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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Changing the shape of circadian rhythms with light no brighter than moonlight

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

Doctor of Philosophy

in

Psychology

by

Jennifer Anne Evans

Committee in charge:

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Sonia Ancoli-Israel
Stuart Brody
Karen Dobkins
Jeffery A. Elliott
James Goodson

2007

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The dissertation of Jennifer Anne Evans is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2007

For my mother

In the right light, at the right time, everything is extraordinary

Aaron Rose

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LIST OF SYMBOLS

τ - Free-running period

α - Duration of the active phase of the circadian cycle

ρ - Duration of the inactive phase of the circadian cycle

$\psi_{L/D}$ - Phase angle of entrainment to the light to dark transition

CT- Circadian time, where CT12 is activity onset

ZT- Zeitgeber time, where ZT12 is the light-to-dark transition

PRC- Phase response curve

SCN- Suprachiasmatic nuclei

LED- Light-emitting diode

LD- Long day photoperiod (e.g., 14L:10D)

SD- Short day photoperiod (e.g., 10L:14D)

T-cycle- non 24 h light:dark cycle (e.g., T26, is a 26 h light:dark cycle)

LDLD- 24 h light:dark:light:dark cycle

DS- Daytime scotophase of LDLD

NS- Nighttime scotophase of LDLD

EP- Evening photophase of LDLD

MP- Morning photophase of LDLD

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ABSTRACT OF THE DISSERTATION

Changing the shape of circadian rhythms with light no brighter than moonlight

by

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Doctor of Philosophy in Psychology

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Day and night are often simulated for nocturnal rodents in the laboratory with 24 h lighting regimes alternating between moderate indoor light levels and complete darkness. Sometimes, complete darkness is replaced by dim illumination to aid human vision, with the assumption that the circadian clock of the rodent is blind to light of extremely low irradiance. However, completely dark nights are not functionally equivalent to dimly lit nights, even when nighttime illumination is below putative thresholds for the circadian visual system. Under a variety of analytical paradigms, dim nighttime illumination increases the plasticity of circadian entrainment in the nocturnal hamster. The present studies are designed to investigate the mechanisms through which dim light operates under entrained conditions.

In my first series of experiments, I demonstrate that canonical effects of dim light on circadian period and phase are insufficient to account for the potency of dim illumination under entrained conditions. Dim illumination is not a stronger zeitgeber

than predicted based on previous research, nor does it directly potentiate stronger zeitgebers. Constant dim illumination increased the period of the daily activity rhythm and increased the duration of the active phase (α) by ~ 3 h relative to that displayed under complete darkness. Specific effects of dim light on α suggest a novel action of dim light on the coupling between oscillators regulating circadian waveform.

To test for effects of dim light on circadian coupling, I employed a novel behavioral assay for studying oscillator interactions. 24 h light:dark:light:dark (LDLD) cycles induce bimodal rhythms, with two daily activity bouts programmed by “split” oscillator groups cycling in antiphase. Nearly 100% of Syrian hamsters split under LDLD with dimly lit nights, compared to only 33% of animals with completely dark nights. Dimly lit nights do not facilitate splitting through mere increases in nonphotic feedback. Instead, dim illumination alters circadian responses to photic and nonphotic stimuli, with specific effects present after entrainment to short night conditions simulating the unsplit state under LDLD. The photoperiod-dependent nature of these effects indicates that dim light alters circadian coupling between oscillators entrained to short night conditions. Interactions between oscillators mediate the fusion of split rhythms after release into constant conditions and the rejoining of split activity components under LDLD. Fusion of LDLD-induced split rhythms under free-running and entrained conditions is likewise modulated by dim illumination. Specific effects on the induction, maintenance, and fusion of LDLD-induced split rhythms are consistent with the hypothesis that dim illumination alters the nature of coupling within the mammalian pacemaker.

Chapter 1

Introduction

Daily rhythmicity is a fundamental organizing principle for life on this planet. Each and every day, the rotation of the Earth around its axis imposes marked changes in sunlight, darkness, temperature, food availability, and predation threat. The highly cyclic nature of the environment is mirrored in the daily rhythms displayed by many species of aquatic and terrestrial organisms, from cyanobacteria, plants, mollusks, insects, reptiles, birds, and mammals. Rather than reflecting a passive response to environmental fluctuations, biological rhythms are programmed by a physiological system for keeping time. Biological rhythms that “free-run” under conditions of constant darkness (DD) with an inherent period (τ) not exactly equal to 24 h are controlled by the circadian system. Free-running circadian rhythms persist in offspring born under DD over many successive generations (Aschoff, 1960) and τ can be influenced by genetic factors in a Mendelian manner (Ralph & Menaker, 1988). Presumably, a physiological system for measuring time increases the chances of survival in a highly rhythmic environment. Under its direction, behavior and physiological events can be programmed to occur at optimal times of the solar cycle, anticipating daily change rather than being passively driven by environmental variations.

The Circadian System

For heuristic purposes, the circadian system can be conceptualized as containing three main components: a pacemaker with a period of about 24 h, output pathways through which the pacemaker controls the behavior and physiology of the organism, and input pathways that synchronize the pacemaker to the environment (Moore & Leak, 2001). This basic model is germane to a wide diversity of organisms, from cyanobacteria to humans. Depending on the organism, these three main components can be found within a single cell or distributed across specialized tissues.

For vertebrate species, circadian rhythmicity is controlled by a central pacemaker within the brain that oscillates with an inherent period close to 24 h (Ralph & Hurd, 1995). In most mammalian species studied to date, the suprachiasmatic nucleus (SCN), a bilateral structure within the anterior hypothalamus, is both necessary and sufficient for circadian rhythms within the brain and body (Klein et al., 1991; Reppert & Weaver, 2002; Weaver, 1998). Lesions of the SCN eliminate many circadian rhythms in behavior and physiology, including those of locomotion, drinking, feeding, and hormone secretion (Meyer-Bernstein et al., 1999; Moore & Eichler, 1972, 1976; Schwartz & Zimmerman, 1991; Stephan & Zucker, 1972). Moreover, behavioral activity rhythms are restored to SCN-lesioned hamsters receiving transplants of SCN tissue, with the period of the restored rhythm matching that of the donor, not the host (Ralph et al., 1990). Even after isolation from the rest of the brain, the SCN display pronounced circadian rhythms in glucose utilization, electrical activity, neuropeptide release, and gene expression (Klein et al., 1991).

Not only does the SCN oscillate at the tissue level, individual neurons within the SCN are self-sufficient oscillators. When synaptic connections within the SCN are disrupted, individual SCN neurons sustain robust circadian rhythms in culture (Herzog et al., 2004; Welsh et al., 1995). Disassociated SCN neurons, however, display differences in inherent period length (τ), thus these cells must interact to synchronize within the network. Without interactions, SCN oscillators with different τ would desynchronize over time, producing arrhythmia at the level of the population (Bouskila & Dudek, 1995; Dudek et al., 1993; Enright, 1980a, 1980b; van den Pol & Dudek, 1993; Winfree, 1967). Desynchrony-induced arrhythmia can be conceptualized as the cacophony that arises when many people are talking or singing out of phase with one another. In this analogy, harmony can be facilitated by the transmission of cues between singers or from a single conductor. Similarly, intercellular interactions within the SCN are likely critical to the formation of a functional pacemaker. Little is known about SCN organization or the mechanisms underlying circadian coupling (Antle & Silver, 2005; Shirakawa et al., 2001), despite having a central role in modern chronobiological theory (Daan & Berde, 1978; Diez-Noguera, 1994; Ueda et al., 2002).

Self-sufficient rhythms in individual SCN neurons demonstrate that all the components required for an oscillator can be found within a single cell. At the molecular level, circadian rhythms are governed by negative and positive feedback loops in “clock” gene transcription and translation (Albrecht, 2002; Ko & Takahashi, 2006; Reppert & Weaver, 2002). Clock genes encode for proteins that ultimately

feedback and inhibit their own transcription, an oscillatory process that takes approximately 24 h. Specifically, two basic helix-loop-helix (bHLH)-PAS-containing transcription factors (CLOCK and BMAL) heterodimerize and dock to the E box binding enhancers within the promoter for three mammalian *period* genes (*mPer1*, *mPer2*, *mPer3*) and two mammalian *cryptochrome* genes (*mCry1*, *mCry2*). Each clock gene is transcribed into the respective mRNA, which translocates into the cytoplasm where it is translated into the associated protein (e.g., mPER1, mCRY1). After post-translation modification (e.g., phosphorylation, subcellular compartmentalization, protein dimerization), PER and CRY proteins translocate back into the nucleus where they inhibit the actions of CLOCK and BMAL and thereby terminate clock gene transcription. A positive feedback loop also regulates the rhythmic transcription of *Bmal* via the repressive actions of *Rev-Erb α* , a gene that is transcribed with *mPer* and *mCry*. Inhibition of CLOCK and BMAL transcription results in a de-repression (activation) of *Bmal* transcription. Clock gene rhythms control the circadian transcription of numerous clock-controlled genes, which constitute the output pathways controlling the circadian rhythms expressed at the cellular level.

Given the central role of the SCN in circadian function, it came as a shock to the field that rhythmic clock gene expression is not limited to cells within the SCN. Autonomous oscillators can be found throughout the brain and body, in the liver, lungs, bone, and even immortalized fibroblasts (Balsalobre, 2002; Nagoshi et al., 2004; Sakamoto et al., 1998; Yamazaki et al., 2000). In contrast to the SCN, circadian rhythms within these other tissues damp over time *in vitro* and ultimately become

arrhythmic, likely reflecting the desynchronization of underlying oscillators (Welsh et al., 2004). The SCN alone appears able to maintain coherent rhythmicity at the tissue level (Yamazaki et al., 2000). Consequently, the SCN is considered the “master” pacemaker, and hierarchical models of the circadian system posit that this structure acts as the conductor for the oscillators within the rest of the brain and body (Balsalobre, 2002; Gachon et al., 2004; Herzog & Tosini, 2001; Moore-Ede et al., 1976; Rosenwasser & Adler, 1986). Synaptic and humoral output pathways from the SCN likely synchronize oscillators within effector tissues to one another and to the environment (Kalsbeek & Buijs, 2002).

Circadian Entrainment to Light and Other Temporal Cues

In order to synchronize with the 24 h world, the circadian pacemaker is reset each day by environmental time cues (i.e., zeitgebers) that correct for deviations in the period of the pacemaker (τ) relative to the period of the external cycle (T). Systematic changes in light across the day are the primary cue synchronizing circadian rhythms in mammals (Meijer & Schwartz, 2003), and rapid changes in photic irradiance during twilight transitions may be the most reliable indicators for phase of the solar cycle (Roenneberg & Foster, 1997). It is of note that many environmental variables (temperature, humidity, food availability predation threat) can be predicted by the phase of the daily light:dark cycle. Nonphotic stimuli, such as exercise and social cues, also influence the function of the mammalian pacemaker, but are less well

characterized relative to responses to light (Mrosovsky, 1995, 1996). Nevertheless, animals under ecological conditions are likely exposed to a variety of time-giving cues.

Synchronization (i.e., entrainment) is achieved when the zeitgeber controls both pacemaker period ($T = \tau$) and the phase of its rhythms so that they are programmed within the appropriate phase of the solar cycle (Moore-Ede et al., 1982). Phase control produces a stable and reproducible relationship between the timing of the entraining zeitgeber and the biological rhythm, which can be quantified as the difference in hours between their respective phase markers (i.e., the phase angle of entrainment, ψ). For example, the onset of sleep in humans might lag 0.5 h behind the light-to-dark transition; yielding a $\psi_{L/D}$ of -0.5 h. True entrainment is typically verified by demonstrating that the free-running phase of the internal rhythm originates from the phase imposed by the environmental cycle.

Circadian responses to external cues are categorized according to the temporal qualities of the stimulus. Discrete pulses (typically < 2 h) elicit “nonparametric” responses, whereas tonic exposure elicits “parametric” responses (Aschoff, 1960; Johnson et al., 2003; Pittendrigh, 1981). Environmental stimuli may also mask the overt expression of circadian rhythmicity without necessarily affecting the central pacemaker itself (Mrosovsky, 1999b). Circadian responses in these three domains most often induce changes in a manner that is dependent on the phase of the internal cycle. By convention, internal time is referred to as circadian time (CT), where the beginning of subjective day and night are defined at CT0 and CT12, respectively.

Below, I discuss nonparametric, parametric, and masking responses in the context of photic stimuli, and follow with a brief description of the corresponding nonphotic responses.

Nonparametric responses to light. The mechanism of nonparametric photoentrainment is derived from a daily rhythm in the light sensitivity of the central pacemaker that is conserved across a wide range of diverse organisms (Johnson, 1999; Johnson et al., 2003). Discrete light pulses reset the phase of the clock in a time-gated manner, which is dependent on the internal phase the light pulse is administered. As represented by the photic phase response curve (PRC), light pulses induce phase delays and advances during early and late subjective night, respectively, but produce negligible phase shifts during subjective day (Daan & Pittendrigh, 1976; Johnson, 1999). The amplitude of the photic PRC can depend on a number of factors, including the species under study, the wavelength, intensity and duration of the light pulse, and the experimental history of the animal (Daan & Pittendrigh, 1976; Pittendrigh, 1981).

It may take several cycles before the steady state phase shift is expressed, with transients cycles being more common after a phase-advancing light pulse (Boulos & Rusak, 1982; Elliott & Tamarkin, 1994; Honma et al., 1985; Illnerova, 1991; Meijer & De Vries, 1995). However, double pulse protocols demonstrate that the photic PRC is immediately altered after a phase shifting stimulus is presented, suggesting that the central pacemaker is shifted to the new phase immediately after the light pulse is administered. Likewise, light pulses that produce significant phase shifts have immediate and direct effects on the SCN, producing upregulation of the immediate

early gene *c-fos* and the central clock genes, *mPer1* and *mPer2* (Kornhauser et al., 1990; Yan & Silver, 2002).

After measuring the inherent free-running period and the photic PRC of a given species, several important features of entrainment can be predicted (Johnson et al., 2003). First, the CT of zeitgeber exposure required to match τ to the external period can be obtained from the photic PRC in a straightforward manner. For instance, to entrain to a 24 h light:dark cycle, a pacemaker with an inherent period of 23 h requires a 1 h delay each day. The CT at which light will produce a 1 h delay can be extracted from the photic PRC and then used to predict ψ , the temporal phase relationship between the zeitgeber and the internal rhythm (e.g., $\psi_{L/D}$). Additionally, the maximum phase advance and phase delay indicated within the photic PRC dictate the lower and upper limits of entrainment to non 24 h light:dark cycles (i.e., T cycles). For example, if a pacemaker with $\tau = 23$ h was characterized by a photic PRC with a maximum advance and delay of 3 and 2 h, then the shortest and longest T-cycle to which it could entrain would be 20 h and 25 h, respectively.

In addition to affecting the phase of the pacemaker, light pulses also affect the velocity of the pacemaker by altering τ , and it has been suggested that entrainment to light pulses involves changes in both phase and period (Aschoff, 1963; Sharma, 2003). Similar to photic resetting, nonparametric responses in τ are dependent on the CT of light administration, as summarized in the τ RC. Typically, light pulses during early subjective night lengthen τ while light pulses during late subjective night shorten τ . Depending on the species of organism, the photic PRC and the photic τ RC may share

a similar waveform and time course, although the correlation between period and phase responses is weak in some mammalian species (Beersma et al., 1999; Pittendrigh & Daan, 1976a).

