, at least in part, signs of bona fide entrainment such as a predictable phase in constant dark and entrained core body temperature rhythms. Dim light at night and bifurcation after-effects were each sufficient to facilitate behavioral entrainment in T18. I have shown that, unlike control animals in T24, flexibly entrained mice in T18 do not show transient misalignment following a phase shift. Combined with the lack of large phase angle of entrainment, this suggests that behavior in T18 is not controlled by a non-parametrically entrained strong circadian oscillator with canonical oscillator characteristics. Additionally, behavioral entrainment does not depend on the traditional clock gene interactions. Despite 18h rhythms in the light:dark cycle, behavior, and core body temperature, clock gene expression rhythm in the SCN, liver, and kidney do not follow an 18 hour rhythm and are instead better explained by a near 24h rhythm. Because in these conditions activity can be scheduled at any time during the day without the need for a long transitional period, entrainment in T18 has the potential to facilitate adaptation in shiftwork-like schedules. Furthermore, the temporal organization of the reproductive axis is altered in T18 compared to T24. This does not, however, result in impaired reproductive function as seen in other non-24h contexts. All together, we have developed an attractive model to investigate under-explored plasticity to the circadian system, mechanisms driving behavior in extreme entrainment and test mechanistic hypotheses of how circadian disruption causes negative health outcomes.

Integration into a working model

Based on the observation of adapted behavior to T18, three main hypotheses can be made on how it is controlled: A) "*Entrained*" - Dim light at night alters the entire circadian system such that its range of entrainment is larger and can adapt to T18 ; B) "*Uncoupled*" - Control of behavior is uncoupled from the SCN. While behavior is controlled by light at 18h, the SCN continues to keep track of 24h time. This could be either in a free-running state or because it is entrained to T24 harmonics; C) "*Dampened*" - The circadian system is completely suppressed. While behavior is controlled by light at 18h, the SCN is dampened to a low amplitude or arrhythmic state and does not continue to keep time (Fig. 6.1).

In Chapter 2, phase of activity in constant darkness was predicted by time of release. If a strong circadian oscillator kept track of 24h time, phase would be predicted by the phase of that clock. If phase was consistent between animals, this would have resulted in clustering at a phase independent of time of release, or in random phases if animals were all free-running independently. The observations of a phase predicted by time of release can not distinguish between an "Entrained" oscillator and one that is "Dampened" but starts back up or is reset-when released in DD. This chapter does, however, discount the "Uncoupled" hypothesis. In Chapter 3, no evidence of a strong circadian oscillator controlling behavior in T18 was observed. At least, not of an oscillator that shares characteristics of the slow-shifting oscillator in T24. In combination with the lack of phase angles, this rejects the "Entrained" hypotheses, but can not distinguish between "Uncoupled" or "Dampened". The gene expression data in Chapter 4, provides strong evidence for a 24h oscillator that is "uncoupled" from behavior. We cannot distinguish between an entrained vs free-running circadian clock, but the 24h oscillation is apparent. In Chapter 5, the lengthened estrous cycle seems to not unambiguously provide evidence rejecting or supporting any of these hypotheses. The fixed timing of mating behavior

relative to the light:dark cycle, on the other hand, strongly suggests that at least part of the hypothalamus might be adapted to 18h rhythms.

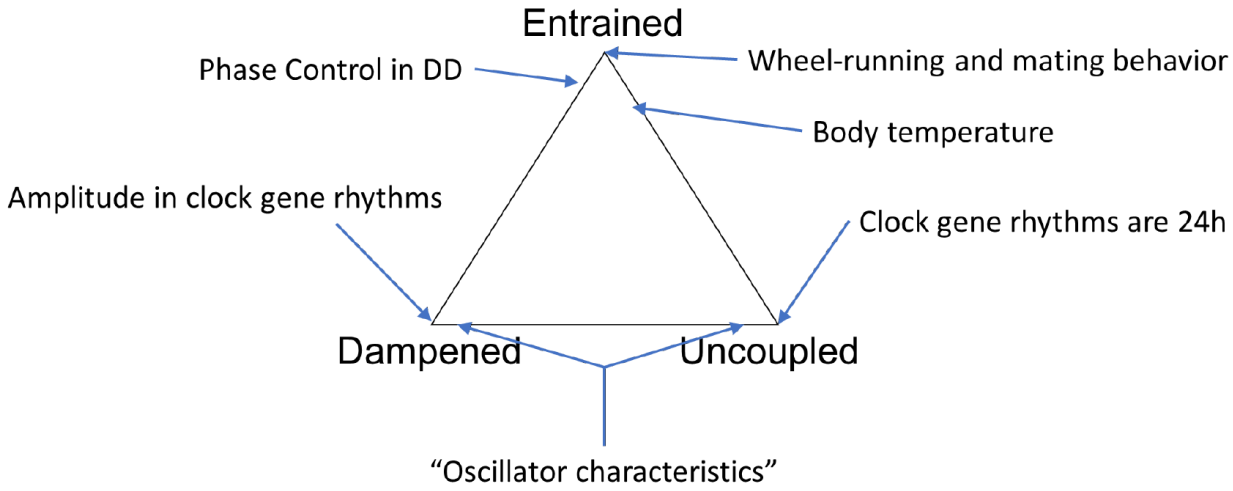

***Figure 6.1**. Summary of experiments. Each of the chapters is mapped onto hypotheses to show which is supported by the data.*

Evidently, different chapters favor different alternative hypotheses and it is not immediately clear how to resolve this seeming conflict. One possibly important factor is the length of time spent in T18. While clock gene expression data was measured after only 10 days in T18 light cycles, animals in all other experiments had been in T18 for at least 2 weeks. Perhaps at first, following an acute transition to T18, behavior becomes uncoupled. Then, over time, the core oscillator dampens. Although untested in these conditions, repeated light exposure at all multiple phases -- as would happen with a free-running SCN in T18 -- theoretically has the ability to drive the SCN to an arrhythmic state (Grone et al. 2011). Unlike in T18, however, the arrhythmic SCN in the cited work led to arrhythmic behavior. Alternatively, not all rhythms in the hypothalamus need to respond in the same way. While (parts of) the SCN might free-run, other areas could adapt to T18 to control mating behavior, for example. Only the characterization of clock gene expression in other areas and at different points in time could differentiate between these working models.

Unresolved questions

Although the work presented in this dissertation provides significant insight into the characteristics, mechanisms, and consequences of enhanced circadian plasticity, several questions remain unanswered. First, for an entrained animal in T18 behavior has 18h rhythmity, while the SCN is at 24h. Therefore, behavior seems uncoupled from control of the SCN. This uncoupling is reliably facilitated by dim light at night, at illuminance levels that have been reported to be too weak to have circadian impact (Brainard et al. 1982; Brainard et al. 1984; Nelson and Takahashi 1991a; Nelson and Takahashi 1991b; Brainard et al. 2001). So far, mechanisms remain unclear, but based on the light intensity rod-mediated pathways might be expected. Additionally, it is unclear what controls behavior, especially after release in constant dark. With an SCN that keeps 24h time, phase in DD would not be expected to be predicted by time of release. As speculated above, it is possible that the SCN become dampened or arrhythmic over time. Alternatively, the SCN might continue to keep time, but become easily resettable, such that the transition to DD resets the phase.

Furthermore, the SCN is thought to be responsible for the circadian gating in ovulatory control. As part of this neuro-endocrine cascade, mating behavior is typically limited to the fertile window following ovulation. Yet, in our experiments, the SCN has 24h rhythmicity while mating behavior appears to be controlled on an 18h basis. The timing of mating behavior relative to light off, however, has been altered. Perhaps, that explains the lack of LH surge around lights off. The mechanisms controlling the temporal organization in the hypothalamus, including the SCN remain to be determined. Extra-SCN hypothalamic nuclei might be responsible for the timing of mating behavior. Understanding these temporal dynamics could additionally provide guidance in finding the timing of an LH surge in T18.

References

Abraham U, Granada AE, Westermark PO, Heine M, Kramer A, Herzel H. 2010. Coupling governs entrainment range of circadian clocks. Mol Syst Biol. 6:438. doi:10.1038/msb.2010.92.

Adams KL, Castanon-Cervantes O, Evans JA, Davidson AJ. 2013. Environmental circadian disruption elevates the IL-6 response to lipopolysaccharide in blood. J Biol Rhythms. 28(4):272–7. doi:10.1177/0748730413494561.

Akerstedt T. 1998. Shift work and disturbed sleep/wakefulness. Sleep Med Rev. 2(2):117–28.

Akerstedt T. 2003. Shift work and disturbed sleep/wakefulness. Occup Med (Chic Ill). 53(2):89–94. doi:10.1093/occmed/kqg046.

An S, Harang R, Meeker K, Granados-Fuentes D, Tsai CA, Mazuski C, Kim J, Doyle FJ, Petzold LR, Herzog ED. 2013. A neuropeptide speeds circadian entrainment by reducing intercellular synchrony. Proc Natl Acad Sci U S A. 110(46):E4355-61. doi:10.1073/pnas.1307088110.

Anglès-Pujolràs M, Díez-Noguera A, Cambras T. 2007. Exposure to T-cycles of 22 and 23 h during lactation modifies the later dissociation of motor activity and temperature circadian rhythms in rats. Chronobiol Int. 24(6):1049–1064. doi:10.1080/07420520701800645.

Aton SJ, Block GD, Tei H, Yamazaki S, Herzog ED. 2004. Plasticity of circadian behavior and the suprachiasmatic nucleus following exposure to non-24-hour light cycles. J Biol Rhythms. 19(3):198–207. doi:10.1177/0748730404264156.

De Bacquer D, Van Risseghem M, Clays E, Kittel F, De Backer G, Braeckman L. 2009. Rotating shift work and the metabolic syndrome: a prospective study. Int J Epidemiol. 38(3):848–54. doi:10.1093/ije/dyn360.

Barclay JL, Husse J, Bode B, Naujokat N, Meyer-Kovac J, Schmid SM, Lehnert H, Oster H. 2012. Circadian desynchrony promotes metabolic disruption in a mouse model of shiftwork. PLoS One. 7(5):e37150. doi:10.1371/journal.pone.0037150.

Benzoni TE. 2017. Circadian Rhythms and Shiftwork Policy Resource and Education Paper. Am Coll Emerg Physicians.

Boulos Z, Macchi MM, Terman M. 2002. Twilights widen the range of photic entrainment in hamsters. J Biol Rhythms. 17(4):353–63. doi:10.1177/074873002129002654.

Brainard GC, Hanifin JP, Greeson JM, Byrne B, Glickman G, Gerner E, Rollag MD. 2001. Action spectrum for melatonin regulation in humans: evidence for a novel circadian photoreceptor. J Neurosci. 21(16):6405–12. doi:21/16/6405 [pii].

Brainard GC, Richardson BA, Hurlbut EC, Steinlechner S, Matthews SA, Reiter RJ. 1984. The influence of various irradiances of artificial light, twilight, and moonlight on the suppression of pineal melatonin content in the Syrian hamster. J Pineal Res. 1(2):105–119. doi:10.1111/j.1600-079X.1984.tb00202.x.

Brainard GC, Richardson BA, Petterborg LJ, Reiter RJ. 1982. The effect of different light intensities on pineal melatonin content. Brain Res. 233(1):75–81. doi:10.1016/0006-8993(82)90931-3.

Brown LA, Fisk AS, Pothecary CA, Peirson SN. 2019. Telling the Time with a Broken Clock:

Quantifying Circadian Disruption in Animal Models. Biology (Basel). 8(1):18. doi:10.3390/biology8010018.

Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N, Schibler U. 2002. Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr Biol. 12(18):1574–1583. doi:10.1016/S0960-9822(02)01145-4.

Byers SL, Wiles M V., Dunn SL, Taft RA. 2012. Mouse estrous cycle identification tool and images. Singh SR, editor. PLoS One. 7(4):e35538. doi:10.1371/journal.pone.0035538.

Cambras T, Chiesa J, Araujo J, Díez-Noguera A. 2004. Effects of photoperiod on rat motor activity rhythm at the lower limit of entrainment. J Biol Rhythms. 19(3):216–225. doi:10.1177/0748730404264201.

Cambras T, Vilaplana J, Campuzano A, Canal-Corretger MM, Carulla M, Díez-Noguera A. 2000. Entrainment of the rat motor activity rhythm: Effects of the light-dark cycle and physical exercise. Physiol Behav. 70(3–4):227–232. doi:10.1016/S0031-9384(00)00241-9.

Carter CS, Witt DM, Manock SR, Adams KA, Bahr JM, Carlstead K. 1989. Hormonal correlates of sexual behavior and ovulation in male-induced and postpartum estrus in female prairie voles. Physiol Behav. 46(6):941–948. doi:10.1016/0031-9384(89)90195-9.

Casiraghi LP, Oda GA, Chiesa JJ, Friesen WO, Golombek DA. 2012. Forced Desynchronization of Activity Rhythms in a Model of Chronic Jet Lag in Mice. J Biol Rhythms. 27(1):59–69. doi:10.1177/0748730411429447.

Castanon-Cervantes O, Wu M, Ehlen JC, Paul K, Gamble KL, Johnson RL, Besing RC, Menaker M, Gewirtz AT, Davidson AJ. 2010. Dysregulation of inflammatory responses by chronic circadian disruption. J Immunol. 185(10):5796–805. doi:10.4049/jimmunol.1001026.

Chiesa JJ, Anglès-Pujolràs M, Díez-Noguera A, Cambras T. 2005. Activity rhythm of golden hamster (Mesocricetus auratus) can be entrained to a 19-h light-dark cycle. Am J Physiol Regul Integr Comp Physiol. 289(4):R998–R1005. doi:10.1152/ajpregu.00139.2005.

Chiesa JJ, Anglès-Pujolràs M, Díez-Noguera A, Cambras T. 2006. History-dependent changes in entrainment of the activity rhythm in the Syrian hamster (Mesocricetus auratus). J Biol Rhythms. 21(1):45–57. doi:10.1177/0748730405283654.

Cho K, Ennaceur A, Cole JC, Suh CK. 2000. Chronic jet lag produces cognitive deficits. J Neurosci. 20(6):RC66.

Christian CA, Mobley JL, Moenter SM. 2005. Diurnal and estradiol-dependent changes in gonadotropin-releasing hormone neuron firing activity. Proc Natl Acad Sci U S A. 102(43):15682–7. doi:10.1073/pnas.0504270102.

Chu A, Zhu L, Blum ID, Mai O, Leliavski A, Fahrenkrug J, Oster H, Boehm U, Storch K-F. 2013. Global but not gonadotrope-specific disruption of Bmal1 abolishes the luteinizing hormone surge without affecting ovulation. Endocrinology. 154(8):2924–35. doi:10.1210/en.2013-1080.

Colwell CS, Matveyenko A V. 2014. Timing is everything: Implications for metabolic consequences of sleep restriction. Diabetes. 63(6):1826–1828. doi:10.2337/db14-0283.

Comas M, Beersma DGM, Spoelstra K, Daan S. 2006. Phase and period responses of the circadian system of mice (Mus musculus) to light stimuli of different duration. J Biol

Rhythms. 21(5):362–72. doi:10.1177/0748730406292446.

Costa G. 1996. The impact of shift and night work on health. In: Applied Ergonomics. Vol. 27. p. 9–16.

Daan S. 2000. Colin Pittendrigh, Jürgen Aschoff, and the Natural Entrainment of Circadian Systems. J Biol Rhythms. 15(3):195–207. doi:10.1177/074873040001500301.

Daan S, Aschoff J. 2001. The Entrainment of Circadian Rhythm. In: Takahashi JS, Turek FW, Moore RY, editors. Handbook of Behavioral Neurobiology; Volume 12 - Circadian Clocks. New York: Springer. p. 7–43.

Daan S, Pittendrigh CS. 1976. A Functional analysis of circadian pacemakers in nocturnal rodents - II. The variability of phase response curves. J Comp Physiol A. 106(3):253–266. doi:10.1007/BF01417857.

Damaggio AS, Gorman MR. 2014. Circadian phase determines effects of repeated ethanol vapor exposure and withdrawal on body temperature and activity rhythms of male mice. Alcohol Clin Exp Res. 38(3):879–888. doi:10.1111/acer.12297.

Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. 2000. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 14(23):2950–2961. doi:10.1101/gad.183500.

Davidson AJ, Sellix MT, Daniel J, Yamazaki S, Menaker M, Block GD. 2006. Chronic jet-lag increases mortality in aged mice. Curr Biol. 16(21):R914-6. doi:10.1016/j.cub.2006.09.058.

Eastman CI. 1990. Circadian rhythms and bright light: Recommendations for shift work. Work Stress. 4(3):245–260. doi:10.1080/02678379008256987.

Edgar DM, Dement WC. 1991. Regularly scheduled voluntary exercise synchronizes the mouse circadian clock. Am J Physiol. 261(4 Pt 2):R928-33. doi:10.1152/ajpregu.1991.261.4.R928.

Endo A, Watanabe T. 1989. Effects of non-24-hour days on reproductive efficacy and embryonic development in mice. Gamete Res. 22(4):435–41. doi:10.1002/mrd.1120220409.

Evans JA, Carter SN, Freeman D a, Gorman MR. 2012. Dim nighttime illumination alters photoperiodic responses of hamsters through the intergeniculate leaflet and other photic pathways. Neuroscience. 202:300–308. doi:10.1016/j.neuroscience.2011.11.037.

Evans JA, Davidson AJ. 2013. Health consequences of circadian disruption in humans and animal models. Prog Mol Biol Transl Sci. 119:283–323. doi:10.1016/B978-0-12-396971-2.00010-5.

Evans JA, Elliott JA, Gorman MR. 2004. Photoperiod differentially modulates photic and nonphotic phase response curves of hamsters. Am J Physiol Regul Integr Comp Physiol. 286(3):R539-46. doi:10.1152/ajpregu.00456.2003.

Evans JA, Elliott JA, Gorman MR. 2009. Dim nighttime illumination accelerates adjustment to timezone travel in an animal model. Curr Biol. 19(4):R156-7. doi:10.1016/j.cub.2009.01.023.

Evans JA, Elliott JA, Gorman MR. 2012. Individual differences in circadian waveform of Siberian hamsters under multiple lighting conditions. J Biol Rhythms. 27(5):410–9. doi:10.1177/0748730412455915.

Evans JA, Gorman MR. 2002. Split circadian rhythms of female Syrian hamsters and their offspring. Physiol Behav. 76(4–5):469–78.

Evans JA, Gorman MR. 2016. In synch but not in step: Circadian clock circuits regulating plasticity in daily rhythms. Neuroscience. 320:259–280. doi:10.1016/j.neuroscience.2016.01.072.

Everett JW, Sawyer CH. 1950. A 24-hour periodicity in the “LH-release apparatus” of female rats, disclosed by barbiturate sedation. Endocrinology. 47(3):198–218. doi:10.1210/endo-47-3-198.

Fahrbach SE, Meisel RL, Pfaff DW. 1985. Preoptic implants of estradiol increase wheel running but not the open field activity of female rats. Physiol Behav. 35(6):985–92. doi:10.1016/0031-9384(85)90270-7.