Parametric responses to light. Conditions of constant light alter the overt expression of circadian rhythms and also change the function of the central pacemaker (Aschoff, 1960, 1979). Typically, parametric circadian responses are proportion to the intensity of constant light, with larger effects produced by brighter light. Relative to constant darkness, constant light alters the amplitude of overt rhythms, their τ , and the shape, or waveform, of circadian rhythms. Circadian waveform, defined as the proportion of the circadian cycle representing subjective day versus subjective night, is commonly quantified by measuring the duration of either the active (α) or inactive (ρ) phase. In rodents, increasing the intensity of constant light increases τ (brighter levels of light lead to longer period), decreases the amplitude of activity rhythms (brighter levels of light suppress activity) and decreases α (brighter levels of light reduce the duration of the active phase), relationships commonly referred to as Aschoff's three "rules" for nocturnal species (Aschoff, 1960, 1979; Pittendrigh, 1960).

Parametric effects of light likely contribute to photoentrainment in nocturnal rodents, but are not thought to be the prime mechanism underlying photoentrainment. Many species of nocturnal rodents sleep in darkened burrows during the day, and under naturalistic laboratory conditions, engage in light sampling behavior at dawn and dusk (Refinetti, 2004). Furthermore, photoentrainment can be maintained under "skeleton" photoperiods, where the full photophase is replaced by two light pulses

(typically 0.15-1 h) positioned at the beginning and end of subjective night (Pittendrigh & Daan, 1976b). When skeleton photoperiods simulate long day lengths; however, entrainment is not stable. Beyond a “minimum tolerable night”, a “phase jump” will occur, where activity abruptly crosses one of the entraining light pulses and realigns into the longer scotophase previously reflecting subjective day (Geetha et al., 1996; Pittendrigh & Daan, 1976b; Sharma et al., 1997; Stephan, 1983). Thus, parametric actions of the full photophase likely stabilize entrainment of nocturnal rodents held under ultra long day lengths.

Masking responses to light. While traditionally viewed as an impediment to rigorous chronobiological study, masking responses can complement the light-induced phase shifts and τ responses that produce photoentrainment (Mrosovsky, 1999b). Both discrete and tonic exposure to light masks the overt expression of circadian rhythms (Mrosovsky, 1999b). When light reduces the expression of a behavior that is normally programmed at that phase, then it is said to produce negative masking. Common instances of negative masking in nocturnal rodents include bright light-induced suppression of melatonin secretion and behavioral activity during subjective night (Mrosovsky et al., 1999; Mrosovsky & Hattar, 2003; Redlin, 2001). In contrast, when light facilitates the expression of a behavior that is not normally programmed at that phase, it is said to produce positive masking. For example, the absence of light during subjective day can trigger wheel running in some species of nocturnal rodents, and very dim levels of light during subjective night enhance the levels of programmed activity (Mrosovsky et al., 1999; Mrosovsky & Hattar, 2003).

Nonphotic responses. Although light is the most commonly studied zeitgeber, the circadian clock can also be entrained by a variety of nonphotic stimuli (e.g., social interactions, melatonin injections, and physical activity produced by cage changing or transfer to novel wheels). In contrast to light, nonphotic stimuli produce phase advances during subjective day and smaller phase delays throughout subjective night, as represented by the nonphotic PRC. Physiologically, exposure to nonphotic stimuli produces high *c-fos* expression within the intergeniculate leaflet (IGL) of the thalamus, but not in the SCN, and downregulation of clock genes in the SCN (Maywood et al., 1999; Maywood et al., 2002; Mead et al., 1992; Mikkelsen et al., 1998). Moreover, discrete and tonic nonphotic stimuli can alter τ (Mrosovsky, 1993). For example, the presence of a running wheel can alter τ in many species of nocturnal rodents (Mrosovsky, 1999a). Furthermore, effects of nonphotic cues may interact with the effects of photic stimuli, producing additive, subtractive, or synergistic effects, depending on the CT of stimulus presentation (Joy & Turek, 1992; Mrosovsky, 1991). In the wild, mammals are likely exposed to photic and nonphotic zeitgebers, and both likely contribute to entrainment under ecological conditions.

The Circadian Visual System

Day and night are often simulated in the laboratory through the use of lighting regimens alternating between moderate indoor light levels (> 100 lux) and complete darkness, which are sufficient to entrain the daily rhythms of most animal species used

to study the circadian system. While some vertebrate species have extraocular photoreceptors that contribute to circadian responses to light, the retina is required for circadian photoreception in mammals (Underwood & Groos, 1982). For example, enucleation in several species of diurnal and nocturnal rodents leads to free-running rhythms under laboratory light:dark cycles (Lucas & Foster, 1999; Nelson & Zucker, 1981). Thus, light cues relayed from the retina are of primary importance for photoentrainment.

Photic stimuli are conveyed from the eye to the SCN through two main retinofugal projections (Meijer & Schwartz, 2003; Moore, 1995; Morin & Allen, 2006). One is a direct pathway, the retinohypothalamic tract (RHT), which arises from the optic chiasm to terminate within the SCN. In rodents, the large majority of RHT axons (~80%) derive from a subset of retinal ganglion cells that contain a newly identified photopigment, melanopsin (Beaulieu et al., 2003; Morin et al., 2003; Sollars et al., 2003). A second visual pathway, the geniculohypothalamic tract (GHT), involves input from the intergeniculate leaflet (IGL) and the vLGN of the thalamus (Harrington, 1997; Muscat & Morin, 2006). The RHT is sufficient for normal photoentrainment, but difficulty severing the RHT without damaging the optic tract precludes complementary studies assessing whether it is necessary. Although the GHT does not contribute significantly to steady-state entrainment, ablating the IGL slows re-entrainment in a number of contexts (Dark & Asdourian, 1975; Freeman et al., 2004; Johnson et al., 1989; Zucker et al., 1976). Additionally, the GHT modulates

masking responses to light pulses (Redlin et al., 1999) and is involved in nonphotic resetting (Hastings et al., 1997; Janik & Mrosovsky, 1992; Mikkelsen et al., 1998).

Electrophysiological studies of rodent SCN visual responses, using retinal illumination and optic tract stimulation, demonstrate that the majority of SCN neurons respond to light and encode luminance by increasing the rate of their electrical discharge within a certain range of light intensities (~10-500 lux in the hamster, ~0.1-500 lux in the rat) typical of twilight transitions (Meijer, 1991; Meijer et al., 1986). Below the lower threshold, no light response is observed, while above the upper bound, the discharge rate saturates. Electrical responses of SCN neurons also reflect temporal aspects of light exposure, with short pulses (< 1 sec) evoking no response and longer light stimuli eliciting a sustained response for the duration of the pulse. Circadian photoentrainment and SCN-mediated photic responses remain intact in transgenic mice lacking both rods and cones, but are eliminated with additional ablation of the gene for melanopsin (Hattar et al., 2003; Lucas et al., 2001). Since melanopsin-containing retinal ganglion cells project directly to the SCN, are intrinsically light sensitive, and display functional properties *in vitro* similar to circadian photic responses studied *in vivo* (see below), they are currently the leading candidate for being the primary photoreceptor of the circadian system (Berson, 2003; Gooley et al., 2003).

Phase resetting and melatonin suppression, two hallmark circadian responses to light, exhibit a monotonic dependence on light intensity that is generally well described by a sigmoid-shaped function in a log-linear plot (Nelson & Takahashi,

1991a, 1991b). Hallmark circadian responses to light are characterized by a spectral tuning curve distinguished from those describing rod and cone photopigments, with the peak in the blue-green ($\lambda = 480\text{-}500$ nm) region of the visible spectrum (Takahashi et al., 1984). The circadian visual system is also able to integrate photons over a much longer temporal window (e.g., an hour) than that for rods and cones (e.g., milliseconds and seconds). For example, in the hamster, a 5 min and 45 min pulse with equivalent photon flux will elicit comparable responses, although the capacity for photon integration may decrease with longer pulses (Dkhissi-Benyahya et al., 2000; Nelson & Takahashi, 1991b, 1999). Lastly, relative to responses mediated by rods and cones, higher light levels are typically required to elicit phase shifts and melatonin suppression in mammals (0.1 – 1 lux), although photic sensitivity does vary by species and ecological niche (Erkert, 2004; Erkert et al., 1976; Erkert & Grober, 1986).

Nocturnal rodents, such as the Syrian hamster, navigate in the field under nighttime illumination as high as 0.04 and 0.3 lux at quarter and full moon, respectively (Biberman et al., 1966; Thorington, 1980). Light of this intensity, while enough for animals to see by (Emerson, 1980), has been shown in the lab and the field to be largely ineffective in producing hallmark circadian responses to light, such as phase resetting and melatonin suppression (Brainard et al., 1982; Nelson & Takahashi, 1991a, 1991b). Comparative studies of light responses among rodents inhabiting canopied forest versus open deserts have suggested that the sensitivity of the circadian visual system in mammals has been titrated so that the central pacemaker is unaffected

by natural nighttime illumination (Brainard et al., 1984; DeCoursey, 1990; Nelson & Takahashi, 1991b).

Photoperiodism

The circadian pacemaker also uses photic stimuli to encode day length (i.e., photoperiod), which permit mammalian species to anticipate seasonal changes in temperature and food availability. Photoperiod-dependent changes in physiology and behavior are also evident in humans under laboratory and naturalistic conditions (Wehr, 2001). In nocturnal rodents, photoperiod alters a suite of diurnal and nocturnal events such that the duration of daily light and dark phases is reflected in circadian waveform (i.e., the duration of subjective night versus subjective day). The duration of several nocturnally expressed markers of circadian phase (e.g., elevated melatonin secretion and behavioral activity, or α) is longer under the long nights of “winter-like” short day photoperiods (SD, e.g., 10 h light:14 h darkness) than under the short nights of “summer-like” long day photoperiods (LD, e.g., 14 h light:10 h darkness) (Elliott & Tamarkin, 1994; Illnerova, 1991; Illnerova et al., 1999; Refinetti, 2002). Proportional changes in subjective day events also occur, and the duration of elevated electrical activity, *mPer*, and spontaneous *c-fos* within the SCN reflect the entraining photophase (de la Iglesia et al., 2004; Messenger et al., 2000; Mrugala et al., 2000; Sumova et al., 2003). Circadian sensitivity to light during subjective night is also altered by photoperiod. SD entrainment increases the fraction of the circadian cycle during which light elicits *c-fos* and *mPer* within the SCN (Sumova & Illnerova, 1998; Travnickova et

al., 1996; Vuillez et al., 1996), and modulates the waveform of the photic PRC (Binkley & Mosher, 1986; Pittendrigh et al., 1984; Pohl, 1983, 1984). After SD entrainment, Syrian hamsters exhibit light-induced phase shifts over a wider range of times (consistent with long α and melatonin secretion), and with peak shifts much greater than after LD entrainment. This photoperiodic difference in phase shift magnitude may reflect a switch from weak (type 1) to strong (type 0) resetting (Lakin-Thomas, 1995). Since myriad biologic events change in proportion to day length, this convergence of results indicates a global change in the central pacemaker itself.

In hamsters, reproductive physiology is maintained under LD by the short melatonin signal associated with this photoperiod, whereas long melatonin signals under SD cause gonadal regression, pelage molt, body weight loss, and changes in immune function (Elliott, 1976; Gorman et al., 2001a). Reproductive and somatic changes presumably increase the chance of surviving the winter months and giving birth under environmental conditions favorable for the survival of offspring. Within many rodent species, however, a subset of animals fails to adopt the typical SD phenotype of reproductive quiescence, and this group of animals is commonly referred to as SD NonResponders (SD-NR) (Nelson, 1987). In Siberian hamsters, the incidence of SD nonresponsiveness has a circadian basis, such that the pacemaker in this species appears “locked” in a summer state (Gorman & Zucker, 1997; Puchalski & Lynch, 1988, 1991a, 1991b, 1994). SD-NR Siberian hamsters display SD phenotypic responses if exogenous melatonin is provided; indicating that peripheral sensitivity is intact but the appropriate SD melatonin signal is absent (Margraf & Lynch, 1993). Siberian SD-NRs express both a short α and a short melatonin signal

under SD, with each rhythm phase locked to dawn in the large majority of animals (Gorman & Elliott, 2004; Gorman & Zucker, 1997; Prendergast & Freeman, 1999; Puchalski & Lynch, 1986). A complementary pattern can be seen: SD-NR display an increase in the duration of the diurnal rhythm of spontaneous electrical activity within the SCN relative to SD-Responders (Margraf et al., 1991). Siberian SD-NRs also display longer τ under DD than their responsive counterparts, which may contribute to aberrant entrainment under SD (Freeman & Goldman, 1997; Kliman & Lynch, 1991). Collectively, these data suggest that in Siberian hamsters the SD-NR phenotype is caused by a fundamental change in the function of the central pacemaker.

Photoperiodic regulation of circadian waveform is not dependent on differences in the amount of light under LD and SD. First, photoperiodic differences in circadian waveform are maintained under skeleton photoperiods that simulate LD and SD cycles while maintaining the total duration of light exposure constant (Elliott, 1976; Elliott, 1981). Furthermore, circadian waveform after release from entrainment into DD is dependent on the photoperiodic history of the animal. Similar to the long subjective night expressed under SD entrainment, animals released from SD into DD display a long α and melatonin signal that is maintained over subsequent weeks. In contrast, animals released from LD into DD, display short α and melatonin immediately upon release, and the duration of these events gradually increase over the following weeks in a systematic manner (Elliott & Tamarkin, 1994; Illnerova, 1991). The expansion of α , for example, occurs as activity onset and offset adopt different τ , shorter and longer than 24 h, respectively (Figure 1.1A). Activity onset advances each cycle while offset delays, and α increases as these phase markers “drift” apart. Advances in the onset of

behavioral activity and melatonin secretion are highly correlated, as are the delays in their offsets, suggesting that a common mechanism underlies expansion of both nocturnal events (Elliott & Tamarkin, 1994). Proportional changes also occur within the SCN itself, with endogenous gene expression profiles being strongly modulated by release into DD (de la Iglesia et al., 2004; Quintero et al., 2003; Sumova et al., 2003; Sumova et al., 2000). When rodent species are released from LD into DD, α and τ typically stabilize within several weeks, and ultimately, animals exposed to different photoperiodic pretreatments display similarities in their free-running rhythms. Stronger “after-effects” of photoperiodic entrainment, lasting several months, may be observed in some species (Binkley & Mosher, 1986; Pittendrigh & Daan, 1976a).

Theoretical Mechanisms Regulating Circadian Waveform

The mechanisms underlying the photoperiodic regulation of circadian waveform are best understood in the context of the dual-oscillator model of Pittendrigh and Daan (Pittendrigh & Daan, 1976b, 1976c). As described above, different phase markers of the pacemaker (e.g., activity onset and offset) adopt different period lengths after release from LD into SD or DD, which is contrary to the notion that a single oscillator can not display simultaneously two different periods. To account for these observations, Pittendrigh and Daan posit that the central pacemaker contains multiple, interacting oscillators (Figure 1.1B). Since events at dusk adopt a short τ upon release into SD or DD, these authors theorized that dusk events are

programmed by an “Evening” oscillator (E) with $\tau_E < 24$ h. Likewise, dawn events are thought to reflect a Morning oscillator (M) with $\tau_M > 24$ h. In nocturnal rodents, it is thus proposed that α reflects the phase angle between E and M (ψ_{EM}). Conversely, the duration of the inactive phase (ρ) is thought to reflect ψ_{ME} .

The dual oscillator model can account for photoperiodic changes in circadian waveform under entrained conditions (Figure 1.1B). Based on nonparametric entrainment theory, E is reset daily via the phase delaying action of light at dusk while M is reset daily via the phase advancing action of light at dawn. Under ultra long day lengths (ULD), the resetting actions of light acting at dusk and dawn reduce ψ_{EM} , which in turn affects the duration of diurnal and nocturnal events (e.g., short α , long *mPer* expression). Transfer to a photoperiod with shorter day lengths (LD or SD), permits E and M to free-run according to their inherent τ until each is re-entrained by the new light-dark transition (Elliott & Tamarkin, 1994; Gorman et al., 1997; Illnerova, 1991; Pittendrigh, 1974). According to the dual oscillator model, photoperiod thereby regulates ψ_{EM} , producing changes in circadian waveform at the level of the animal (e.g., changes in melatonin secretion and α).

Under entrained conditions, the resetting actions of light at dawn and dusk could synchronize E and M and regulate ψ_{EM} , without requiring oscillator interactions. Under constant dark conditions, however, non-interacting oscillators with different intrinsic periods would continue to free-run independently, producing a “beating” pattern in overt rhythms monitored over many cycles. In other words, without any coupling, E and M oscillators would not free-run synchronously unless $\tau_E = \tau_M$. As

discussed earlier, it is likely that the mammalian pacemaker contains a *population* of many oscillators running at different speeds (Herzog et al., 1998; Honma et al., 1998; Ohta et al., 2005; Quintero et al., 2003; Schaap et al., 2003; Welsh et al., 1995; Yamaguchi et al., 2003), and even modest discrepancies in τ would ultimately produce arrhythmia at the level of the population. Since overt rhythms under DD adopt a steady state τ and waveform (e.g., $\alpha:p$), underlying oscillators are thought to interact, or couple, in such a way that prevents arrhythmia at the level of the population and the animal.

Interactions between circadian oscillators are modeled mathematically in terms of shifts in the rhythm of a given oscillator that are induced by the partner oscillator at a certain phase in its daily cycle (Daan & Berde, 1978; Pavlidis, 1973; Shinbrot & Scarbrough, 1999). Thus, the basis of oscillator-oscillator synchronization is conceptualized in a manner analogous to the mechanisms that permit an organism to entrain to the environmental cycle. Functions describing circadian coupling mechanisms can be envisioned as phase response curves for oscillator interactions (i.e., coupling response curves), where positive and negative values represent the shifted oscillator is phase advanced and delayed, respectively (Oda & Friesen, 2002; Oda et al., 2000). A different coupling response curve may be constructed where coupling influences τ , rather than phase.

Formal properties of these coupling response curves will be influenced by the assumptions on which the mathematical model is based. If it is assumed as above that the free-running period of each oscillator is inherently different (e.g., $\tau_E < \tau_M$), then

the increase in α (and ψ_{EM}) is produced when E and M free-run upon release into DD and “drift” apart from one another. As discussed above, expansion of α (and ψ_{EM}) is checked when $\psi_{EM} > \sim 12$ h, and $\tau_E = \tau_M$. Rather than adopting either τ_E or τ_M , the τ of the synchronized system (τ_{EM}) is an intermediate of τ_E and τ_M . This suggests that E and M are phase shifting one another in complementary ways to produce synchrony when $\psi_{EM} > \sim 12$ h (i.e., the faster oscillator speeds up the slower one, and vice versa). Thus, the strength of this interaction is predicted to increase as ψ_{EM} increases.

Alternatively, the increase in ψ_{EM} may reflect interactions between E and M that cause these oscillators to “repel” one another (i.e., the faster oscillator slows the slower oscillator, and vice versa) until $\psi_{EM} > 12$ h. In this latter model, the strength of the coupling would be predicted to actually decrease as ψ_{EM} increases. Mathematical models incorporating either function are able to simulate a wide range of experimental findings, and both potentially describe coupling mechanisms operating within the central pacemaker.

One of the primary appeals of multi-oscillatory models of the central pacemaker is the theoretical application to many different types of experimental findings. Multi-oscillator models, both formal and mathematical, have been used to account for many phenomena, including transients in resetting, photoperiodic after-effects on τ , and individual differences in circadian entrainment. For instance, in the Siberian hamster, individual differences in the plasticity of circadian waveform during SD re-entrainment have been attributed to differences in coupling between E and M (Puchalski & Lynch, 1991a). It has been argued that ψ_{EM} is not plastic in the SD-

NonResponders that maintain summer-like activity profiles and physiology despite prolonged exposure to SD, whereas in SD-Responders, E and M may adopt different phase relations. Individual differences in the plasticity of ψ_{EM} in this species may be produced by changes in the properties of underlying oscillators and discrepancies in circadian coupling mechanisms. However, it is difficult to speculate the manner in which coupling might be affected in a given population or after a given treatment, since the precise mechanisms underlying coupling are ill defined. If expansion of α reflects the “drift” of E and M oscillators, then stronger interactions would be predicted to limit increases in ψ_{EM} in SD-NonResponders relative to SD-Responders. In contrast, if expansion of α reflects E and M “repelling” one another, then SD-NonResponders could be viewed as having weaker coupling not able to increase ψ_{EM} .

In mammals, each lobe of the SCN function as a pair of redundant pacemakers (Davis & Gorski, 1984; Davis & Viswanathan, 1996), but distinct neural groups can be localized to each lobe (Antle et al., 2003; Antle & Silver, 2005). Progress in understanding the functional and structural organization of the central pacemaker has been hindered by the complexity of the mammalian nervous system. Bilaterally distributed pacemakers exist in the brains of invertebrate species (e.g., mollusks and insects), and insights gleaned from studies on circadian coupling within invertebrates may serve to demonstrate principles operating within mammals.

Circadian Coupling

To establish that invertebrate pacemakers are interacting, overt rhythms displayed by the distinct pacemakers should adopt a stable ψ under constant dark conditions. The degree to which ψ is stable reflects the strength of the coupling between these pacemakers, with highly reproducible and persistent ψ indicating strong oscillator interactions. Further, a stable ψ should be reestablished after experimentally produced desynchrony (i.e., phase shift or change in τ of one pacemaker) through a series of transient phase shifts in the rhythms of both pacemakers. If oscillators instead display desynchronized rhythms under constant conditions or after experimental manipulations, weak coupling mechanisms may cause one pacemaker to modulate the τ and amplitude of rhythms expressed by the other pacemaker in a phase dependent manner.

In several species of mollusks, each eye contains a distinct pacemaker, which can be physically isolated from the contralateral eye and brain to investigate coupling mechanisms within the system. Comparative studies in different species of mollusks reveal differences in circadian organization and coupling between bilaterally distributed retinal pacemakers. In two species of marine snail, *Bulla gouldiana* and *Bursatell leachi plei*, electrical rhythms between the eyes maintain a stable ψ under constant conditions, whereas ψ is less stable in the sea slug, *Aplysia californica* (Roberts & Block, 1983; Roberts et al., 1987). Phase shifting or altering τ of one pacemaker in the sea snail changes the rhythmicity of the contralateral pacemaker, which is prevented after dissection of the cerebral commissure (Page & Nalovic, 1992; Roberts & Block, 1983; Roberts et al., 1987). In contrast, retinal pacemakers in

Aplysia do not resynchronize after experimental manipulations of one eye, but phase-dependent modulation of τ and overt expression as each retinal rhythm beats in and out of phase with the other suggests the presence of weak coupling mechanisms.

Within several species of insects, a pacemaker has been localized to the base of each optic lobe, and each bilateral pacemaker can be exposed to different photic environments. After unilateral optic nerve dissection, which “blinds” the optic lobe on the ipsilateral side, 70% of crickets and cockroaches under constant light (LL) display two free running components, one lengthened by LL and the other free running as if in complete darkness (Tomioka, 1993; Tomioka et al., 1991; Wiedenmann, 1983). Like studies conducted on mollusks, phase dependent modulation of τ and overt expression occurs as components free-run in and out of phase with one another. Lesions of the “sighted” or “blind” optic lobe eliminate the free-running component displaying the LL and DD rhythm, respectively. Similar effects of unilateral optic nerve dissection in crickets also occur under T-cycles at the limits of entrainment, with the sighted pacemaker entraining and the blinded pacemaker free-running. T-cycles using dim light photophases increase the incidence of the desynchrony between optic lobes in cricket species, suggesting that bilateral pacemakers exchange photic information (Tomioka, 1993).

For both mollusks and crickets, it is likely that the identified bilateral pacemakers are functionally redundant, since similar effects occur after manipulations of either the left or right side of the brain. In the fruit fly, *Drosophila melanogaster*, six functionally distinct clock groups within each optic lobe coordinate circadian

rhythms displayed by the animal (Blanchardon et al., 2001; Rieger et al., 2006). Behavioral activity rhythms in fruit flies are crepuscular, and the activity peak at dawn and dusk is controlled respectively by the M-cells and E-cells within the lateral neurons of the optic lobes (Grima et al., 2004; Peng et al., 2003; Stoleru et al., 2004). These two groups of cells were named after the Morning and Evening oscillators of the dual oscillator model of the central pacemaker. Pigment-dispersing factor has been identified as a daily resetting signal maintaining synchrony amongst M-cells and E-cells within *Drosophila* (Lin et al., 2004). Recent genetic advances in the fruit fly permit tissue-specific genetic manipulations of τ in six different oscillatory regions of the fly brain, which together appear to be organized into two main neuronal circuits for keeping time (Stoleru et al., 2005).

Progress in understanding the functional organization and coupling with the invertebrate circadian systems has been facilitated by the localization and manipulation of discrete pacemaker subunits. It remains a challenge to physically isolate and manipulate clock subgroups in mammals; however, environmental manipulations can be used to disassociate oscillators within the SCN. The disassociated system can then be studied formally to characterize the organization of the pacemaker and the nature of its interactions.

Split Rhythms in Mammals

In several species of mammals, bimodal, or “split,” rhythms emerge when animals are held under constant bright light conditions (LL) (Cheung & McCormack, 1983; Pittendrigh, 1960; Pittendrigh & Daan, 1976c; Turek et al., 1982). Incidence of LL-induced splitting increases as the intensity of light under LL increases (Pickard et al., 1993); but see (Meijer et al., 1990). After chronic exposure to LL, two distinct activity bouts emerge, free run with different circadian periods until ~12 h apart, where upon they synchronize and establish a new antiphase configuration. After transfer from LL into DD, the two split activity components rapidly rejoin by adopting different period lengths (Earnest & Turek, 1982). Under the premise that a single oscillator can not express two different period lengths simultaneously, many have suggested that LL-induced splitting reflects the presence of at least two interacting oscillators within the mammalian SCN. In addition to the activity rhythm, other behavioral and physiological rhythms are likewise split by LL, indicating a temporal reorganization of the pacemaker rather than a mere change in the overt expression of a single rhythm (Mason, 1991; Ohta et al., 2005; Pickard et al., 1984; Shibuya et al., 1980; Swann & Turek, 1982; Swann & Turek, 1985; Zlomanczuk et al., 1991). It is now known that after LL-induced split rhythms emerge, electrophysiological and gene expression rhythms controlled by the left and right sides of the SCN oscillate in antiphase (de la Iglesia et al., 2000; de la Iglesia et al., 2003; Ohta et al., 2005). There is also recent data that suggest there are further subdivisions induced within each lobe of the SCN (Yan et al., 2005).

LL temporally disassociates central oscillators within the SCN so that oscillator groups are cycling 12 h out of phase with one another; however, this behavioral paradigm has been limited in application for several reasons. One notable impediment is that LL-induced splitting is a long process over which there is little experimental control. First, it is impossible to predict in advance which animals will split and how many weeks after transfer to LL the split rhythms will emerge. Since the system is free running in LL, the formal methods for understanding functional differences between split oscillators are limited. Furthermore, constant exposure to bright LL acutely masks many overt rhythms (e.g., melatonin secretion), limiting generalizations applicable to photoperiodic paradigms using incompatible experimental conditions. In fact, it is unlikely that oscillators disassociated under LL correspond to those that encode photoperiod (Davis & Gorski, 1984; de la Iglesia et al., 2004; Hastings et al., 1987).

Recently, an entrainment paradigm has been developed for generating split rhythms rapidly and with more experimental control (Evans & Gorman, 2002; Gorman, 2001; Gorman & Elliott, 2003; Gorman & Lee, 2001; Gorman & Steele, 2006; Gorman et al., 2001b). Under 24 h light:dark:light:dark cycles (LDLD), some animals entrain in a conventional manner, with behavioral activity confined to one of the two daily scotophases (Figure 1.2A). Other individuals, however, display two activity bouts per 24 h, one entrained to each daily scotophase (Figure 1.2A). After release into constant conditions, LDLD-induced split activity bouts rejoin in a series of transients lasting 2-7 days, suggesting the presence of two groups of interacting

oscillators. Additional rhythms beside activity (e.g., body temperature, melatonin) are likewise expressed in a bimodal fashion (Gorman et al., 2001b; Rosenthal et al., 2005), suggesting that the central pacemaker has been functionally reorganized. However, animals split under a variety of protocols using LDLD cycles do not exhibit the left: right asymmetry displayed by animals split under LL (Edelstein et al., 2003; Gorman et al., 2001b; Watanabe et al., 2007), and ongoing work seeks to identify the physiological reorganization of cells within the SCN.

Both nonphotic and photic stimuli contribute to the induction of split rhythms under LDLD cycles (Gorman, 2001; Gorman et al., 2003; Gorman & Lee, 2001). For example, when “novel wheel running” (NWR) is repeatedly scheduled during subjective day, animals that engage in robust NWR later exhibit split rhythms under LDLD cycles, whereas less active “sluggards” do not (Evans & Gorman, 2002; Gorman & Lee, 2001; Gorman et al., 2001b; Mrosovsky & Janik, 1993; Sinclair & Mistlberger, 1997). In other experiments using LDLD, split rhythms appear to be triggered by activity induced by transfer to a wheel running cage or by a cage change (Gorman et al., 2003). These results are consistent with a hypothesis that nonphotic phase shifts of distinct oscillator populations contribute to the emergence of split rhythms under LDLD (Gorman, 2001; Gorman et al., 2003; Gorman & Lee, 2001).

Bright light is likewise implicated in splitting under LDLD since split rhythms are not sustained under constant darkness (i.e., activity components rapidly rejoin) (Gorman & Elliott, 2003; Gorman & Lee, 2001). Additionally, an inductive role for the bright light of LDLD cycles is suggested by the spontaneous emergence of a

second activity bout when the duration of the scotophase entraining nocturnal activity is sufficiently reduced (Gorman, 2001). The emergence of these LDLD-induced split rhythms bears some resemblance to phase jumps under skeleton photoperiod simulating increasing day lengths (Pittendrigh & Daan, 1976b; Stephan, 1983). Both phase jumps and LDLD-induced split rhythms emerge after bright light compresses the duration of subjective night, thereby challenging circadian entrainment. Beyond a “minimum tolerable night” (Pittendrigh & Daan, 1976b; Stephan, 1983), the resulting expression of a phase jump, or a split rhythm, may depend on the duration of the alternative scotophase (Gorman et al., 2003).