Filipski E, Delaunay F, King VM, Wu M-W, Claustrat B, Gréchez-Cassiau A, Guettier C, Hastings MH, Francis L. 2004. Effects of chronic jet lag on tumor progression in mice. Cancer Res. 64(21):7879–85. doi:10.1158/0008-5472.CAN-04-0674.

Flôres DEFL, Bettilyon CN, Yamazaki S. 2016. Period-independent novel circadian oscillators revealed by timed exercise and palatable meals. Sci Rep. 6(1):21945. doi:10.1038/srep21945.

Gamble KL, Motsinger-Reif AA, Hida A, Borsetti HM, Servick S V., Ciarleglio CM, Robbins S, Hicks J, Carver K, Hamilton N, et al. 2011. Shift Work in Nurses: Contribution of Phenotypes and Genotypes to Adaptation. Goel N, editor. PLoS One. 6(4):e18395. doi:10.1371/journal.pone.0018395.

Gamble KL, Resuehr D, Johnson CH. 2013. Shift work and circadian dysregulation of reproduction. Front Endocrinol (Lausanne). 4:92. doi:10.3389/fendo.2013.00092.

Glickman GL, Harrison EM, Elliott JA, Gorman MR. 2014. Increased photic sensitivity for phase resetting but not melatonin suppression in Siberian hamsters under short photoperiods. Horm Behav. 65(3):301–7. doi:10.1016/j.yhbeh.2014.01.002.

Glickman GL, Webb IC, Elliott J a, Baltazar RM, Reale ME, Lehman MN, Gorman MR. 2012. Photic sensitivity for circadian response to light varies with photoperiod. J Biol Rhythms. 27(4):308–18. doi:10.1177/0748730412450826.

Gorman MR. 2001. Exotic photoperiods induce and entrain split circadian activity rhythms in hamsters. J Comp Physiol - A Sensory, Neural, Behav Physiol. 187(10):793–800. doi:10.1007/s00359-001-0250-1.

Gorman MR, Elliott JA. 2003. Entrainment of 2 subjective nights by daily light:dark:light:dark cycles in 3 rodent species. J Biol Rhythms. 18(6):502–12. doi:10.1177/0748730403260219.

Gorman MR, Elliott JA. 2004. Dim nocturnal illumination alters coupling of circadian pacemakers in Siberian hamsters, Phodopus sungorus. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 190(8):631–9. doi:10.1007/s00359-004-0522-7.

Gorman MR, Elliott JA. 2019. Exceptional Entrainment of Circadian Activity Rhythms With Manipulations of Rhythm Waveform in Male Syrian Hamsters. Yale J Biol Med. 92(2):187–199.

Gorman MR, Elliott JA, Evans JA. 2003. Plasticity of hamster circadian entrainment patterns

depends on light intensity. Chronobiol Int. 20(2):233–48. doi:10.1081/CBI-120018576.

Gorman MR, Evans JA, Elliott JA. 2006. Potent circadian effects of dim illumination at night in hamsters. Chronobiol Int. 23(1–2):245–250. doi:10.1080/07420520500521905.

Gorman MR, Harrison EM, Evans JA. 2017. Circadian Waveform and Its Significance for Clock Organization and Plasticity. In: Biological Timekeeping: Clocks, Rhythms and Behaviour. New Delhi: Springer India. p. 59–79.

Gorman MR, Kendall M, Elliott JA. 2005. Scotopic illumination enhances entrainment of circadian rhythms to lengthening light:dark cycles. J Biol Rhythms. 20(1):38–48. doi:10.1177/0748730404271573.

Gorman MR, Steele NA. 2006. Phase angle difference alters coupling relations of functionally distinct circadian oscillators revealed by rhythm splitting. J Biol Rhythms. 21(3):195–205. doi:10.1177/0748730406287665.

Gorman MR, Yellon SM, Lee TM. 2001. Temporal reorganization of the suprachiasmatic nuclei in hamsters with split circadian rhythms. J Biol Rhythms. 16(6):552–63. doi:10.1177/074873001129002240.

Granada AE, Bordyugov G, Kramer A, Herzel H. 2013. Human chronotypes from a theoretical perspective. PLoS One. 8(3):e59464. doi:10.1371/journal.pone.0059464.

Gray GDD, Söderstein P, Tallentire D, Davidson JMM. 1978. Effects of lesions in various structures of the suprachiasmatic-preoptic region on LH regulation and sexual behavior in female rats. Neuroendocrinology. 25(3):174–91. doi:10.1159/000122739.

Grone BP, Chang D, Bourgin P, Cao V, Fernald RD, Heller HC, Ruby NF. 2011. Acute light exposure suppresses circadian rhythms in clock gene expression. J Biol Rhythms. 26(1):78–81. doi:10.1177/0748730410388404.

Ha M, Park J. 2005. Shiftwork and metabolic risk factors of cardiovascular disease. J Occup Health. 47(2):89–95.

Hansen J. 2001. Light at night, shiftwork, and breast cancer risk. J Natl Cancer Inst. 93(20):1513–1515. doi:10.1093/jnci/93.20.1513.

Harrison E, Carmack S, Block C, Sun J, Anagnostaras S, Gorman MR. 2017. Circadian waveform bifurcation, but not phase-shifting, leaves cued fear memory intact. Physiol Behav. 169:106–113. doi:10.1016/j.physbeh.2016.11.033.

Harrison EM, Gorman MR. 2012. Changing the waveform of circadian rhythms: considerations for shift-work. Front Neurol. 3(May):72. doi:10.3389/fneur.2012.00072.

Harrison EM, Gorman MR. 2015. Rapid Adjustment of Circadian Clocks to Simulated Travel to Time Zones across the Globe. J Biol Rhythms. 30(6):557–562. doi:10.1177/0748730415598875.

Harrison EM, Walbeek TJ, Sun J, Johnson J, Poonawala Q, Gorman MR. 2016. Extraordinary behavioral entrainment following circadian rhythm bifurcation in mice. Sci Rep. 6:38479. doi:10.1038/srep38479.

Haus EL, Smolensky MH. 2013. Shift work and cancer risk: potential mechanistic roles of circadian disruption, light at night, and sleep deprivation. Sleep Med Rev. 17(4):273–84. doi:10.1016/j.smrv.2012.08.003.

Hughes ATL, Piggins HD. 2012. Feedback actions of locomotor activity to the circadian clock. Prog Brain Res. 199:305–36. doi:10.1016/B978-0-444-59427-3.00018-6.

Karatsoreos IN, Bhagat S, Bloss EB, Morrison JH, McEwen BS. 2011. Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. Proc Natl Acad Sci U S A. 108(4):1657–1662. doi:10.1073/pnas.1018375108.

Kaur G, Thind R, Glass JD. 2009. Brief constant light accelerates serotonergic re-entrainment to large shifts of the daily light/dark cycle. Neuroscience. 159(4):1430–40. doi:10.1016/j.neuroscience.2009.02.016.

Kerin A, Aguirre A. 2005. Improving health, safety, and profits in extended hours operations (shiftwork). Ind Health. 43(1):201–8.

Kessler EJ, Sprouse J, Harrington ME. 2008. NAN-190 potentiates the circadian response to light and speeds re-entrainment to advanced light cycles. Neuroscience. 154(4):1187–94. doi:10.1016/j.neuroscience.2008.04.054.

Kiessling S, Eichele G, Oster H. 2010. Adrenal glucocorticoids have a key role in circadian resynchronization in a mouse model of jet lag. J Clin Invest. 120(7):2600–2609. doi:10.1172/JCI41192.

Klett NJ, Allen CN. 2017. Intracellular Chloride Regulation in AVP+ and VIP+ Neurons of the Suprachiasmatic Nucleus. Sci Rep. 7(1):10226. doi:10.1038/s41598-017-09778-x.

Knutsson A, Hammar N, Karlsson B. 2004. Shift workers' mortality scrutinized. Chronobiol Int. 21(6):1049–53.

Kopp C, Ressel V, Wigger E, Tobler I. 2006. Influence of estrus cycle and ageing on activity patterns in two inbred mouse strains. Behav Brain Res. 167(1):165–174. doi:10.1016/J.BBR.2005.09.001.

de la Iglesia HO, Cambras T, Schwartz WJ, Díez-Noguera A. 2004. Forced desynchronization of dual circadian oscillators within the rat suprachiasmatic nucleus. Curr Biol. 14(9):796–800. doi:10.1016/j.cub.2004.04.034.

Legan SJ, Coon GA, Karsch FJ. 1975. Role of estrogen as initiator of daily LH surges in the ovariectomized rat. Endocrinology. 96(1):50–6. doi:10.1210/endo-96-1-50.

Legan SJ, Karsch FJ. 1975. A daily signal for the LH surge in the rat. Endocrinology. 96(1):57–62. doi:10.1210/endo-96-1-57.

Leise TL, Goldberg A, Michael J, Montoya G, Solow S, Molyneux P, Vetrivelan R, Harrington ME. 2018. Recurring circadian disruption alters circadian clock sensitivity to resetting. Eur J Neurosci. doi:10.1111/ejn.14179.

Lightfoot JT. 2008. Sex hormones' regulation of rodent physical activity: a review. Int J Biol Sci. 4(3):126–32. doi:10.7150/ijbs.4.126.

Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods. 25(4):402–408. doi:10.1006/METH.2001.1262.

Loh DH, Navarro J, Hagopian A, Wang LM, Deboer T, Colwell CS. 2010. Rapid changes in the light/dark cycle disrupt memory of conditioned fear in mice. PLoS One. 5(9). doi:10.1371/journal.pone.0012546.