Relative to other behavioral paradigms, LDLD-induced splitting is a strong paradigm with which to study circadian coupling since reorganization is rapid and subject to more experimental control (Gorman & Steele, 2006). Moreover, there are a number of ways in which LDLD can be used to investigate putative interactions between oscillators, as represented by Figure 1.2B. In this model, short night entrainment of the unsplit rhythm under LDLD alters oscillator interactions such that a distinct oscillator subgroup re-entrains to the new daytime scotophase (represented by curved arrow), through nonphotic resetting or phase jumping mechanisms. These mechanisms are investigated in Chapter 3. Furthermore, after release into constant conditions, interactions between split oscillators cause the rapid rejoining of activity components (represented by the double headed arrow), which are characterized rigorously for the first time in Chapter 4. Lastly, bright light during daily photophases is thought to prevent oscillators from rejoining (represented by each X), since

photophase deletions and de-masking dark pulses affect the entrained phase of each split activity component. These latter interactions are investigated in Chapter 5.

Dim Nighttime Illumination

During the course of studies examining the formal bases of LDLD-induced split rhythms, we discovered that re-entrainment under LDLD is markedly influenced by the presence of dim nighttime illumination during the daily scotophases (Gorman & Elliott, 2004; Gorman et al., 2003). Prior to their use in LDLD-induced splitting studies, green light-emitting diodes (LEDs, peak $\lambda = 560$ nm) were installed within environmental chambers to aid human vision during experimental and husbandry regimens. These LEDs emit nighttime illumination comparable in intensity to dim moonlight or starlight (~ 0.01 lux), which is largely ineffective at eliciting phase shifts in the Syrian hamster. However, this dim nighttime illumination is far from being biologically inefficacious. Only 33% of animals will split under LDLD using bright light photophases (~ 100 lux) and completely dark scotophases (i.e., green LEDs extinguished). In contrast, nearly 100% of animals will exhibit LDLD-induced split rhythms when provided bright light photophases and dimly lit scotophases (i.e., green LEDs illuminated).

Across a variety of behavioral assays, circadian entrainment in the nocturnal hamster is markedly altered by dim nighttime illumination (Gorman et al., 2006). Despite conventional wisdom that the circadian pacemaker is blind to dim light

comparable to moonlight and starlight, completely dark nights are not functionally equivalent to dimly lit nights, even when this nighttime illumination is below putative thresholds for the circadian visual system. Convergent effects of dim light across diverse paradigms in multiple species attest to its potency as a modulator of circadian rhythms and reveal a latent plasticity in the mammalian circadian system.

In Siberian hamsters, dim nighttime illumination increases the incidence of LDDL-induced splitting (Gorman & Elliott, 2004). In addition, photoperiodic responses to SD entrainment (e.g. expansion of α , gonadal regression, and body weight loss) are accelerated in this species where the daily dark periods are dimly lit rather than completely dark (Figure 1.3A). Moreover, SD with dimly lit nights reduces the incidence of SD “nonresponsiveness” by 20% relative to that observed under SD with completely dark nights (Gorman & Elliott, 2004). Thus, in this species, dim nighttime illumination increases the plasticity of circadian entrainment to SD photoperiods in two ways. Through effects on α , dim light markedly alters circadian waveform under this behavioral paradigm.

Dimly lit nights also increase the ability of the central pacemaker to entrain to non-24 hour light:dark cycles (i.e., T-cycles). In Syrian hamsters held under lengthening T-cycles, dimly lit nights increase the upper range of entrainment by ~ 4 h relative to animals that are housed under non-24 T-cycles with completely dark nights (Gorman et al., 2005). Specifically, animals with completely dark nights fail to synchronize to T-cycles longer than 26 h, whereas more than 50% of animals held under dimly lit nights entrain to 30 h T-cycles (Figure 1.3C and 1.3D). Moreover, dim

nighttime illumination under T-cycles increases α relative to that displayed by animals held under T-cycles with completely dark nights. The change in circadian waveform produced by exposure to dimly lit nights may be mechanistically involved in enhancing the limits of entrainment since in the Syrian hamster there is a positive correlation between α and the amplitude of the PRC to bright light pulses (Pittendrigh et al., 1984; Refinetti, 2006; Shimomura & Menaker, 1994).

Dim nighttime illumination, below established thresholds for phase shifting and melatonin suppression, can nonetheless modulate biological rhythmicity. While these data challenge current assumptions about the photic sensitivity of the circadian pacemaker, this is not without precedent. In addition to its acute effect on activity levels (Edelstein & Mrosovsky, 2001; Erkert & Grober, 1986; Gorman et al., 2003; Mrosovsky et al., 1999), dim light influences circadian behavior in other nocturnal mammals (Erkert et al., 1976; Meijer et al., 1990). Kavanau reported that white-footed mice will entrain to dim:dark cycles (< 0.02 lux: 0 lux cycles), although this lacked rigorous quantification (Kavanau, 1967). Additionally, dim illumination has been used under the context of studies with twilight transitions, which widen the range of photic entrainment in both hamsters and mice (Boulos et al., 2002; Kavanau, 1968).

Potential mechanisms underlying the potency of dim nighttime illumination

Potent effects of dim nighttime illumination call into question the prevailing view that the central pacemaker is buffered from dim light like that experienced at

night in the wild. Instead, the central pacemaker appears to be more sensitive to photic input than is perhaps currently appreciated. But in what manner can dim illumination, a seemingly innocuous stimulus, be influencing circadian entrainment?

It seems unlikely that these effects are produced by nonparametric responses of the circadian visual system given previous research indicating that phase shifts and melatonin suppression are not elicited by light of this intensity. However, prior studies may have underestimated the photic sensitivity of the circadian pacemaker through the nearly exclusive use of short, discrete light pulses (typically 0.25 h in duration). If the central pacemaker is able to integrate photons over many hours, circadian responses to dim light may emerge after longer exposure, like that experienced during laboratory scotophases and in the wild. Thus, the phase and expression of circadian rhythms may be influenced by cumulative light exposure over a longer timescale than previously tested. While this idea is intriguing, studies using 3 h dim light pulses to suppress melatonin support results obtained with shorter light pulses (Brainard et al., 1984). Thus, it is likely that the fundamental action of dim illumination lies elsewhere.

Since dim nighttime illumination has potent actions under behavioral paradigms indicative of coupled oscillators, the marked changes in circadian waveform under dim light may be induced by a change in the interactions between central oscillators (Gorman & Elliott, 2004; Gorman et al., 2003). Lacking a direct assay of circadian coupling, the present studies employ a two-pronged approach for addressing whether dim nighttime illumination influences circadian coupling mechanisms. First, I begin by rigorously testing alternative hypotheses involving

hallmark circadian responses to light that do not require a pacemaker comprised of multiple oscillators. Second, I analyze how dim light facilitates LDLD-induced splitting, since it is thought that the induction, maintenance, and fusion of split activity rhythms reflect the actions of interacting oscillators (Figure 1.2B). Knowledge of how dim illumination influences each of these processes will aid in testing the putative effect of dim light on circadian coupling.

Specific Aims

The experiments reported here investigate potential mechanisms through which dim nighttime illumination might influence circadian entrainment. Specifically:

1) Is dim illumination a strong zeitgeber when provided on its own? The present studies are designed to detect even a subtle response requiring long dim light exposure more characteristic of that experienced in the laboratory and in nature.

2) Does dim illumination alter an intrinsic property of the central pacemaker as measured under free-running conditions (e.g., circadian period, waveform, or amplitude)?

3) Does dim illumination potentiate circadian responses to stronger zeitgebers (e.g., bright light and nonphotic stimuli) by directly augmenting the strength of circadian input pathways?

4) How does dim light facilitate the induction of split rhythms under LDLD? Does dim nighttime illumination interact with the nonphotic and photic zeitgebers that trigger splitting under LDLD in the hamster?

5) Does dim illumination influence the way split oscillators fuse together after release from LDLD into free-running conditions?

6) Does dim nighttime illumination maintain the stability of the split state once oscillators have been disassociated under LDLD? Since bright light photophases are required to maintain the split state, does dim light interact with bright light to inhibit oscillator interactions that would otherwise cause the system to rejoin?

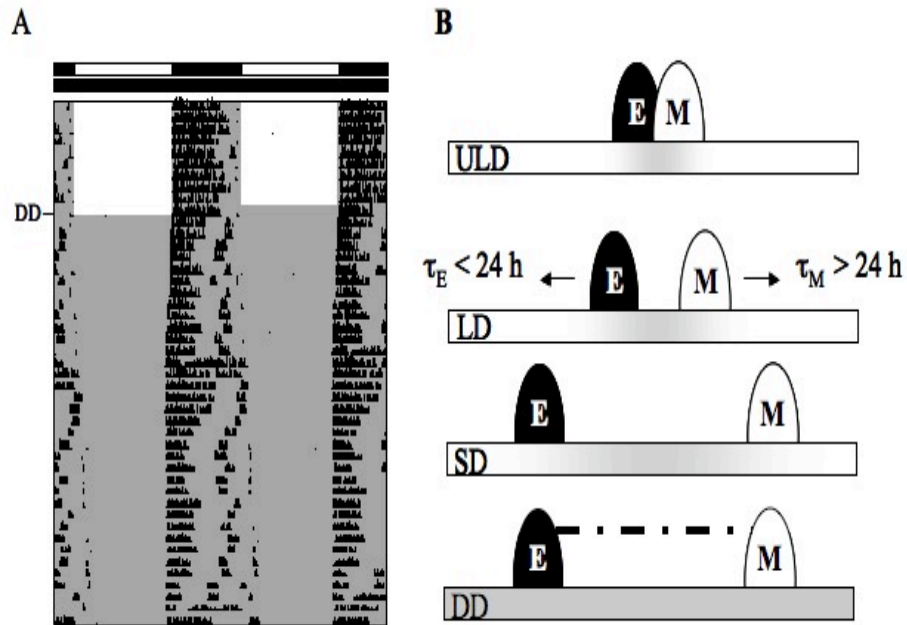


Figure 1.1: Photoperiodic regulation of circadian waveform and dual oscillator model of the central pacemaker. A). Representative double-plotted wheel running actogram of a hamster released into constant darkness from entrainment. White and shaded bars above the actogram represent the light and dark conditions, respectively and are referred to as light:dark bars in subsequent figures. Dark phases are also represented by the internal shading within the actogram. B). According to the dual oscillator model, adjustments in the duration of subjective night, as measured by activity duration (α), reflect changes in the phase relation (ψ_{EM}) between evening (E) and morning (M) oscillators. After transfer from long day photoperiods (LD) to short day photoperiods (SD), inherent differences in free-running period (τ) cause E and M to drift apart, producing increases in activity duration. After chronic exposure to constant dark conditions (DD), ψ_{EM} and circadian waveform stabilize due to interactions between E and M (dashed line). ULD: Ultra Long Day photoperiod, LD: Long Day photoperiod, SD: Short Day photoperiod, DD: Constant Darkness.

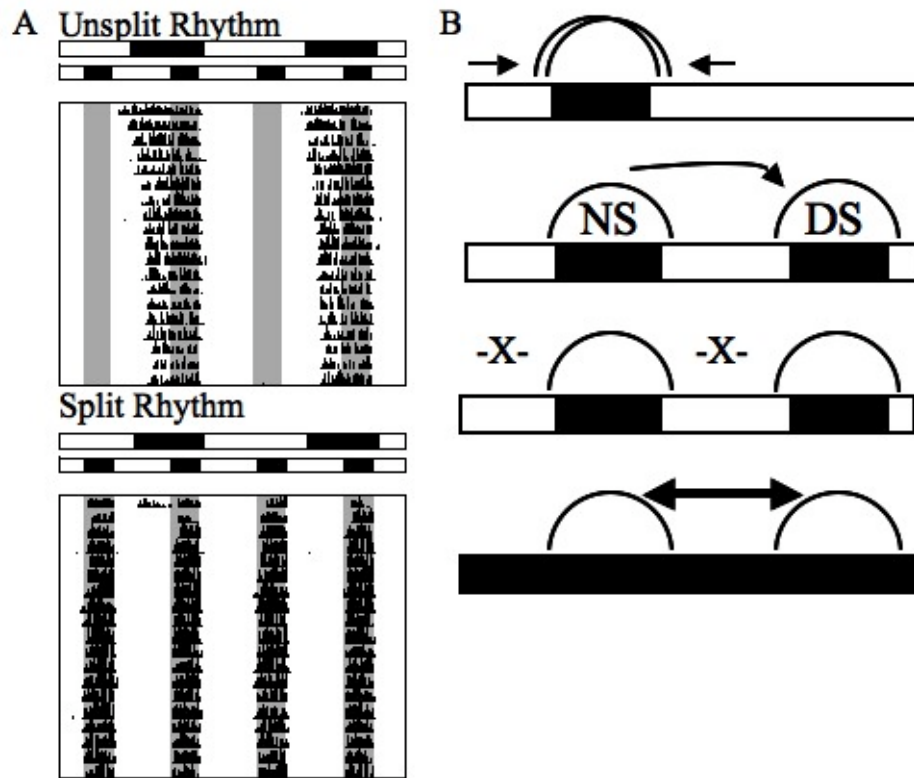


Figure 1.2: Split rhythms and conceptual model for understanding the interactions between oscillators split under 24 h light:dark:light:dark cycles (LDLD). A) Representative double-plotted wheel running actograms of hamsters displaying unsplit (top) and split rhythms (bottom). Light:dark bars above each actogram represent the lighting conditions in place prior to (top bars) and during the experiment (bottom bars). B) Conceptual model of oscillators split under LDLD. An oscillator is illustrated as the upper crest of a sine wave, and only two are represented for clarity. Entrainment to ultra long day photoperiods compresses the duration of subjective night by reducing the phase angle between underlying oscillators. Exposure to LDLD causes a subset of oscillators to re-entrain to the daytime scotophase (DS). The remainder of oscillators remains entrained to the nighttime scotophase (NS) and the split state is maintained under LDLD via an inhibition of interactions that would otherwise cause the system to rejoin like after release into constant dark conditions.

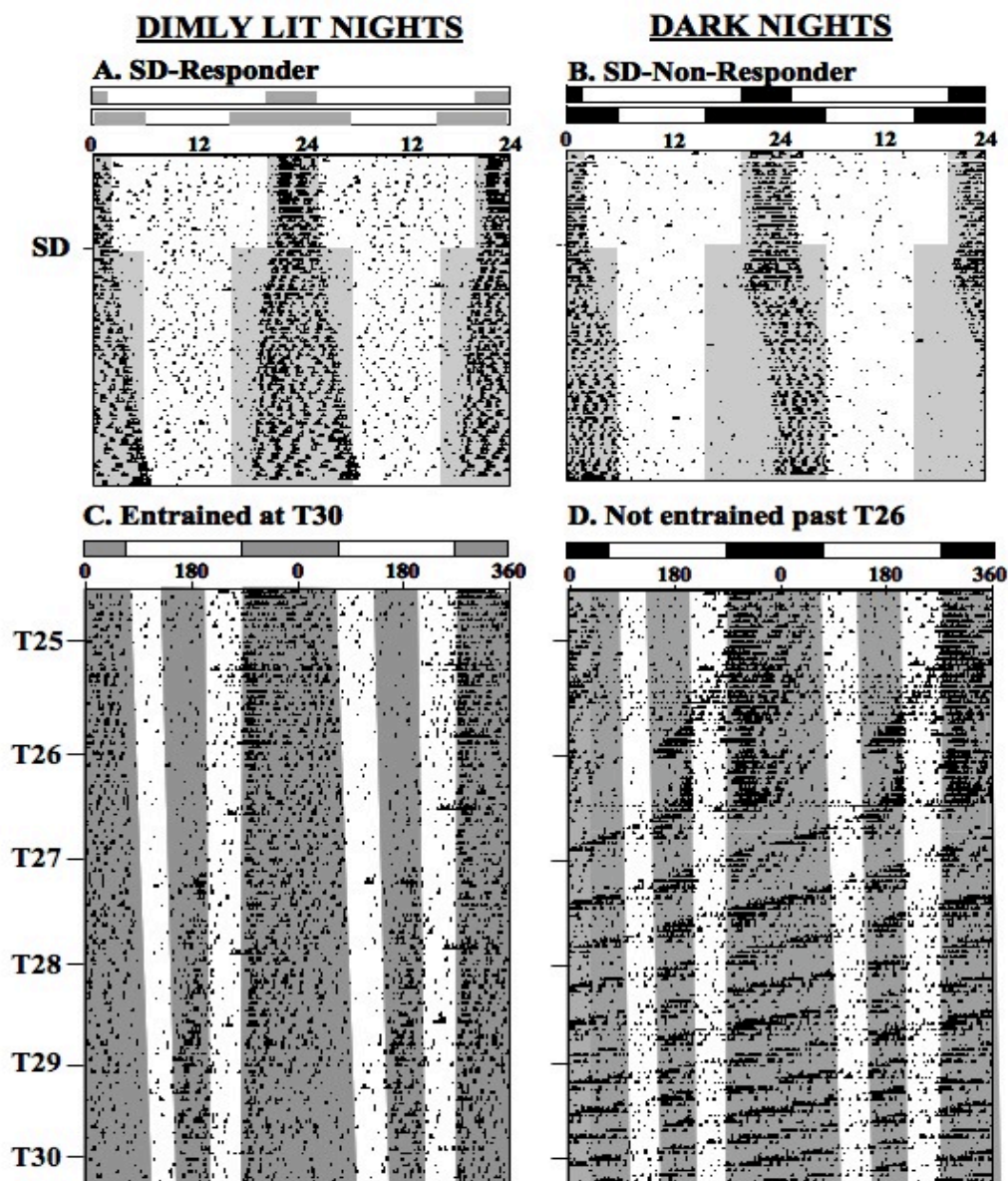


Figure 1.3: Effects of dim nighttime illumination on circadian entrainment. Representative double-plotted actograms from hamsters entrained to conditions with dimly lit nights (left) or completely dark nights (right). Black bars within light:dark bars above each actogram indicate complete darkness while gray bars represent dim light. A-B) Male Siberian hamsters were transferred from a long day photoperiod to a short day photoperiod (SD). Dimly lit nights promote the rapid increase in the duration of behavioral activity. E-F) Male Syrian hamsters held under lengthening T cycles. T-cycle length indicated to the left of leftmost actogram. Actograms are plotted by angular degree rather than h due to changing cycle length. Loss of entrainment is indicated by a break from the vertical alignment of activity onsets.

Chapter 2

Experiment 1: Circadian responses to dim light pulses and dimly lit conditions

The present studies assess which of three non-exclusive hypotheses may account for the potency of dim illumination under entrained conditions (i.e., LDLD, T-cycles, and SD photoperiods). According to the first hypothesis, dim illumination acts through a hallmark circadian response to light (e.g., phase resetting or melatonin suppression) and some feature of this stimulus causes it to be a more potent zeitgeber than predicted from published fluence-response curves (e.g., its duration or wavelength). Second, dim light could influence re-entrainment by modulating the magnitude of phase resetting by stronger zeitgebers, such as bright light or nonphotic stimuli. Alternatively, dim light may alter an intrinsic property of the circadian pacemaker (e.g., period (τ), activity duration (α), or amplitude).

Since estimates of τ can be confounded by a history of phase resetting, the effects of constant dim light and complete darkness on free-running rhythms were assessed first. Manipulations designed to assess phase resetting to dim light, bright light, and nonphotic stimuli followed. Lastly, a separate group of animals was used to assess whether dim light pulses suppress pineal melatonin secretion.

General method

Animals and husbandry. Syrian hamsters (*Mesocricetus auratus*) were bred from stock originally purchased from Harlan (HsdHan; AURA, Indianapolis, IN).

Animals were group-housed without running wheels inside polypropylene cages (48 x 27 x 20 cm) located on open racks and raised under a 14 h light: 10 h dark cycle (14L:10D, lights on: 0300 PST, lights off: 1700 PST, photophase: 100-300 lux, scotophase: 0 lux). Ambient temperature was maintained at $22 \pm 2^\circ \text{C}$, and animals had ad libitum access to water and food (Purina Rodent Chow #5001, St Louis, MO).

Experiment 1A: Effects of dim light on free-running circadian rhythmicity

Methods and Procedures

At 8 weeks of age, male Syrian hamsters ($N = 59$) were transferred to individual polypropylene cages (48 x 27 x 20 cm) located within light-tight environmental chambers (9-10 cages/chamber). Each cage contained a 17 cm diameter running-wheel with an opaque, plastic guard woven through the rungs to prevent injuries associated with running. Half revolutions of home cage wheels triggered closures of a magnetic reed switch, which were recorded and compiled into 6 min bins by VitalView software (Mini-Mitter, Sun River, OR). Actograms were prepared and analyzed using ClockLab software (Actimetrics, Evanston, IL) for experiments in the present and subsequent chapters.

Under entrained conditions, photophase light intensity was ~ 100 lux, and scotophases were dimly lit with green LEDs (12V, Product#LH1049-3702, Arcolectric, Thousand Palms, CA) mounted externally and facing the back wall of each cage. These LEDs emit a peak transmission λ of 560 nm, with a half maximum

bandwidth of 23 nm (Ocean Optics PS1000 spectrometer; Dunedin, FL). LEDs were outfitted with neutral density filters to approximate irradiances used in previously published studies. Dim illumination was measured with the photometer sensor (IL1700 Radiometer system, International Light, Newburyport, MA) placed within the running-wheel and oriented towards the LED to estimate dim light levels experienced by hamsters while active in the brightest area of the home cage. Average luminance across positions within an environmental chamber was 0.01 ± 0.001 lux, which is equivalent to an irradiance of 1.3×10^{-6} $\mu\text{W}/\text{cm}^2$ and a photon flux of 3.7×10^9 photons/ cm^2 sec. Previously reported facultative effects of dim nighttime illumination on LDLD-induced splitting (Evans et al., 2005; Gorman & Elliott, 2004; Gorman et al., 2003) were replicated under the present conditions (DIM nights: 8/8 animals split, DARK nights: 2/8 animals split; Fisher's Exact Test $p < 0.005$, data not shown).

Because prior studies indicated that the effects of dim light may depend on photoperiodic history (Evans et al., 2005), animals were pre-entrained to either a Long Day photoperiod (LD; 14L:10D, lights-on: 0300, $n = 29$) or a Short Day photoperiod (SD; 8L:16D, lights-on: 0600, $n = 30$). After five weeks under LD and SD, the house lights in each environmental chamber were permanently extinguished at the lights-off transition (zeitgeber time 12, ZT12), as illustrated in Figure 2.1. Additionally, scotophase illumination was either extinguished at ZT12 (DARK; $n = 20$ /photoperiod) or retained (DIM; $n = 9-10$ /photoperiod). Cage changes were scheduled at 2-3 wk intervals for specific circadian times (c.f., Experiment 1B) and performed with the aid of a dim red headlamp (exposure < 2 min/animal). Animals remained relatively undisturbed for at least

6 weeks after release from entrainment, after which DARK animals received 2 h dim light pulses during Weeks 6-13 of constant conditions (c.f., Experiment 1B).

Data Collection and Analyses

τ was measured by the slope of a regression line fit to 5-7 consecutive activity onsets, excluding the first 4 days after a cage change. Activity onset was defined each day as the first 6 min bin above a threshold value of 15 counts, preceded by at least one hour of inactivity and followed immediately by two consecutive bins above threshold. Activity offset was determined by a similar but opposite rule. α was calculated each day as the difference between activity offset and onset, and the median α for each animal for every week of analysis was recorded. Analyses using mean α and maximum α yielded similar results as analyses using median α . Total number of wheel revolutions per circadian cycle (WR/cycle) was also quantified for each week of analysis. Further analyses designed to examine differences in the distribution of activity were conducted by assessing the number of activity bouts per circadian cycle (Bouts/cycle) during Weeks 2 and 12 under constant conditions. Using day-to-day bout analyses (ClockLab), distinct bouts were defined as episodes of wheel-running activity surpassing 15 counts, lasting at least 30 min (5 bins), and separated by more than 60 min (10 bins) of subthreshold activity.

Continuously varying circadian measures under free-running conditions were assessed using repeated measures ANOVA with Bonferroni post hoc tests (Factors: Time

under constant conditions (Time), Scotopic condition (SC), Photoperiodic pre-treatment (PP), Time*SC, Time*PP, Time*SC*PP).

Results

Free-running period. During each week under constant conditions, ~75% of DARK animals exhibited $\tau < 24$ h while ~75% of DIM animals exhibited $\tau > 24$ h. Relative to DARK cohorts, DIM increased τ in SD animals by Week 2, and in LD animals by Week 4 (Figure 2.2). By Week 6 under constant conditions, DIM had lengthened τ by ~0.2 h relative to DARK. Constant DIM continued to lengthen τ over subsequent weeks, while τ remained essentially unchanged in DARK animals exposed to intermittent 2 h dim light pulses (c.f., Experiment 1B). Photoperiodic pretreatment did not significantly influence τ , nor did it interact with any other factor.

Circadian waveform. For the first two weeks after release from entrainment, SD animals had longer α than LD animals, regardless of whether constant conditions were DIM or DARK (Figure 2.2). Over subsequent weeks, DIM-LD and DARK-LD animals lengthened α by ~4 h and ~1.5 h, respectively (Figure 2.3, inset top). Unexpectedly, the long α produced by SD was maintained under DIM, but not under DARK conditions (Figure 2.2, inset bottom). DIM-SD animals increased α by ~1.5 h from initial free running values, whereas DARK-SD animals decreased α by ~2 h. Consequently, α values corresponded to scotopic condition and not photoperiodic history after six weeks under constant DIM and DARK conditions.

Wheel-running amplitude. Release from entrainment led to marked decreases in wheel running (WR/cycle), which was influenced thereafter by photoperiodic history and not scotopic condition (Figure 2.2). For the first few weeks after release, WR/cycle was lower for LD groups than SD groups. After ~10 weeks under constant conditions, SD animals then increased WR/cycle, likely in response to gonadal recrudescence. LD animals similarly increased WR/cycle ~5 weeks later (data not shown).

Distribution of activity. Bout analyses characterizing the distribution of activity under constant conditions indicate that DIM promotes the fragmentation of the active phase (Figure 2.3). At Week 12, hamsters under DIM displayed more Bouts/cycle than their DARK counterparts (SC: $F(1,55) = 10.26$, $p < 0.005$) and there were no differences based on photoperiodic history (PP and SC*PP: $p > 0.1$). At Week 2, SD animals displayed more Bouts/cycle than LD animals, and DIM animals displayed more Bouts/cycle compared to DARK animals (PP: $F(1,55) = 27.63$, $p < 0.0001$; SC: $F(1, 55) = 7.8$, $p < 0.01$, SC*PP: $p > 0.3$). Background illumination thus altered the structure of the active phase before its duration, since the latter did not manifest until Week 4 of constant DIM.

Experiment 1B: Effects of dim light pulses and phase resetting under dimly lit background conditions

Methods and Procedures

Dim light resetting. To assess whether dim light is a strong zeitgeber, each free-running DARK animal from Experiment 1A was exposed to a 2 h dim light pulse once every 21 days during Weeks 6-13 of constant conditions (3 pulses/animal). Under the control of an external electronic timer, all LEDs in a given chamber were simultaneously powered for 2 h. These dim light pulses thus fell over a broad range of circadian phases since animals had been free running for at least six weeks. Two of four environmental chambers (one/photoperiod pre-treatment) were pulsed with dim light, and the remaining two chambers were left unpulsed as controls. 10-11 days later, this arrangement was reversed. Data from this light pulsing protocol were also used to assess whether dim illumination can suppress or augment wheel running (see below).

Nonphotic resetting. To study effects of dim illumination on nonphotic resetting, I calculated phase shifts to cage changes performed under DIM and DARK conditions during Experiment 1A (c.f., Figure 2.2). To minimize effects on free-running rhythms, initial cage changes (CC#1-3) were scheduled exclusively during subjective night. Subjective day data were obtained during CC#4-5.

Bright light-induced resetting. To assess whether dim light potentiates bright light-induced resetting, a photic phase response curve (PRC) to a 15 min, 100-lux light pulse was collected against a background condition of complete darkness or dim illumination. To avoid producing confounding changes in α and τ , animals were pretreated with dim light for only one week. One week was sufficient to observe marked effects of dim illumination in a prior study (Gorman et al., 2003). Seventeen weeks after release from entrainment, LEDs were re-powered for two of the four

DARK chambers (DARK/DIM, one/photoperiod pretreatment). After one week, DARK/DIM chambers and the remaining two DARK chambers were given bright light pulses. Hamsters within each chamber were pulsed simultaneously to sample the full circadian cycle, and then left undisturbed for one week. LEDs were extinguished for two weeks, and then this protocol was repeated with the reversed arrangement.

Seventeen weeks after release from entrainment, LEDs were permanently extinguished for DIM animals (DIM/DARK, $n = 20$) to assess whether changes in circadian rhythmicity under constant dim light conditions (c.f., Experiment 1A) were due to masking of the activity rhythm. Changes in τ , α , and WR/cycle were quantified for DIM/DARK, DARK/DIM, and DARK controls.

Data Collection and Analyses

Phase shifts in response to dim light, bright light, and nonphotic stimuli were calculated identically (Figure 2.4). A phase shift was determined for each animal by the displacement between regression lines fit to 5-7 consecutive activity onsets before and after the presentation of the stimulus, excluding the first 4 days to allow for transient cycles. Regression lines fit before and after stimulus presentation were also used to calculate τ during these periods and determine the magnitude of changes in τ . For phase and τ response curve analyses, CT of stimulus presentation was coded as a categorical variable in 4 h bins and time-gated changes in phase and τ were assessed with full factorial ANOVA (Factors: CT, SC, CT*SC). ANOVA analyses of phase resetting rhythms were supplemented with a recently developed PRC bisection test

that permits tests of PRC robustness and between-group comparisons of PRC amplitude (Kripke et al., 2003).

Paired t tests were used to test whether circadian rhythmicity was altered by the scotopic manipulations that occurred seventeen weeks after release from entrainment (e.g., DIM/DARK). To assess behavioral masking during 2 h dim light pulses during Weeks 6-13 of constant conditions, total wheel-running activity exhibited by each animal during the 2 h dim light pulse was subtracted from that expressed on the previous day at the same clock time. Change values were compared between pulsed and control groups using factorial ANOVA binned according to circadian time (CT, where CT12 is activity onset). A second test for behavioral masking was also performed, which normalizes individual differences in activity levels. Wheel running levels on the day before and of the dim light pulse were correlated with one another, and the residuals were then plotted by CT and assessed with factorial ANOVA. Identical analyses were performed for unpulsed controls.