Lowden A, Öztürk G, Reynolds A, Bjorvatn B. 2019. Working Time Society consensus statements: Evidence based interventions using light to improve circadian adaptation to working hours. Ind Health. 57(2):213–227. doi:10.2486/indhealth.SW-9.

Mahoney MM. 2010. Shift work, jet lag, and female reproduction. Int J Endocrinol. 2010:813764. doi:10.1155/2010/813764.

Marquié J-C, Tucker P, Folkard S, Gentil C, Ansiau D. 2014. Chronic effects of shift work on cognition: findings from the VISAT longitudinal study. Occup Environ Med. 72(4):258–64. doi:10.1136/oemed-2013-101993.

McClintock MK. 1978. Estrous synchrony and its mediation by Airborne chemical communication (Rattus norvegicus). Horm Behav. 10(3):264–276. doi:10.1016/0018-506X(78)90071-5.

McGowan NM, Coogan AN. 2013. Circadian and behavioural responses to shift work-like schedules of light/dark in the mouse. J Mol psychiatry. 1(1):7. doi:10.1186/2049-9256-1-7.

McLean AC, Valenzuela N, Fai S, Bennett SAL. 2012. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. J Vis Exp.(67):e4389. doi:10.3791/4389.

Meijer JH, Robbers Y. 2014. Wheel running in the wild. Proceedings Biol Sci. 281(1786). doi:10.1098/rspb.2014.0210.

Miller BH, Olson SL, Turek FW, Levine JE, Horton TH, Takahashi JS. 2004. Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. Curr Biol. 14(15):1367–73. doi:10.1016/j.cub.2004.07.055.

Mohawk JA, Green CB, Takahashi JS. 2012. Central and peripheral circadian clocks in mammals. Annu Rev Neurosci. 35(1):445–62. doi:10.1146/annurev-neuro-060909-153128.

Mohawk JA, Takahashi JS. 2011. Cell autonomy and synchrony of suprachiasmatic nucleus circadian oscillators. Trends Neurosci. 34(7):349–58. doi:10.1016/j.tins.2011.05.003.

Mohren DCL, Jansen NWH, Kant IJ, Galama J, van den Brandt PA, Swaen GMH. 2002. Prevalence of common infections among employees in different work schedules. J Occup Environ Med. 44(11):1003–11.

Molyneux PC, Dahlgren MK, Harrington ME. 2008. Circadian entrainment aftereffects in suprachiasmatic nuclei and peripheral tissues in vitro. Brain Res. 1228:127–34. doi:10.1016/j.brainres.2008.05.091.

Moreno CRC, Marqueze EC, Sargent C, Wright Jr KP, Ferguson SA, Tucker P. 2019. Working Time Society consensus statements: Evidence-based effects of shift work on physical and mental health. Ind Health. 57(2):139–157. doi:10.2486/indhealth.SW-1.

Nachreiner F. 1998. Individual and social determinants of shiftwork tolerance. Scand J Work Environ Health. 24 Suppl 3(SUPPL. 3):35–42.

Nelson DE, Takahashi JS. 1991a. Sensitivity and integration in a visual pathway for circadian entrainment in the hamster (Mesocricetus auratus). J Physiol. 439(1):115–145. doi:10.1113/jphysiol.1991.sp018660.

Nelson DE, Takahashi JS. 1991b. Comparison of visual sensitivity for suppression of pineal melatonin and circadian phase-shifting in the golden hamster. Brain Res. 554(1–2):272–

277. doi:10.1016/0006-8993(91)90200-F.

Noguchi T, Harrison EM, Sun J, May D, Ng A, Welsh DK, Gorman MR. 2018. Circadian rhythm bifurcation induces flexible phase resetting by reducing circadian amplitude. Eur J Neurosci. doi:10.1111/ejn.14086.

Novak CM, Burghardt PR, Levine JA. 2012. The use of a running wheel to measure activity in rodents: relationship to energy balance, general activity, and reward. Neurosci Biobehav Rev. 36(3):1001–1014. doi:10.1016/j.neubiorev.2011.12.012.

Oike H, Nagai K, Fukushima T, Ishida N, Kobori M. 2011. Feeding Cues and Injected Nutrients Induce Acute Expression of Multiple Clock Genes in the Mouse Liver. Yamazaki S, editor. PLoS One. 6(8):e23709. doi:10.1371/journal.pone.0023709.

Oike H, Sakurai M, Ippoushi K, Kobori M. 2015. Time-fixed feeding prevents obesity induced by chronic advances of light/dark cycles in mouse models of jet-lag/shift work. Biochem Biophys Res Commun. 465(3):556–61. doi:10.1016/j.bbrc.2015.08.059.

Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB. 2002. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell. 109(3):307–20. doi:10.1016/s0092-8674(02)00722-5.

Patton DF, Mistlberger RE. 2013. Circadian adaptations to meal timing: neuroendocrine mechanisms. Front Neurosci. 7:185. doi:10.3389/fnins.2013.00185.

Pendergast JS, Yamazaki S. 2017. Extra-SCN circadian pacemakers. Biol Clocks, Honma, K, Honma, S, eds.:141–152.

Penev PD, Kolker DE, Zee PC, Turek FW. 1998. Chronic circadian desynchronization decreases the survival of animals with cardiomyopathic heart disease. Am J Physiol. 275(6 Pt 2):H2334-7.

Petrov ME, Clark CB, Molzof HE, Johnson RL, Cropsey KL, Gamble KL. 2014. Sleep Strategies of Night-Shift Nurses on Days Off: Which Ones are Most Adaptive? Front Neurol. 5:277. doi:10.3389/fneur.2014.00277.

Pezük P, Mohawk JA, Wang LA, Menaker M. 2012. Glucocorticoids as entraining signals for peripheral circadian oscillators. Endocrinology. 153(10):4775–83. doi:10.1210/en.2012-1486.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29(9):e45. doi:10.1093/nar/29.9.e45.

Pittendrigh CS, Daan S. 1976a. A functional analysis of circadian pacemakers in nocturnal rodents. J Comp Physiol ? A. 106(3):223–252. doi:10.1007/BF01417856.

Pittendrigh CS, Daan S. 1976b. A functional analysis of circadian pacemakers in nocturnal rodents - V. Pacemaker structure: A clock for all seasons. J Comp Physiol A. 106(3):333–355. doi:10.1007/BF01417860.

Pittendrigh CS, Elliott JA, Takamura T. 1984. The Circadian Component in Photoperiodic Induction. In: Porter R, Collins GM, editors. The Circadian Component in Photoperiodic Induction, in Ciba Foundation Symposium 104 - Photoperiodic Regulation of Insect and Molluscan Hormones. Pittman London. p. 26–47.

Raiewski EE, Elliott JA, Evans JA, Glickman GL, Gorman MR. 2012. Twice daily melatonin

peaks in Siberian but not Syrian hamsters under 24 h light:dark:light:dark cycles. Chronobiol Int. 29(9):1206–15. doi:10.3109/07420528.2012.719965.

Ramkisoensing A, Gu C, van Engeldorp Gastelaars HMD, Michel S, Deboer T, Rohling JHT, Meijer JH. 2014. Enhanced phase resetting in the synchronized suprachiasmatic nucleus network. J Biol Rhythms. 29(1):4–15. doi:10.1177/0748730413516750.

Reppert SM, Weaver DR. 2002. Coordination of circadian timing in mammals. Nature. 418(6901):935–941. doi:10.1038/nature00965.

Ritonja J, Aronson KJ, Matthews RW, Boivin DB, Kantermann T. 2019. Working Time Society consensus statements: Individual differences in shift work tolerance and recommendations for research and practice. Ind Health. 57(2):201–212. doi:10.2486/indhealth.SW-5.

Rosenthal SL, Vakili MM, Evans JA, Elliott JA, Gorman MR. 2005. Influence of photoperiod and running wheel access on the entrainment of split circadian rhythms in hamsters. BMC Neurosci. 6:41. doi:10.1186/1471-2202-6-41.

Saderi N, Báez-Ruiz A, Azuara-Álvarez LE, Escobar C, Salgado-Delgado RC. 2019. Differential Recovery Speed of Activity and Metabolic Rhythms in Rats After an Experimental Protocol of Shift-Work. J Biol Rhythms. 34(2):154–166. doi:10.1177/0748730419828534.

Saksvik IB, Bjorvatn B, Hetland H, Sandal GM, Pallesen S. 2011. Individual differences in tolerance to shift work--a systematic review. Sleep Med Rev. 15(4):221–35. doi:10.1016/j.smrv.2010.07.002.

Samson WK, McCann SM. 1979. Effects of suprachiasmatic nucleus lesions on hypothalamic LH-releasing hormone (LHRH) content and gonadotropin secretion in the ovariectomized (OVX) female rat. Brain Res Bull. 4(6):783–788. doi:10.1016/0361-9230(79)90012-1.

Schmal C, Myung J, Herzel H, Bordyugov G. 2015. A theoretical study on seasonality. Front Neurol. 6:94. doi:10.3389/fneur.2015.00094.

Schoeller EL, Clark DD, Dey S, Cao N V, Semaan SJ, Chao LW, Kauffman AS, Stowers L, Mellon PL. 2016. Bmal1 Is Required for Normal Reproductive Behaviors in Male Mice. Endocrinology. 157(12):4914–4929. doi:10.1210/en.2016-1620.

Schwartz WJ, Tavakoli-Nezhad M, Lambert CM, Weaver DR, de la Iglesia HO. 2011. Distinct patterns of Period gene expression in the suprachiasmatic nucleus underlie circadian clock photoentrainment by advances or delays. Proc Natl Acad Sci U S A. 108(41):17219–24. doi:10.1073/pnas.1107848108.

Schwartz WJ, Zimmerman P. 1990. Circadian timekeeping in BALB/c and C57BL/6 inbred mouse strains. J Neurosci. 10(11):3685–3694.

Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, et al. 2000. Interacting molecular loops in the mammalian circadian clock. Science. 288(5468):1013–9. doi:10.1126/science.288.5468.1013.

Shearman LP, Zylka MJ, Weaver DR, Kolakowski LF, Reppert SM. 1997. Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron. 19(6):1261–9.

Siegel HI, Bast JD, Greenwald GS. 1976. The effects of phenobarbital and gonadal steroids on periovulatory serum levels of luteinizing hormone and follicle-stimulating hormone in the hamster. Endocrinology. 98(1):48–55. doi:10.1210/endo-98-1-48.

Smith MR, Eastman CI. 2012. Shift work: health, performance and safety problems, traditional countermeasures, and innovative management strategies to reduce circadian misalignment. Nat Sci Sleep. 4:111–32. doi:10.2147/NSS.S10372.

Snell GD, Fekete E, Hummel KP, Law LW. 1940. The relation of mating, ovulation and the estrous smear in the house mouse to time of day. Anat Rec. 76(1):39–54. doi:10.1002/ar.1090760105.

Solberg LC, Horton TH, Turek FW. 1999. Circadian rhythms and depression: effects of exercise in an animal model. Am J Physiol. 276(1):R152-61. doi:10.1152/ajpregu.1999.276.1.R152.

Sosniyenko S, Parkanová D, Illnerová H, Sládek M, Sumová A. 2010. Different mechanisms of adjustment to a change of the photoperiod in the suprachiasmatic and liver circadian clocks. Am J Physiol Integr Comp Physiol. 298(4):R959–R971. doi:10.1152/ajpregu.00561.2009.

Stephens SBZ, Di Giorgio NP, Liaw RB, Parra RA, Yang JA, Chahal N, Lux-Lantos VA, Kauffman AS. 2018. Estradiol-Dependent and -Independent Stimulation of Kiss1 Expression in the Amygdala, BNST, and Lateral Septum of Mice. Endocrinology. 159(9):3389–3402. doi:10.1210/en.2018-00583.

Stokkan K-A, Yamazaki S, Tei H, Sakaki Y, Menaker M. 2001. Entrainment of the Circadian Clock in the Liver by Feeding. Science (80-). 291(5503):490–493. doi:10.1126/science.291.5503.490.

Straif K, Baan R, Grosse Y, Secretan B, El Ghissassi F, Bouvard V, Altieri A, Benbrahim-Tallaa L, Cogliano V, WHO International Agency For Research on Cancer Monograph Working Group. 2007. Carcinogenicity of shift-work, painting, and fire-fighting. Lancet Oncol. 8(12):1065–6. doi:10.1016/S1470-2045(07)70373-X.

Summa KC, Vitaterna MH, Turek FW. 2012. Environmental perturbation of the circadian clock disrupts pregnancy in the mouse. PLoS One. 7(5):e37668. doi:10.1371/journal.pone.0037668.

Sun J, Joye DAM, Farkas AH, Gorman MR. 2019. Photoperiodic Requirements for Induction and Maintenance of Rhythm Bifurcation and Extraordinary Entrainment in Male Mice. Clocks & Sleep. 1(3):290–305. doi:10.3390/clockssleep1030025.

Tonsfeldt KJ, Schoeller EL, Brusman LE, Cui LJ, Lee J, Mellon PL. 2019. The Contribution of the Circadian Gene Bmal1 to Female Fertility and the Generation of the Preovulatory Luteinizing Hormone Surge. J Endocr Soc. 3(4):716–733. doi:10.1210/js.2018-00228.

Voigt RM, Forsyth CB, Keshavarzian A. 2013. Circadian disruption: potential implications in inflammatory and metabolic diseases associated with alcohol. Alcohol Res. 35(1):87–96.

Walbeek TJ, Gorman MR. 2017. Simple Lighting Manipulations Facilitate Behavioral Entrainment of Mice to 18-h Days. J Biol Rhythms. 32(4):309–322. doi:10.1177/0748730417718347.

Walbeek TJ, Harrison EM, Soler RR, Gorman MR. Enhanced circadian entrainment in mice and its utility under human shiftwork schedules. Manuscript under Review.

Walbeek TJ, Joye DAM, Mishra I, Gorman MR. 2019. Physiological, behavioral and environmental factors influence bifurcated circadian entrainment in mice. Physiol Behav. 210:112625. doi:10.1016/j.physbeh.2019.112625.

Watanabe T, Naito E, Nakao N, Tei H, Yoshimura T, Ebihara S. 2007. Bimodal clock gene expression in mouse suprachiasmatic nucleus and peripheral tissues under a 7-hour light and 5-hour dark schedule. J Biol Rhythms. 22(1):58–68. doi:10.1177/0748730406295435.

Weinert D, Waterhouse J. 1998. Diurnally changing effects of locomotor activity on body temperature in laboratory mice. Physiol Behav. 63(5):837–843. doi:10.1016/S0031-9384(97)00546-5.

Welsh DK, Takahashi JS, Kay SA. 2010. Suprachiasmatic nucleus: cell autonomy and network properties. Annu Rev Physiol. 72:551–577. doi:10.1146/annurev-physiol-021909-135919.

Williams WP, Jarjisian SG, Mikkelsen JD, Kriegsfeld LJ. 2011. Circadian control of kisspeptin and a gated GnRH response mediate the preovulatory luteinizing hormone surge. Endocrinology. 152(2):595–606. doi:10.1210/en.2010-0943.

Williams WP, Kriegsfeld LJ. 2012. Circadian control of neuroendocrine circuits regulating female reproductive function. Front Endocrinol (Lausanne). 3:60. doi:10.3389/fendo.2012.00060.

Wintermantel TM, Campbell RE, Porteous R, Bock D, Gröne H-J, Todman MG, Korach KS, Greiner E, Pérez CA, Schütz G, et al. 2006. Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. Neuron. 52(2):271–80. doi:10.1016/j.neuron.2006.07.023.

Wright KP, Hull JT, Hughes RJ, Ronda JM, Czeisler CA. 2006. Sleep and wakefulness out of phase with internal biological time impairs learning in humans. J Cogn Neurosci. 18(4):508–21. doi:10.1162/jocn.2006.18.4.508.

Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB. 2016. MetaCycle: an integrated R package to evaluate periodicity in large scale data. Bioinformatics. 32(21):3351–3353. doi:10.1093/bioinformatics/btw405.

Yan L, Silver R, Gorman MR. 2010. Reorganization of suprachiasmatic nucleus networks under 24-h LDLD conditions. J Biol Rhythms. 25(1):19–27. doi:10.1177/0748730409352054.

Zarrinpar A, Chaix A, Panda S. 2016. Daily Eating Patterns and Their Impact on Health and Disease. Trends Endocrinol Metab. 27(2):69–83. doi:10.1016/j.tem.2015.11.007.

Appendix A

Supplemental material to Chapter 2

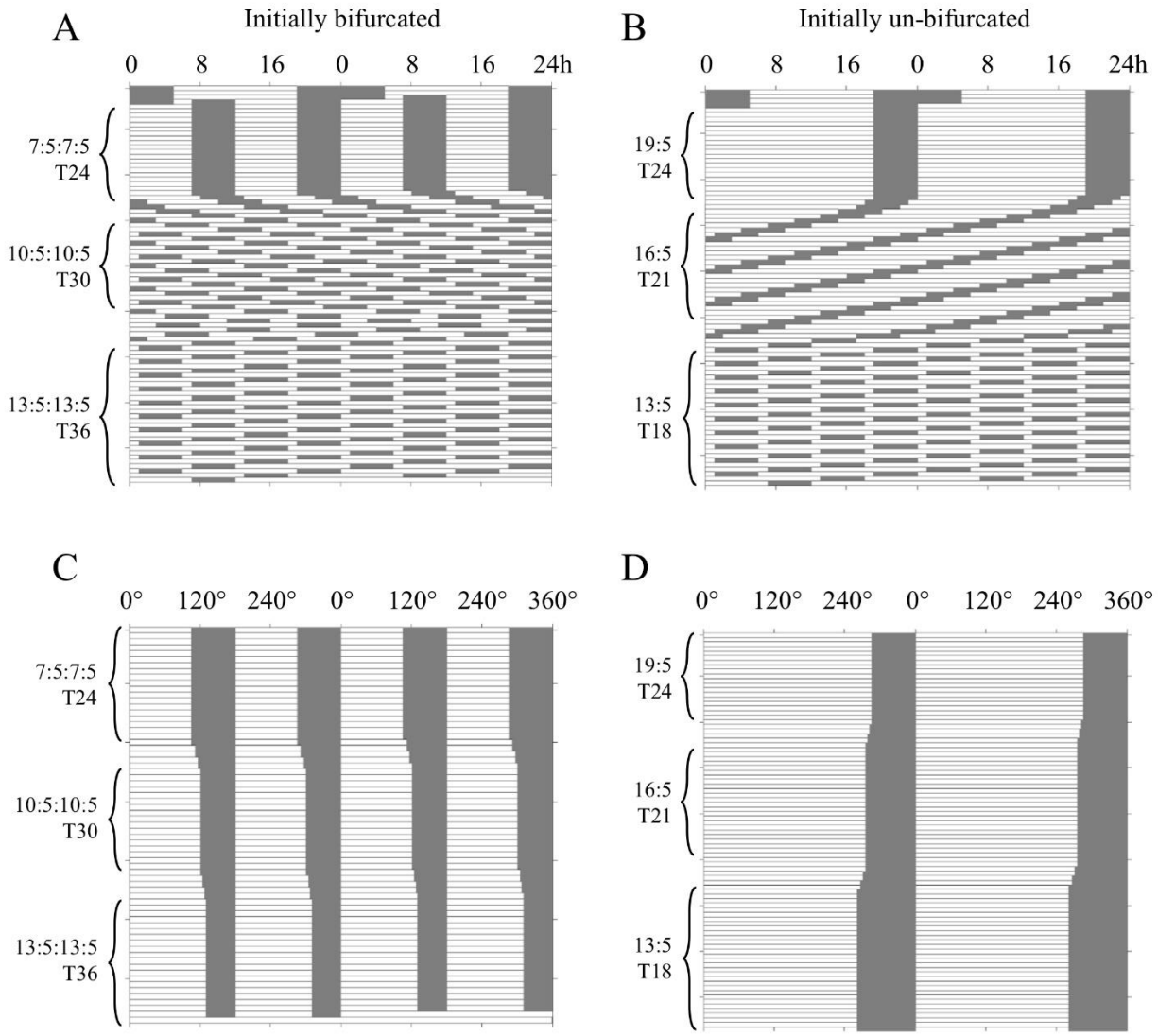

Figure S1*. Schematic light schedules in (A, B) hours and (C, D) angular degrees. Left and right panels depict Bif and nBif conditions, respectively. Gray represents night periods, all of which were dimly illuminated. Photophases are unshaded.*