Results

Dim light-induced PRC and τ RC. Exposure to 2 h dim light pulses yielded a statistically robust PRC (Figure 2.5A; PRC Bisection test, $p < 0.05$), with a 45 min difference between peak advances and delays. Significant phase advances were elicited during late subjective night (CT20-24: ANOVA LSM contrasts, $p < 0.05$), and the PRC for dim light pulses was similar in shape to that obtained with 15 min bright

light pulses (Figure 2.5B). Relative to unpulsed DARK controls, animals given 2 h dim light pulses did not display significant changes in τ (Figure 2.6A; $p > 0.1$).

Bright light-induced PRC and τ RC. Bright light PRCs collected against DIM and DARK conditions were both statistically significant (Figure 2.5B; PRC Bisection test, $p < 0.0001$ in both cases). Whether the background condition was completely dark or dimly lit did not significantly alter PRC amplitude or shape (PRC Bisection test, $p > 0.1$; ANOVA, $p > 0.2$). Bright light pulses also caused significant changes in τ (ANOVA, $p = 0.06$, CT: $F(5, 64) = 3.35$, $p < 0.01$); however, background condition did not influence the shape of the τ RC (Figure 2.6B; SC, CT*SC: $p > 0.15$).

Cage change-induced PRC and τ RC. Under both DIM and DARK, cage changes produced significant nonphotic PRCs (PRC Bisection test, $p < 0.01$ in both cases) characterized by large phase advances during subjective day (Figure 2.5C). PRC amplitude was not influenced by background condition (PRC bisection test, $p > 0.05$), although the shape of the nonphotic PRC was affected (SC*CT: $F(5,237) = 2.97$, $p < 0.05$). Cage changes conducted during late subjective day elicited significant phase advances from animals in DIM, but not DARK (CT9-12, ANOVA LSM contrasts, $p < 0.009$). Additionally, cage changes conducted during early subjective day tended to produce phase advances from animals in DARK, but not DIM (CT1-4, ANOVA LSM contrasts, $p < 0.03$), although this test did not meet the criteria for multiple comparisons. Periodic cage changes also produced significant changes in τ (ANOVA, $p < 0.05$, CT: $F(5, 273) = 5.1$, $p < 0.0005$), and the τ RC was modulated by background conditions (Figure 2.6C; SC: $F(1, 273) = 8.3$, $p < 0.05$). Cage changed

conducted during early subjective day increased τ in DIM animals more than in DARK animals (CT1-4, ANOVA LSM contrast, $p < 0.009$).

Behavioral masking to dim light pulses. Visual inspection of activity during 2 h dim light pulses did not reveal a robust change in wheel running levels (c.f., Figure 2.4). Relative to unpulsed controls, animals given 2 h dim light pulses did not display a significant change in wheel running levels relative to the preceding day. This result was obtained when analyses included all data (ANOVA, $p > 0.05$) or when analyses were restricted to 4 h CT bins during the active phase of the circadian cycle (ANOVA, $p > 0.1$). Wheel-running revolutions displayed by animals during 2 h dim light pulses were significantly correlated with those exhibited at the same time on the preceding day ($R^2 > 0.72$, $p < 0.0001$; Day of Pulse = $124 + 0.75\text{DayBefore}$), as were activity levels displayed by unpulsed controls ($R^2 > 0.92$, $p < 0.0001$; Day of Pulse = $27 + 0.99\text{DayBefore}$). In the linear regression model for pulsed animals, but not controls, the slope is significantly less than one, which was caused by an increase in wheel running during the 2 h dim light pulse in animals with low wheel-running levels on the preceding day. To assess phase-dependent changes in activity levels, the residuals of the model for pulsed animals were plotted by the CT of the 2 h dim pulse. The residual error did not vary significantly across the circadian cycle ($\chi^2(1) = 5.9$, $p > 0.3$), even when these analyses were restricted to the active phase during subjective night ($\chi^2(1) = 1.6$, $p > 0.4$). Thus, even with this second test of behavioral masking, there was little evidence that 2 h dim light pulses influenced activity levels.

Responses to acute changes in scotopic condition. Extinguishing LEDs after seventeen weeks under DIM did not significantly decrease τ or α , or alter WR/cycle (Figure 2.7). Similarly, the week of dim light provided to DARK animals did not significantly alter any of these parameters (Figure 2.7). Thus, masking did not produce DIM-induced changes in free-running rhythms but instead these effects reflect intrinsic changes in the circadian pacemaker itself.

Experiment 1C: Melatonin suppression in response to dim light pulses

Methods and Procedures

Group-housed, female hamsters (N = 63, 8 wks of age) were weaned and gradually re-entrained to a reversed 14L:10D cycle (lights-on: 2000; photophase illumination: > 100 lux, scotophase illumination: 0 lux). Cage changes occurred once a week during the photophase. After four weeks under the reversed 14L:10D cycle, the completely dark scotophase (DARK-) was either retained, or LEDs were powered to provide dim illumination (DIM-) on a single night (Figure 2.8). Blood samples were collected at two points during the scotophase (ZT17 and ZT20), and thus, DIM animals received either a 5 h or 8 h dim light pulse (DIM-5h and DIM-8h, respectively; n = 12-16/ZT). Three weeks later, this protocol was repeated in a counterbalanced manner. Additionally, a separate group of animals received a 2 h dim light pulse from ZT 18-20 (DIM-2h; n = 9). Three weeks later, a subset of DIM- and DARK- animals received a 2 h, 100-lux light pulse (n = 8/ZT) as a positive control for melatonin suppression.

Data Collection and Analyses

Before blood collection, animals were anesthetized with sodium-pentobarbital (55 mg/kg), which was supplemented with isoflurane. Plasma samples were collected via retinal-orbital bleeds using heparinized caraway micro blood collecting tubes (Fisherbrand) and transferred to test tubes containing 50 μ L of heparin (1000 units/mL). Melatonin concentration was measured using radioimmunoassay (RIA) kits, employing an enzymatic pretreatment step (Buhlmann Melatonin Direct RIA, ALPCO, Ltd., Windham, NH). Experimental samples, controls and standard curve calibrators were incubated with the anti-melatonin antibody and 125 I-melatonin for 20 h at 2-8° C. Second antibody was added before a 15-min incubation at 2-8° C. After centrifugation at 18-28° C, the unbound supernatant was discarded, and the antibody-bound precipitate was counted via Gamma-counter (Titertek Instruments, Inc., Huntsville, AL). Lastly, immunoassay curve-fitting software (Isodata Software; Titertek Instruments, Inc.) used the standard curves to calculate a best fitting smoothed curve from which sample potency estimates (pg/ml) were obtained algebraically. Kuskall-Wallis nonparametric tests were used to compare DIM and DARK melatonin levels at each ZT. Posthoc nonparametric tests were also used to compare melatonin levels after 2 h light pulses to DARK controls at the corresponding ZT.

Results

During the first round of blood collection, 8 h DIM light pulses significantly suppressed melatonin secretion relative to levels exhibited by DARK controls at ZT20 (Figure 2.9; $\chi^2(1) = 4.6$, $p < 0.05$). At ZT17, melatonin levels were lower than at ZT20 and were not significantly different under DIM and DARK conditions ($\chi^2(1) = 1.6$, $p > 0.1$).

2 h bright pulses delivered at the end of the experiment yielded low melatonin values at ZT20, which was significantly lower than levels displayed by DARK-ZT20 animals during each earlier round of collection (Figure 2.9; post-hoc comparison, $\chi^2(2) = 9.8$, $p < 0.01$). In contrast, melatonin levels after 2 h bright light pulses delivered at ZT17 were significantly lower than levels displayed by DARK-ZT17 animals during the second (Figure 2.9; post-hoc comparison, $\chi^2(1) = 15.6$, $p < 0.0001$), but not the first round of collection (Figure 2.9, post-hoc comparison, $p > 0.05$).

During the second round of blood collection, DARK controls at ZT17 displayed an increase in melatonin levels relative to those displayed three weeks earlier (Figure 2.10), whereas melatonin levels at ZT20 collected from DARK controls during each round did not differ from one another. Since the increase in melatonin levels during the second round of blood collection interacted with the order of the scotopic conditions (Figure 2.10), a matched t test was performed for each group. Animals that were provided 5 h or 8 h DIM pulses during the first round of collection displayed an increase in melatonin levels while serving as DARK controls during the second round of collection (Figure 2.10). In contrast, animals that served as DARK

controls during the first round of collection did not display an increase in melatonin levels after the 5 h or 8 h DIM light pulse during the second round of collection.

During the second round of blood collection, neither 8 h nor 5 h DIM light pulses significantly suppressed melatonin secretion relative to levels exhibited by DARK controls (Figure 2.9, $p > 0.1$). After 2 h DIM light pulses, melatonin levels showed no evidence of suppression and were not different from DARK controls sampled during the second round of blood collection ($\chi^2(1) = 0.15$, $p > 0.5$). The within subject comparisons suggest that detecting the melatonin suppressing effect of DIM light pulses during the second round of blood collection was inhibited by the nonspecific increase in melatonin levels at this later time in the experiment.

Discussion

The present studies confirm that dim light is only a very weak zeitgeber when given on its own, producing at most a 30 min phase advance with a 2 h pulse. After dim light pulses of more standard duration (e.g., 0.25 h) the phase shift would be expected to be non-detectable. Indeed, 1 h pulses failed to produce statistically significant changes in phase (Evans, unpublished observations). Further, dimly lit versus completely dark background conditions do not directly augment the phase-shifting capacities of bright light or cage changes. Instead, constant dim light and darkness cause the circadian pacemaker to exhibit markedly different free-running properties. As discussed further below, these latter actions provide insight into

potential mechanisms underlying the effects of dim illumination under entrained conditions.

Circadian phase resetting exhibits a monotonic dependence on light intensity that is generally well described by a sigmoid-shaped function in a log-linear plot (Nelson & Takahashi, 1991a). Using monochromatic light of 503 nm, Nelson and Takahashi (Nelson & Takahashi, 1991b) estimate the threshold for phase resetting in the nocturnal hamster at 10^{11} photons/cm²sec, a value 27 times greater than the irradiance used here. Accounting for the fact that phase-shifting mechanisms are roughly 8 times more sensitive to photons of ~503 nm than ~560 nm (Takahashi et al., 1984), the effective irradiance of this dim light can be calculated as ~2.3 log units below their estimated threshold. However, the definition of absolute sensitivity can be difficult without large sample sizes that would distinguish between small and null effects. A complementary approach for comparing the present stimulus with the published fluence-response curves is to extract, from Naka-Rushton equations, the irradiance predicted to produce phase shifts equivalent to those presently elicited by dim light (i.e., 30 min phase advances). With 5 min pulses of 500 nm light, an irradiance of 7.5×10^{10} photons/cm²sec is required (Nelson & Takahashi, 1991b). Again accounting for its longer wavelength, this dim light stimulus has an effective irradiance 2.2 log units lower. With perfect temporal summation of photons, the long duration of these dim light pulses (2 h) could account for as much as 1.4 log units of this discrepancy, although previous work demonstrates that photons are less efficiently summated over 1 h than over 5 min (Nelson & Takahashi, 1991b, 1999). The remaining discrepancy may relate to the longer time that animals in the present study were kept in

constant conditions before being pulsed with light, since larger phase shifts are commonly observed in this species after prolonged exposure to constant darkness (Pittendrigh et al., 1984; Shimomura & Menaker, 1994). Additional methodological differences between present and previous reports (e.g., pulsing procedures and light sources) may also temper absolute comparisons across studies.

Light-induced melatonin suppression is likewise characterized by a sigmoidal relationship between light intensity and response magnitude but this curve is shifted to lower irradiances than that for phase resetting (Nelson & Takahashi, 1991a). In the present study, melatonin secretion was suppressed by 50% after 8 h of dim light with photon flux approximately 1/3 of that required to suppress melatonin by a similar amount with 5 min, 503 nm pulses. Once again accounting for the 8-fold lower efficacy of the longer wavelength stimulus used here, the effective irradiance is 1.4 log units lower. This discrepancy is reasonably accounted for by the much longer pulses used here (90 fold), even given a marked reduction in temporal summation during long light pulses (Dkhissi-Benyahya et al., 2000; Nelson & Takahashi, 1991b). The very low amplitude PRC and modest melatonin suppression in response to dim light pulses thus afford little direct insight into why animals entrain so differently in dimly lit versus completely dark nights. Indeed, any melatonin suppression accomplished by dim nighttime lighting is insufficient to disrupt the interpretation of the melatonin signal, since dimly lit nights do not compromise gonadal regression in Siberian hamsters held under short day lengths (Gorman & Elliott, 2004).

In previous studies reporting large effects of dim light versus darkness, hamsters were also regularly exposed to bright light and, by necessity, periodic cage changes, raising the possibility that the potency of dim illumination depended on an interaction with these other well-studied zeitgebers (Evans et al., 2005; Gorman & Elliott, 2004; Gorman et al., 2005). The complete bright light and cage-changing PRCs reveal that the amplitude of phase resetting to each zeitgeber is not differentially affected by dimly lit and completely dark background conditions (c.f., Figures 2.5), and only the cage-changing curve showed any change in shape. This latter effect may represent a delay in the phase of subjective day rather than an absolute change in the shape of the nonphotic PRC. Nevertheless, dim illumination does not directly increase the amplitude of inputs for bright light and nonphotic stimuli, and thus, the potentiation hypothesis does not provide a general explanation for the effects of dim illumination observed under entrained conditions. Similarly, providing a background of dim illumination did not significantly alter the τ RC for bright light pulses, although it did modulate the τ RC for cage changes.

In nocturnal rodents, constant light increases τ and decreases α , relationships commonly referred to as Aschoff's first and second rules (Aschoff, 1960, 1979; Pittendrigh, 1960). Consistent with this first "rule", a rule to which Aschoff himself reported variances at low light levels (Aschoff, 1979), constant dim light lengthened τ relative to complete darkness by 0.3 h. As discussed in previous reports, a slight increase in τ by itself is insufficient to account for the ~ 4 h increase in the upper limit of entrainment, (Gorman et al., 2005), enhanced entrainment to short T-cycles (Chiesa

et al., 2005), or accelerated short day re-entrainment (Gorman & Elliott, 2004).

Contrary to Aschoff's second rule, which is based on fewer studies, constant dim illumination increased α by 3 h, and this proved to be a robust effect maintained over many weeks despite periodic cage changes and dim light pulses. Thus, DIM and DARK conditions produced different steady states, and despite initial differences in α , LD and SD groups converged to common values determined by lighting condition- with α even decreasing in SD animals released into constant darkness.

Previous studies have shown that dim light can increase activity levels (Mrosovsky, 1999b), but I found no evidence that the effect of dim light on α was produced by positive masking. First, 2 h pulses and constant dim illumination did not increase wheel revolutions relative to animals held under dark conditions. Further, masking would not account for the gradual increase in α under constant conditions, and α did not decrease during the week after the constant dim light was extinguished. Lastly, analyses of wheel-running patterns demonstrate that the longer α under dimly lit conditions stems from the redistribution of activity rather than an increase in activity levels. Collectively, these results indicate that masking does not account for the effects of dim light on circadian waveform under entrained or free-running conditions.