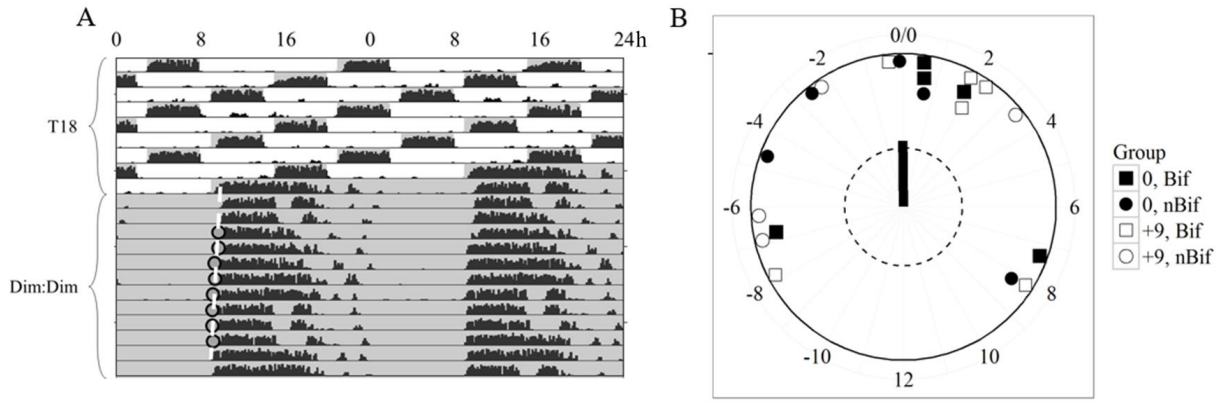

***Figure S2**. (A) Representative actogram around transition from T18 to constant D_{im}. Circles show activity onset and the white dashed lines represent the regression fit to project onset of day of release. (B) Angular plots of projected activity onset at release in $D_{im}D_{im}$. Phase angles are relative to onset of constant conditions. Radial line represents the mean vector.*

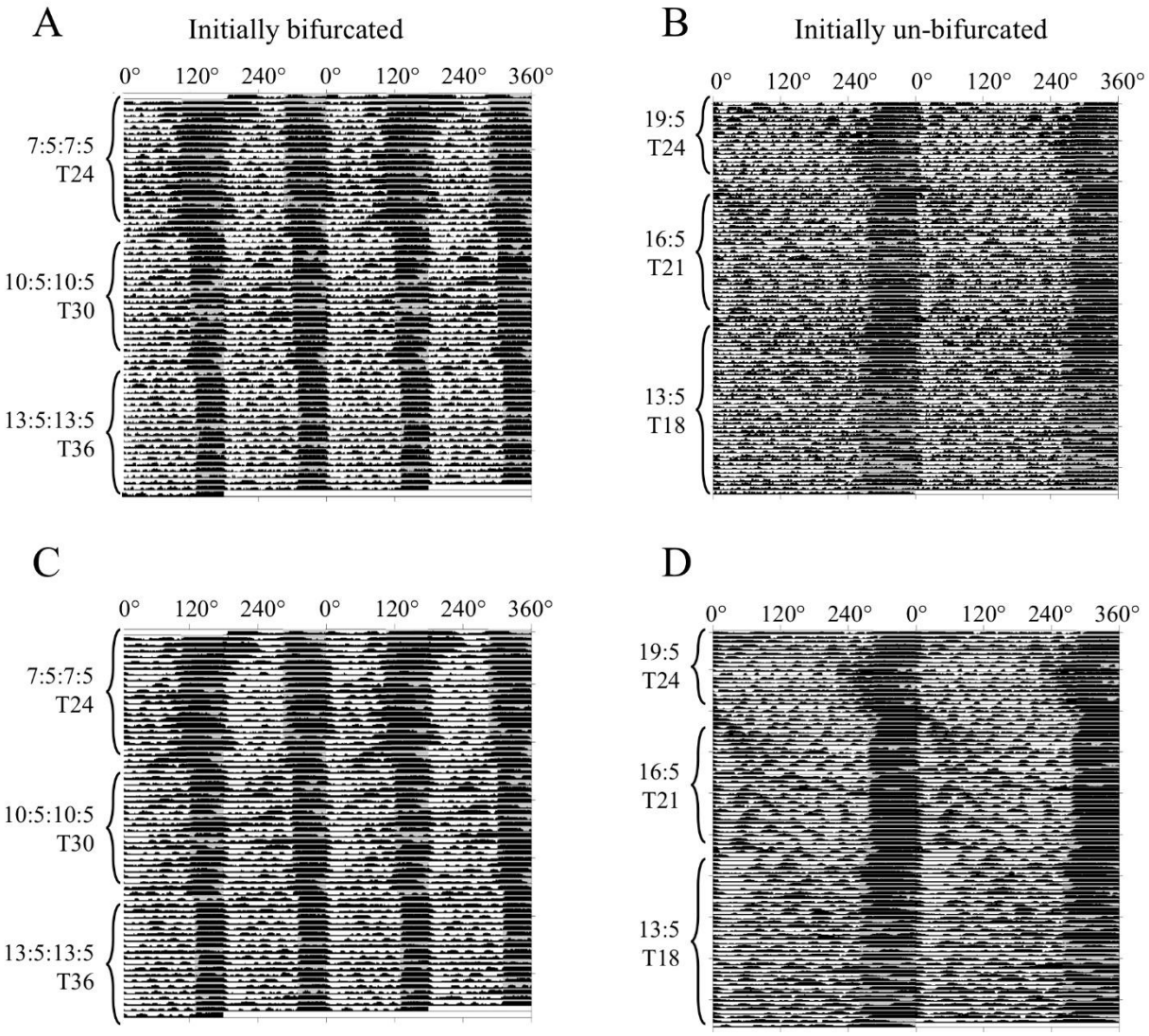

Figure S3. *Representative double-plotted actograms of (A, B) General Locomotor Activity and (C, D) recorded Body Temperature from Experiment 1. Every cycle is divided into 360 degrees. In every line, dark represents high activity/temperature and white represents low activity/temperature. Gray bars indicate lighting schedule, with gray being night and white being the light phase.*

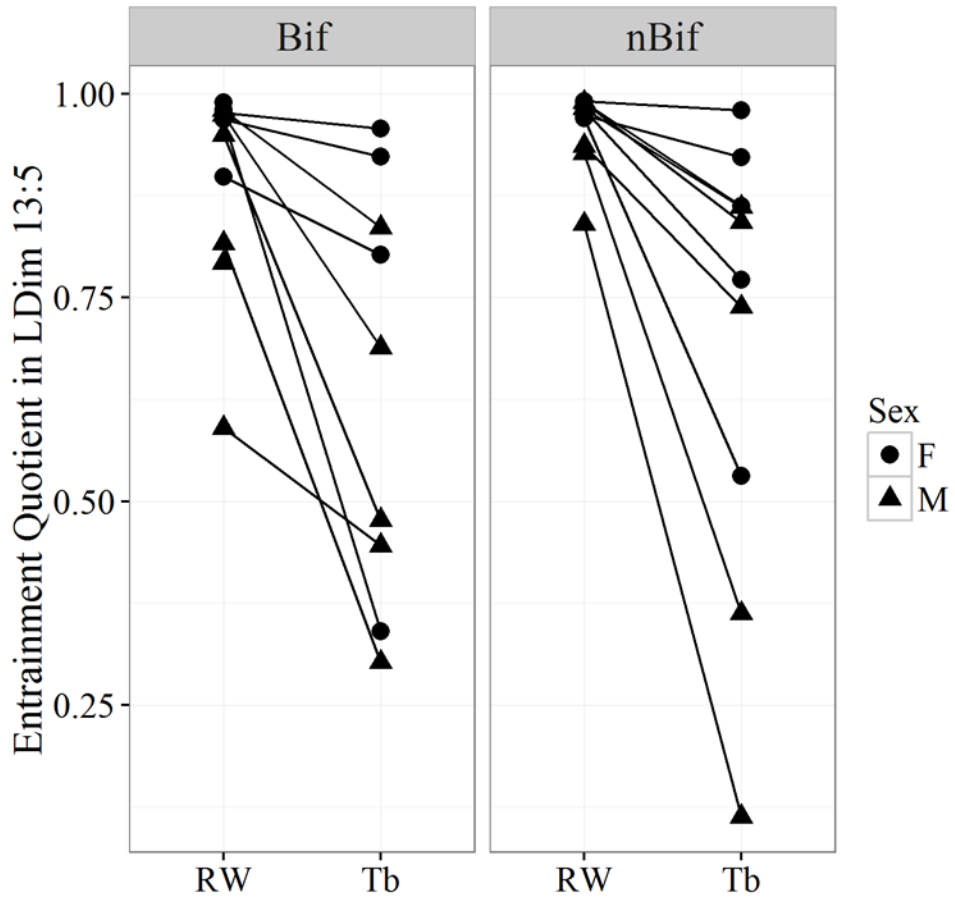

***Figure S4**. Entrainment quotients in LD_{im} 13:5 for running wheel (RW) and body temperature (Tb) data for initially bifurcated and non-bifurcated animals from Experiment 1.*