This basic change in circadian waveform helps us to understand effects of dim nighttime illumination under entrained conditions. For example, the amplitude of the bright light PRC is proportional to α (Pittendrigh et al., 1984). Therefore, animals held in DIM long enough for α to increase would be expected to have expanded limits

of entrainment, as previously reported (Chiesa et al., 2005; Gorman et al., 2005). In this manner, dim light may affect entrainment to bright light regimens without itself being a strong zeitgeber or direct potentiator of bright light. When scotopic condition is manipulated for only a short period of time (e.g., a week as in Experiment 1B), α is not yet altered (DIM: 9.08 ± 0.32 h, DARK: 10.54 ± 0.32 h) and these groups display similarities in the amplitude and shape of the bright light PRC. Empirical studies where α is increased through longer exposure to dim illumination will be critical in assessing whether an increase in the bright light PRC can account for the increase in the upper limit of entrainment in this species. If the effect of dim light on α can be generalized to other species, this may also explain the accelerated response to short photoperiods in *Phodopus*. This finding, however, does not suggest an immediate explanation for the increased incidence of split rhythms of both species in LDLD (see below).

How does prolonged dim illumination increase α ? To account for photoperiodic effects on α , Pittendrigh and Daan (Pittendrigh & Daan, 1976b, 1976c) posit the existence of “Evening” and “Morning” oscillators with different intrinsic free-running periods. According to the dual oscillator model, the phase relation between these oscillators determines the length of subjective night (c.f., Figure 1.1). But wholly independent oscillators with different intrinsic periods would be expected to drift in and out of phase with one another over time in constant conditions. Some manner of coupling is therefore needed to account for the fact that a stable long α typically develops in constant darkness and no “criss-crossing” of activity components

generally occurs (but see, (Meijer et al., 1990). If the steady state α is indeed a reflection of oscillator coupling, then differences in α produced by dim illumination may result from altered oscillator coupling.

An effect on circadian coupling might also be inferred from the splitting of activity rhythms under LDLD (Evans et al., 2005; Gorman & Elliott, 2004; Gorman et al., 2003). Indeed, the case for coupling is most strongly made by demonstrating convergent actions of dim light in multiple experimental paradigms for which coupling has been invoked (Gorman et al., 2006). Considering no direct assay of circadian coupling has been developed yet, one approach for assessing whether dim illumination influences the coupling between circadian oscillators is to investigate the role of dim light under behavioral paradigms where changes in circadian waveform reflect interactions between oscillators.

LDLD-induced splitting presents a unique opportunity to study the putative influence of dim illumination on circadian coupling mechanisms. First, the effect of dim nighttime illumination under LDLD cannot be accounted for by alternative explanations that do not involve changes in the interactions between underlying oscillators (e.g., effect on τ , or bright light induced phase resetting). Additionally, dim light-induced changes in circadian coupling may influence the response to LDLD in several discrete ways (c.f., Figure 1.2). Specifically, dim illumination could influence LDLD-induced splitting by altering the interactions that promote the emergence, the maintenance, and the fusion of LDLD-induced split rhythms.

The text of Chapter 2 comprises material published in the Journal of Biological Rhythms: Evans, Jennifer A.; Elliott, Jeffery A.; & Gorman, Michael R. Circadian effects of light no brighter than moonlight, J Biol Rhythms, in press. I was the primary investigator and first author of this paper. My co-authors listed in these publications directed and supervised the research that forms the basis for these chapters, and both have granted permission for its use.

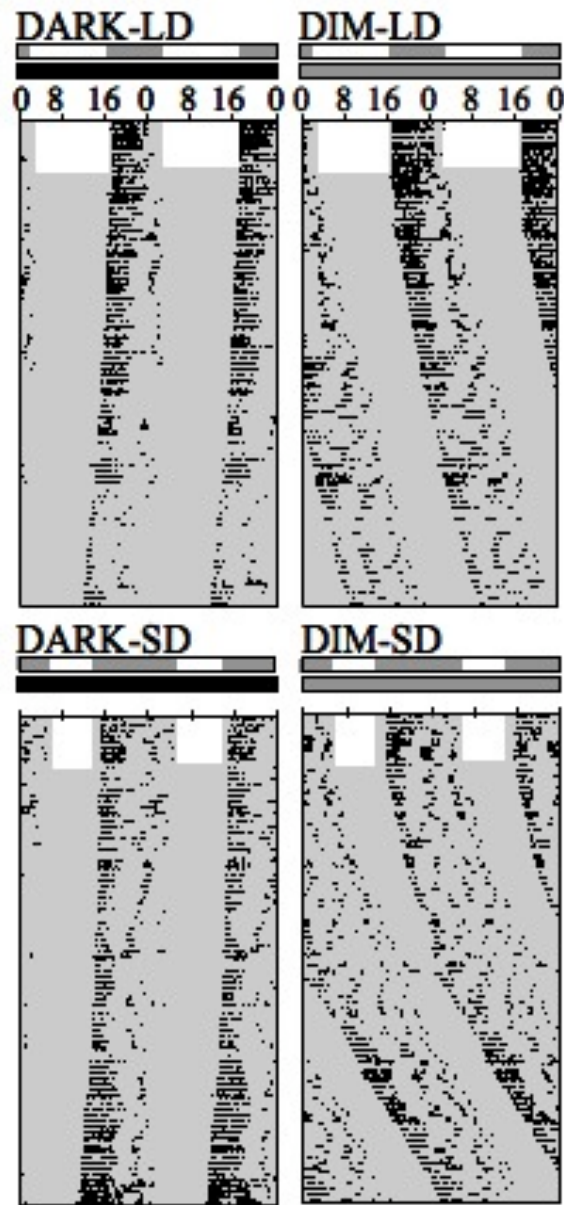


Figure 2.1: Representative double-plotted actograms for free-running rhythms under constant dim illumination (DIM) or complete darkness (DARK). Animals were entrained to Long Day (LD) or Short Day (SD) photoperiods, then released into either DIM or DARK conditions. White and shaded bars above each actogram illustrate the lighting conditions in place during entrainment (top) and after release into constant conditions (bottom). Shading within each actogram depicts the change in lighting conditions. Note only the last two weeks of entrainment are shown. Other conventions as in Figure 1.1.

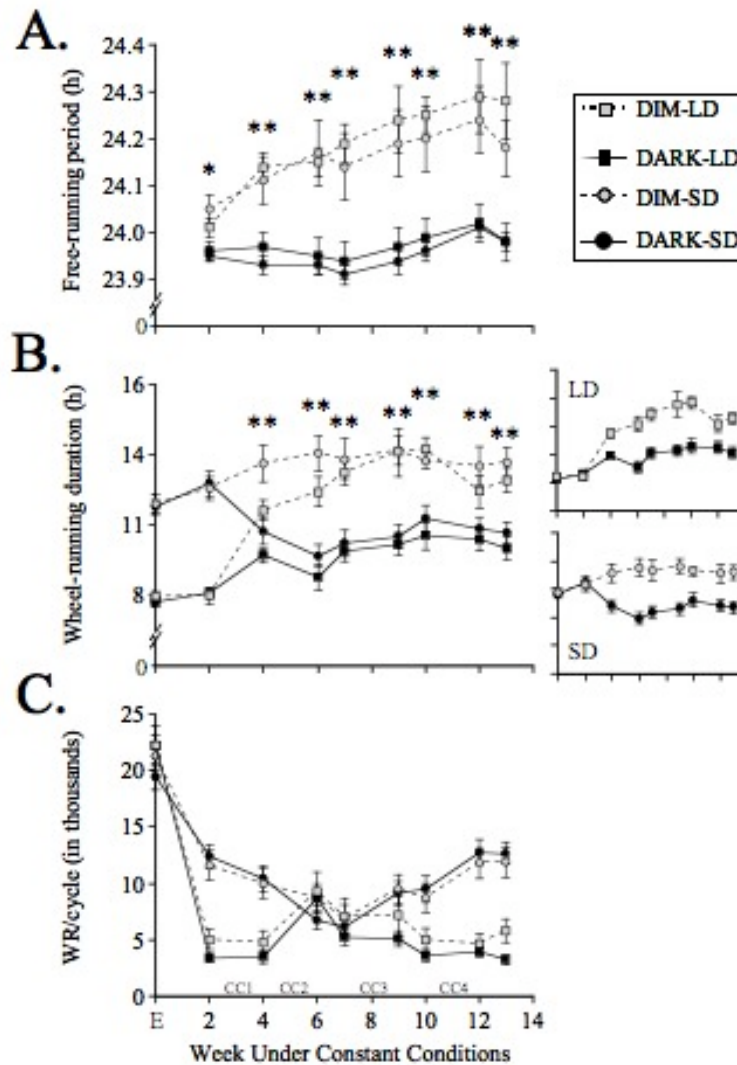


Figure 2.2: Free-running rhythmicity under constant dim illumination (DIM) or complete darkness (DARK). Free-running period (τ), activity duration (α), and wheel revolutions per cycle (WR/cycle). τ and α were influenced by scotopic condition (SC) and Time after release into constant conditions (Time), whereas WR/cycle was affected by photoperiodic pretreatment (PP) and Time. B) τ : SC- F (1, 45) = 38.8, Time- F (8, 38) = 6.5, SC*Time- F (8, 36) = 4.2, $p < 0.001$; PP-, SC*PP-, PP*Time-, SC*PP*Time-, $p > 0.1$. C) α : SC- F (1, 52) = 35.1, PP- F (1, 52) = 11.3, PP*Time- F (10, 43) = 11.1, SC*Time- F (10, 43) = 8.0, Time- F (10, 43) = 5.3, $p < 0.001$; SC*PP, SC*PP*Time, $p > 0.8$. D) WR/cycle: PP- F (1, 54) = 22.5, T- F (8, 47) = 22.1, PP*T- F (8, 47) = 11.3, $p < 0.0001$; SC*PP*T- F (8, 47) = 2.7, $p < 0.05$; SC, SC*PP, SC*T, $p > 0.1$). On the abscissa: E is the last week of entrainment and CC# is the cage change number since release into constant conditions. * DIM-SD versus DARK-SD, $p < 0.005$. ** DIM versus DARK for both photoperiodic groups, $p < 0.005$.

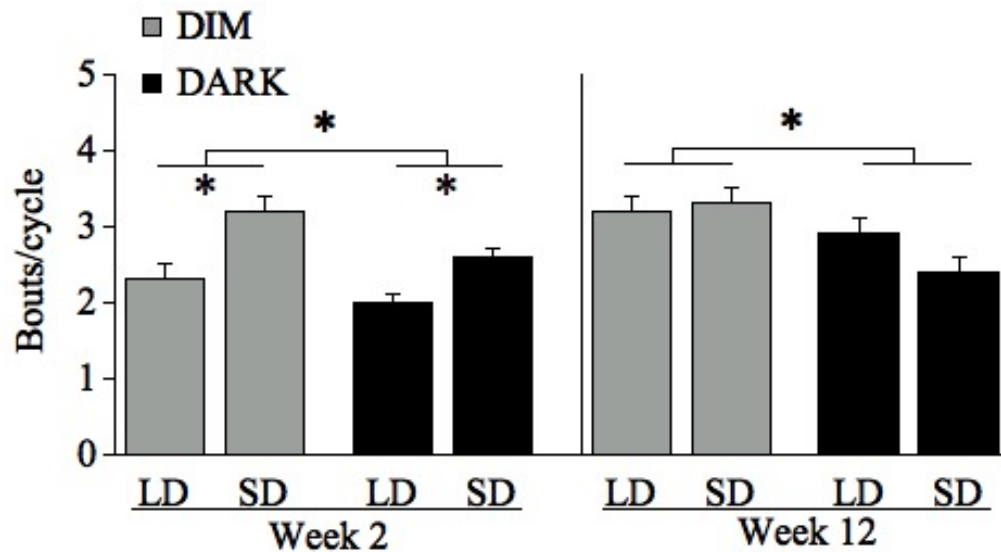


Figure 2.3: Mean (\pm SEM) number of activity bouts displayed per circadian cycle (Bouts/cycle), either 2 or 12 weeks after release from LD or SD into constant conditions. During Week 2 of constant conditions, Bouts/cycle were greatest in SD and DIM animals (PP: $F(1,55) = 27.6$, $p < 0.0001$; SC: $F(1,55) = 7.8$, $p < 0.01$; SC*PP, $p > 0.3$). During Week 12, Bouts/cycle were greatest in animals under DIM conditions (SC: $F(1,55) = 10.26$, $p < 0.01$; PP, SC*PP, $p > 0.1$). * $p < 0.01$.

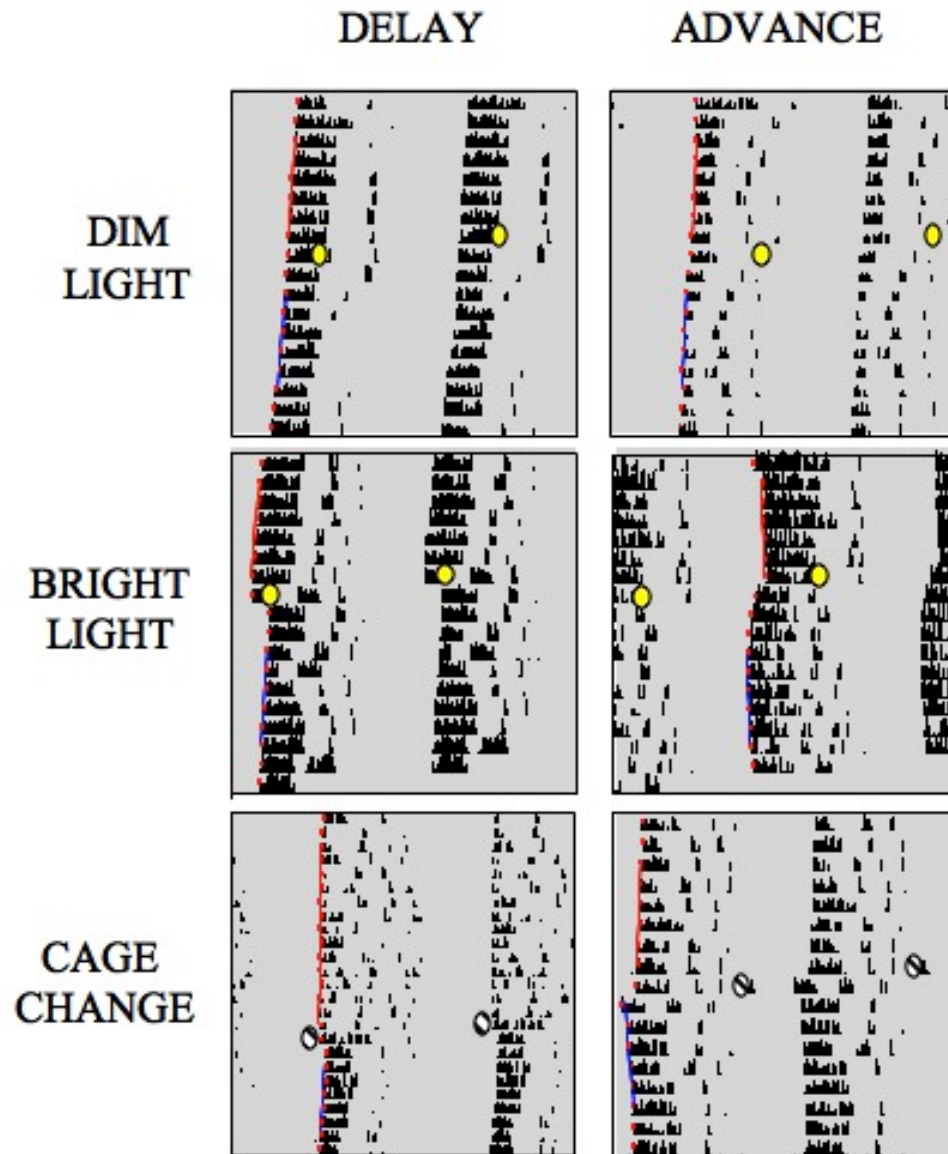


Figure 2.4: Representative double-plotted actograms for phase shifts caused by 2 h dim light pulses (top), 5 min bright light (100 lux) pulses (middle), or cage changes (bottom). Both phase delays (left column) and phase advances (right column) are represented. Red and blue lines are linear regression lines fit to pre- and post-pulse activity onsets, respectively. The large circle embedded within each actogram depicts the timing of zeitgeber presentation. Note this circle is not drawn to scale and is enlarged for clarity.