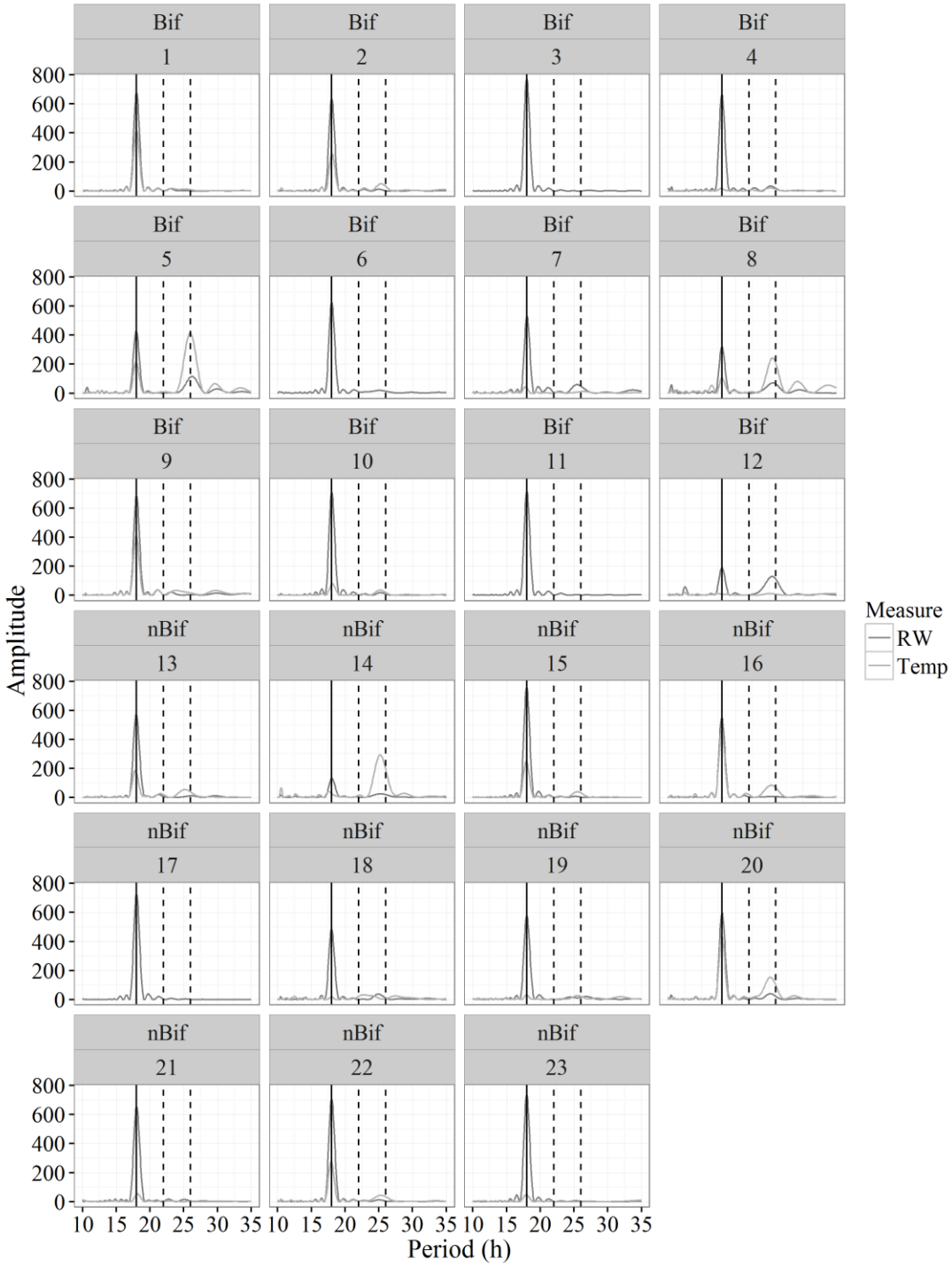

Figure S5*. Individual periodograms for RW and Temp for all animals from Experiment 1. Solid vertical line indicates 18h period and dashed lines indicate the circadian range used to find the circadian peak.*

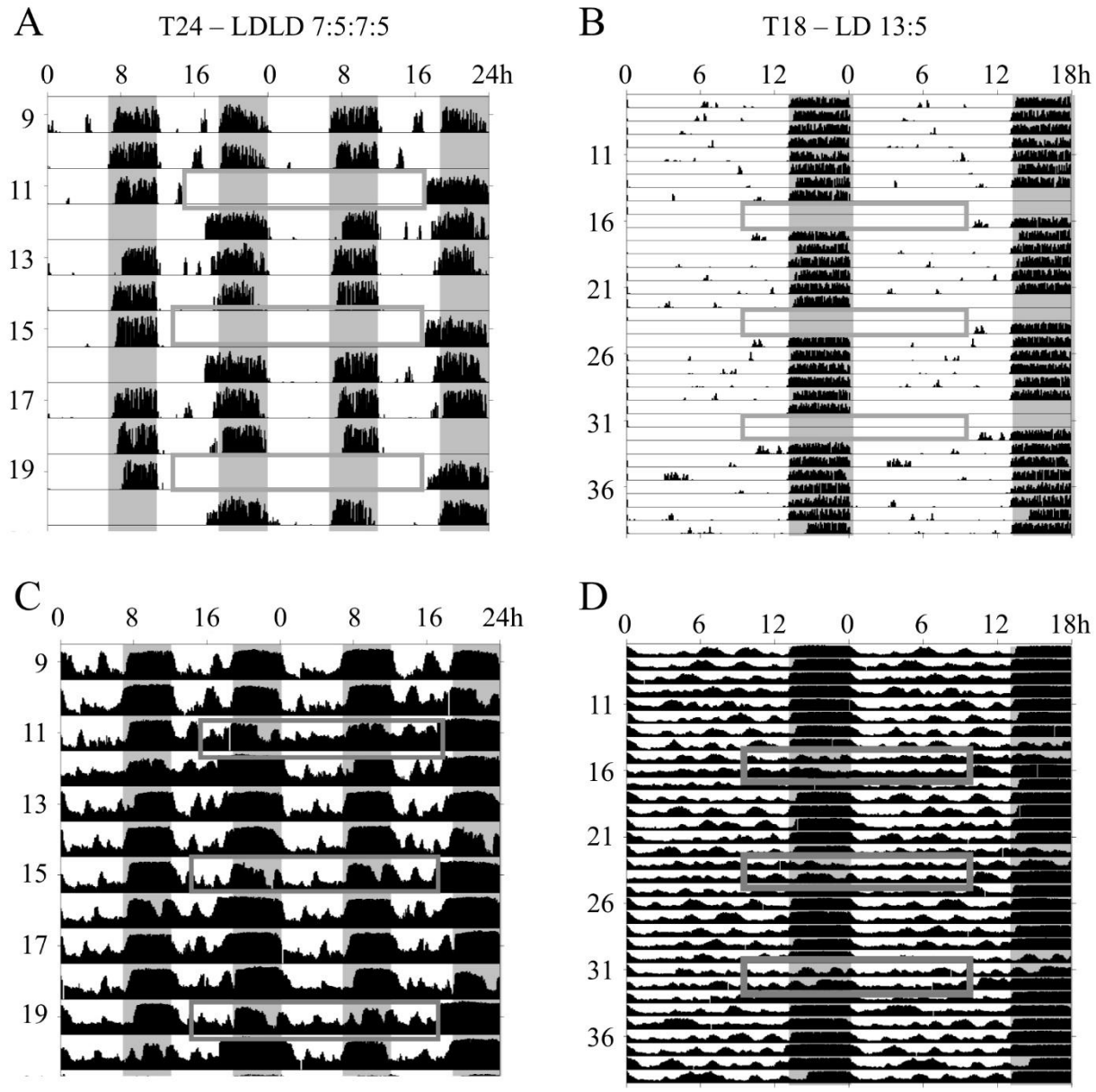

***Figure S6**. Representative actograms of (A) RW and (C) Tb in $LD_{im}LD_{im}$ 7:5:7:5 from Experiment 1. Wheel blocking indicated by rectangles starting at 11th day of Phase 1. B and D show data from the same animal of (B) RW and (D) Tb in LD_{im} 13:5. Wheels were blocked starting on cycle 15. All data are from the same animal. Conventions as in Figure 2.1.*

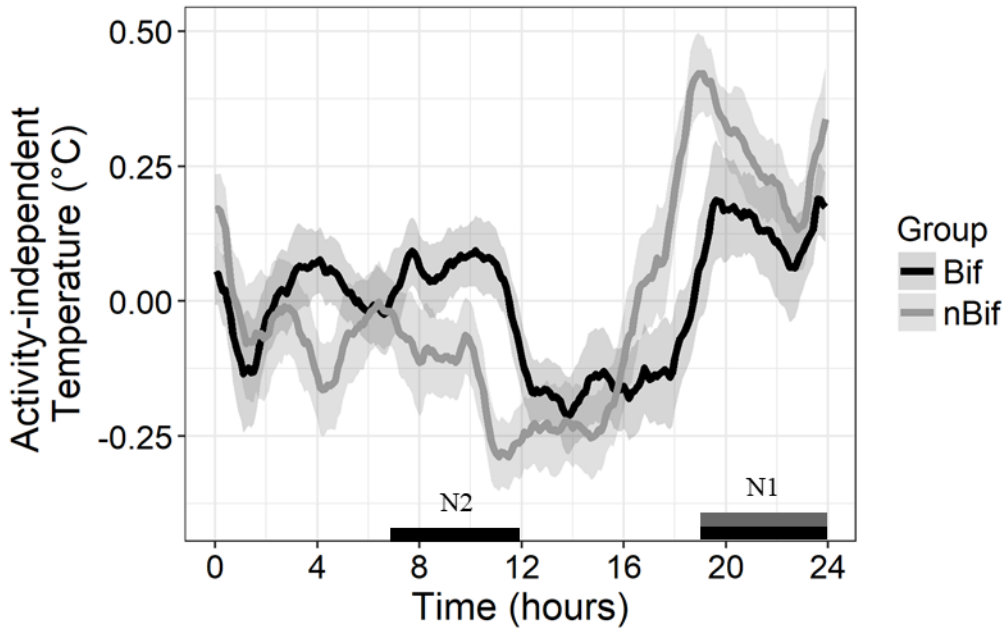

***Figure S7**. Tb' averaged across 72 hours of wheel blocking in 24h conditions in Experiment 1. Conventions as in Figure 2.3*

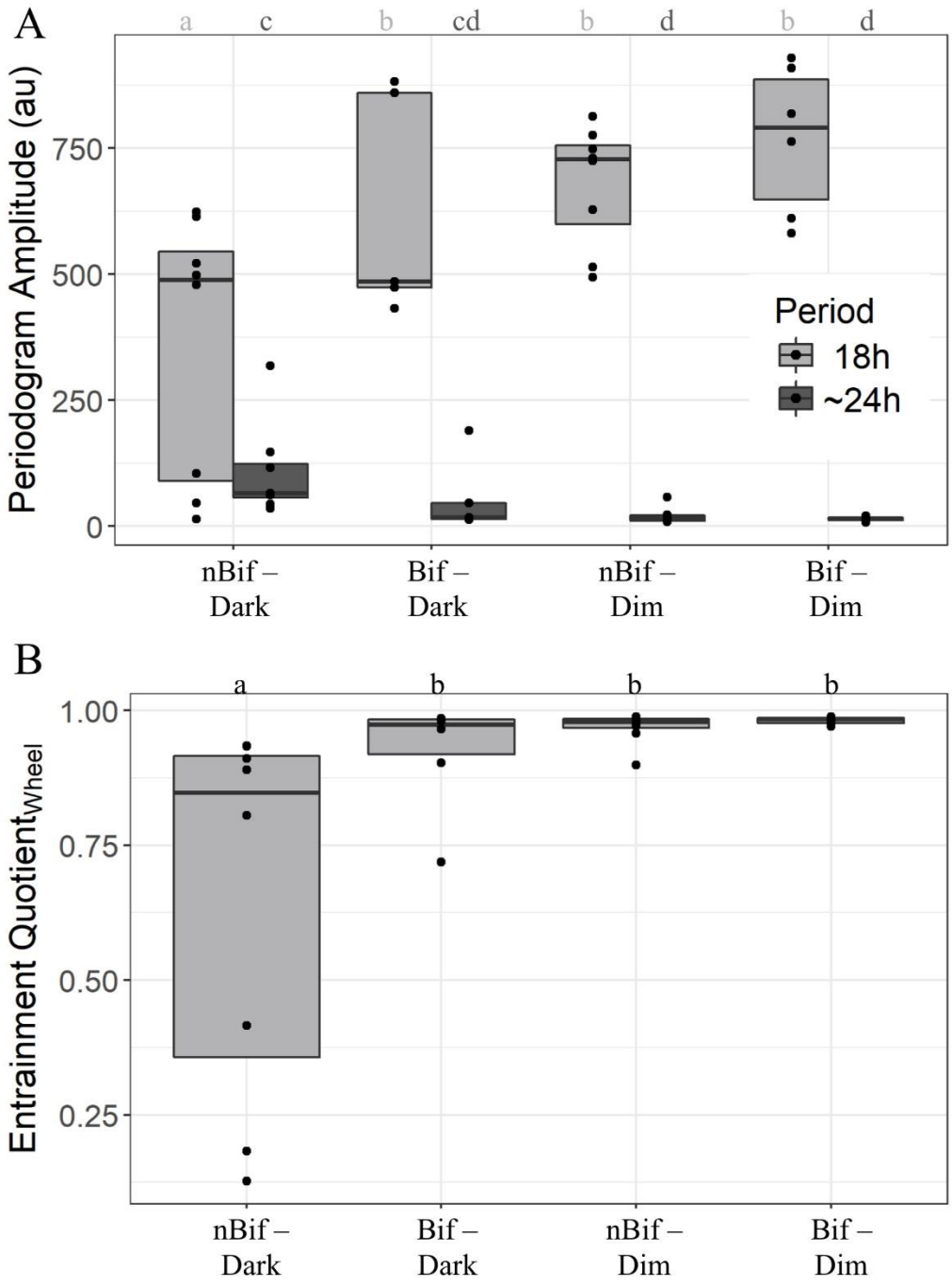

Figure S8. *(A). Periodogram amplitude at 18h and in the circadian range for animals in LD13:5, from Experiment 2. (B) Entrainment quotient in LD13:5. Boxes represent interquartile range and median.*

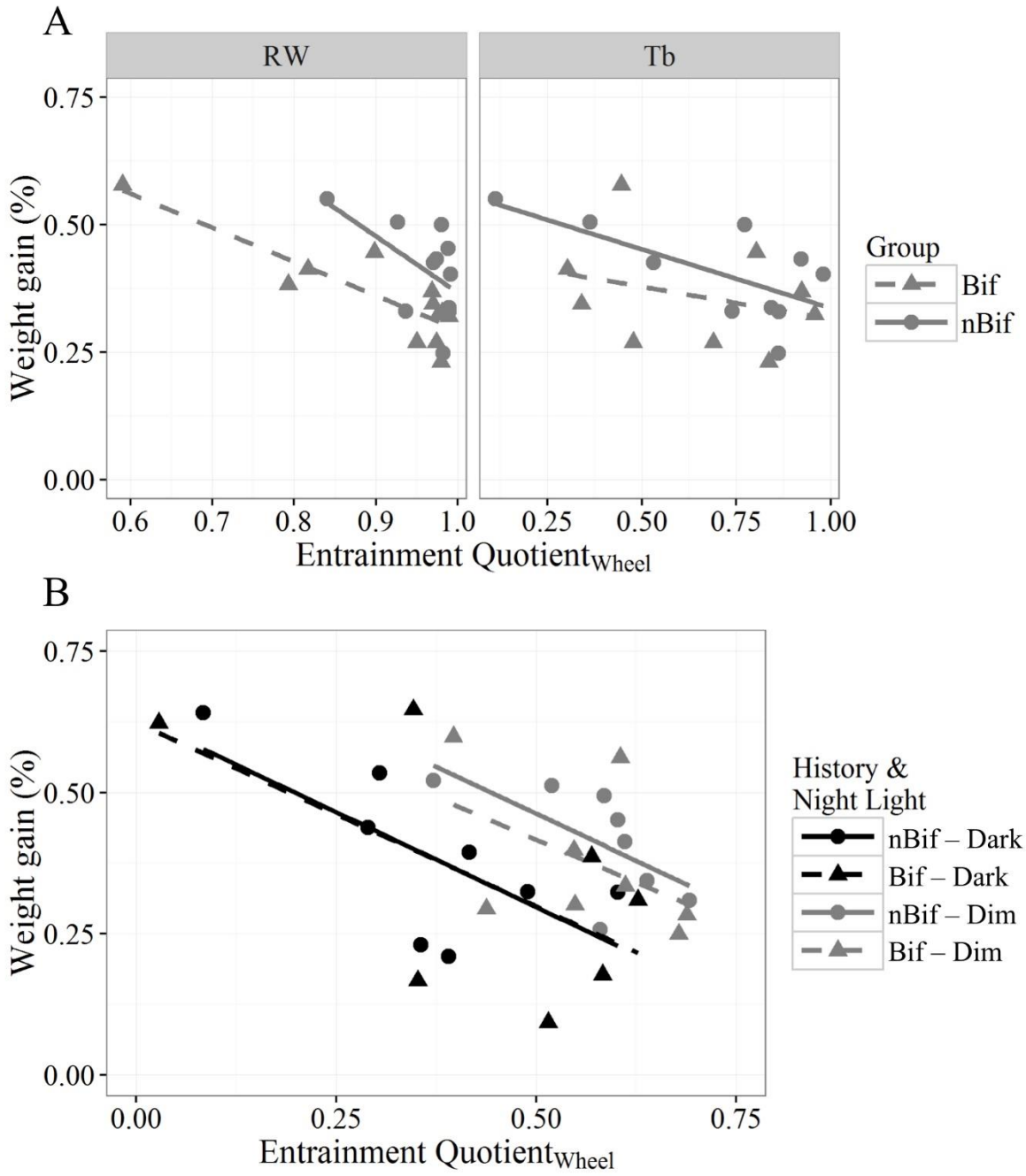

***Figure S9**. Linear regressions between entrainment quotient and total weight gain in (A) Experiment 1 and (B) Experiment 2. Lines represent best linear fit. Weight gain = (BW_{after} – BW_{before}) / BW_{before}.*