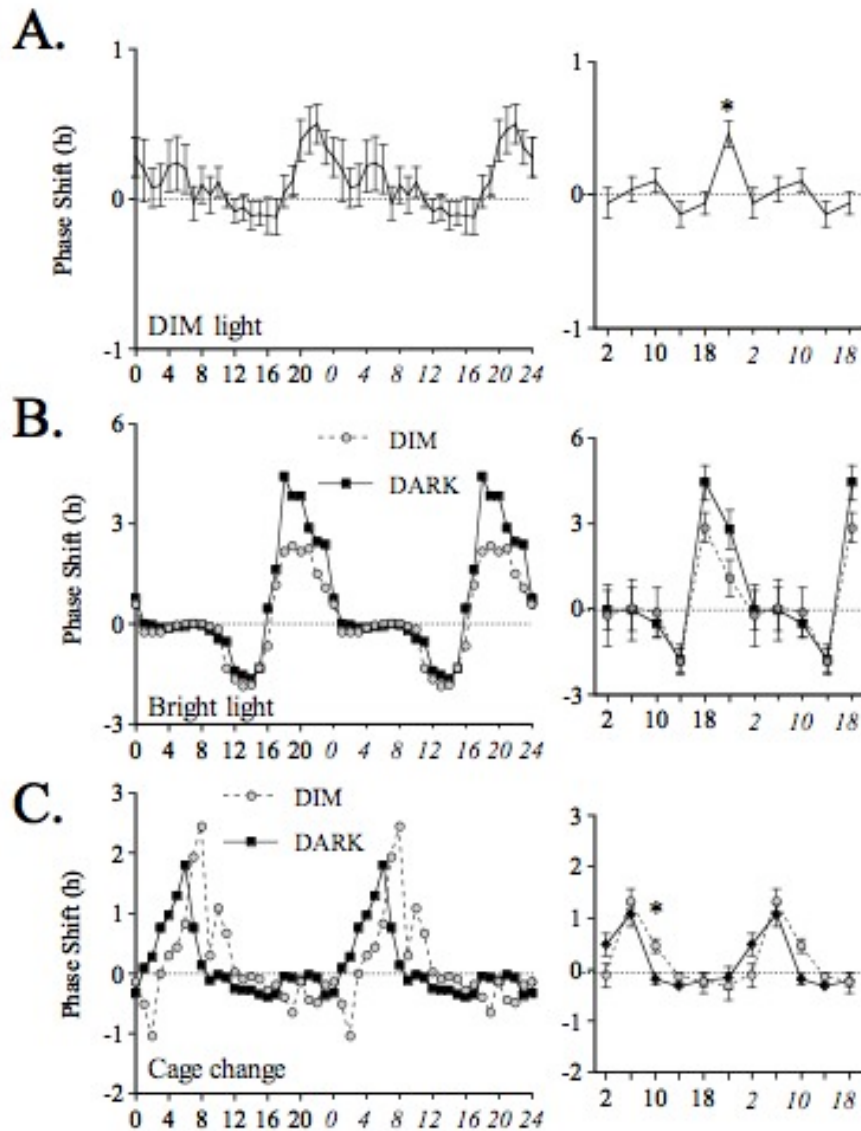


Figure 2.5: Phase response curves (PRC) for photic and nonphotic stimuli as drawn with a 3h moving average (left) or as grouped in 4 h bins for quantitative analyses (right). A) PRC for 2 h dim light pulses provided against complete darkness. Stippled line at zero represents mean values of controls maintained in darkness. B) Bright light PRC for 5 min, 100 lux light pulse collected against DARK or DIM background conditions. Prior to bright light pulses, animals were exposed to dim illumination for one week. Note the difference in the scale of the y-axis relative to the DIM light PRC. C) Nonphotic PRC for cage changes performed under constant DIM and DARK conditions once every 2-3 weeks. Error bars have been removed from smoothed bright light and nonphotic PRCs for clarity. * $p < 0.009$.

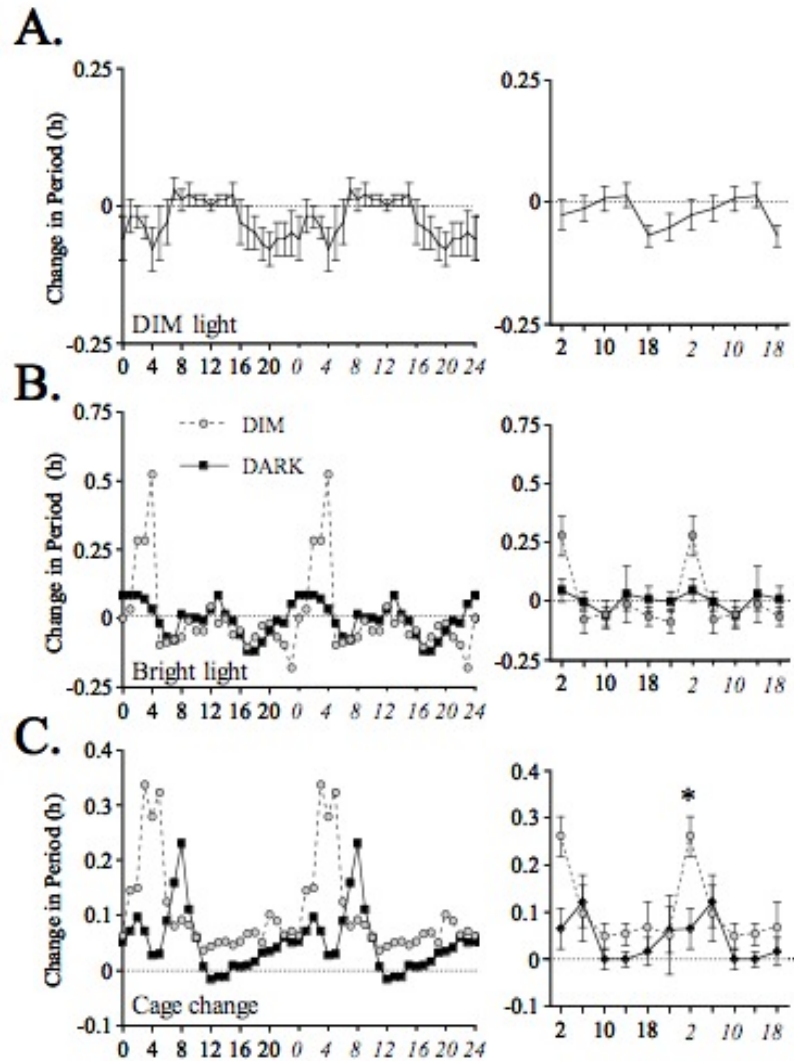


Figure 2.6: Tau response curves (τ RC) for photic and nonphotic stimuli as drawn with a 3h moving average (left) or as grouped in 4 h bins for quantitative analyses (right). A) τ RC for 2 h dim light pulses provided against complete darkness. Stippled line at zero represents mean values of controls maintained in darkness. B) Bright light τ RC for 5 min, 100 lux light pulse collected against DARK or DIM background conditions. Prior to bright light pulses, animals were exposed to dim illumination for one week. C) Nonphotic τ RC for cage changes performed under constant DIM and DARK conditions once every 2-3 weeks. Error bars have been removed from smoothed bright light and nonphotic τ RC s for clarity. * $p < 0.009$.

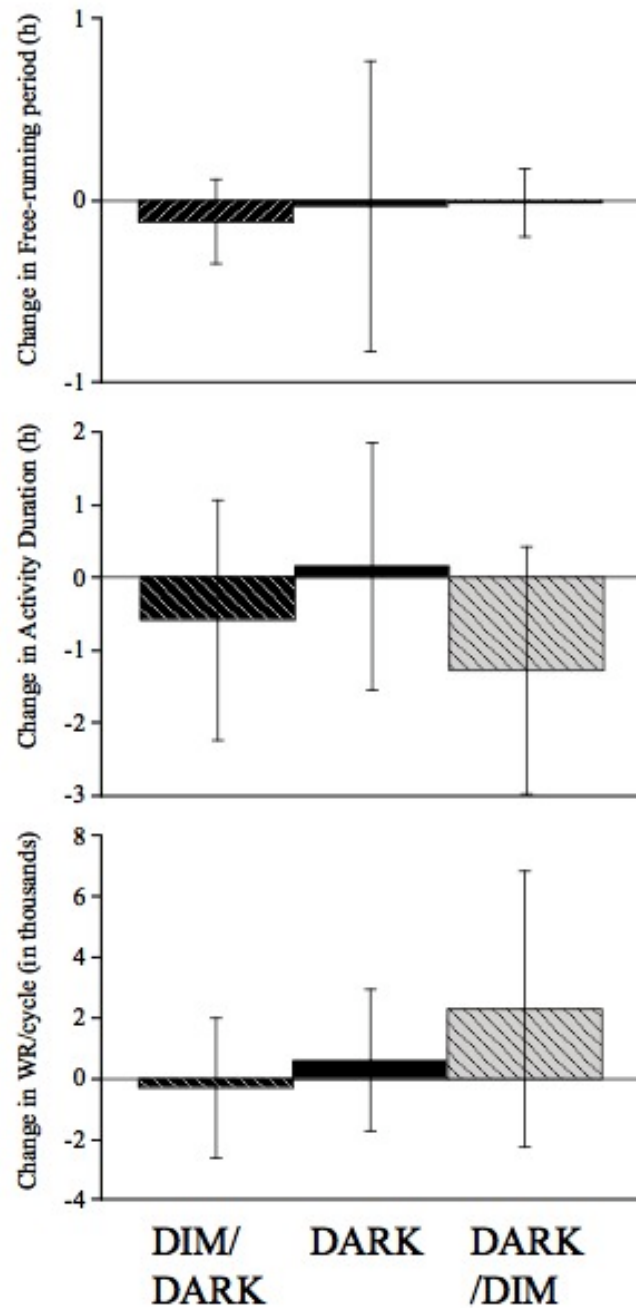


Figure 2.7: Changes in free-running period (τ), activity duration (α), and wheel revolutions per cycle (WR/cycle) during the week after the changes in scotopic conditions that occurred seventeen weeks after release into constant conditions. Animals were either retained under completely dark conditions (DARK), or were transferred from dimly lit to completely dark conditions (DIM/DARK), or from completely dark to dimly lit conditions (DARK/DIM).

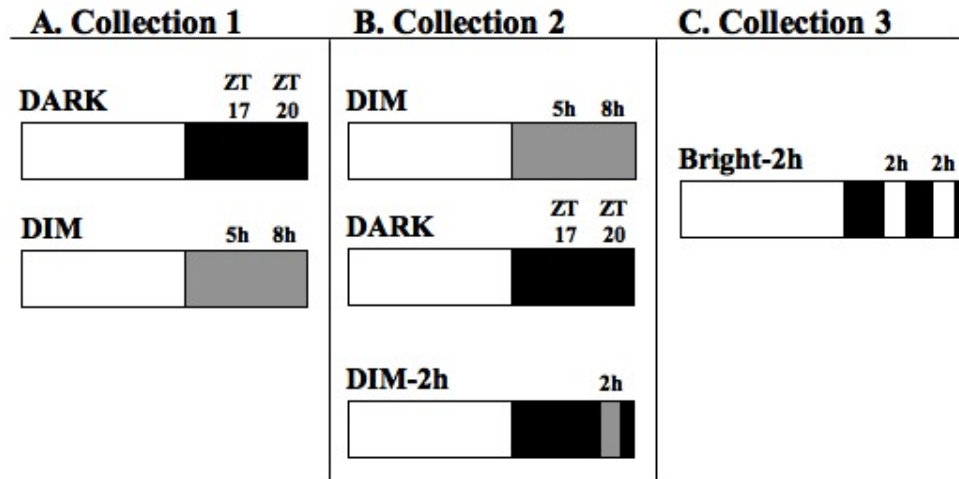


Figure 2.8: Schematic of experimental protocol used in Experiment 1C. Animals were entrained to a light:dark cycle with 14 hours of light and completely dark nights. A) After five weeks, animals were either retained under dark nights (DARK, top) or provided with dim nighttime illumination for one night only (DIM, bottom). Blood was collected at two timepoints relative the light-to-dark transition (zeitgeber time 12, ZT12), either 5 h or 8 h after ZT 12 (ZT17 and ZT20, respectively). Thus, DIM animals received either a 5 h or 8 h dim light pulse. B) Three weeks later, blood was again collected in animals at the same ZT as Collection 1, but now in a manner that was counterbalanced for scotopic condition. Additionally, a separate group of animals were given a 2 h dim light pulse from ZT 18-20 (DIM-2h). C) Three weeks later, a subset of animals used for both Collection 1 and 2 was provided a 2 h bright light (100 lux) pulse from ZT15-17 or ZT18-20 (Bright-2h).

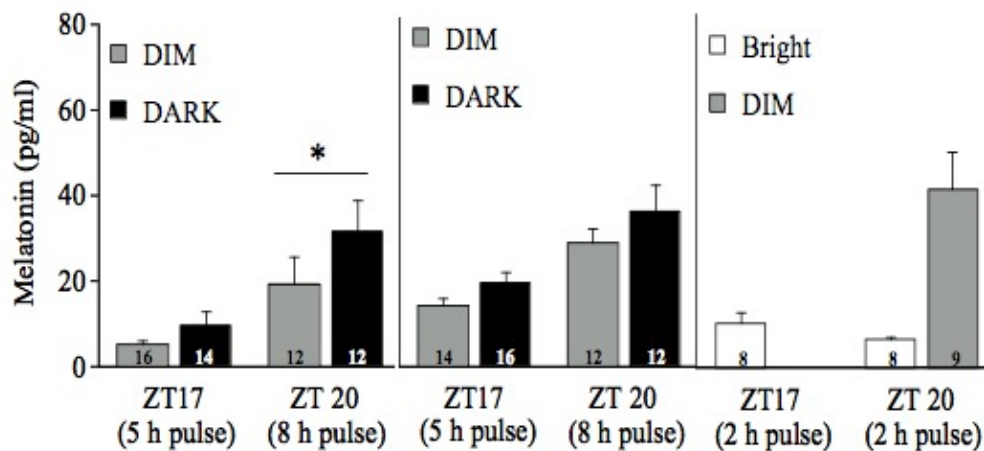


Figure 2.9: Mean (\pm SEM) serum melatonin concentrations of hamsters sampled under dim or dark conditions at different phases of a 14L:10D cycle. During Collection 1 (leftmost graph), DIM light pulses 8 h long, but not 5 h long, produced significant melatonin suppression. In contrast, no significant melatonin suppression was observed during Collection 2 (center graph), although the pattern of the results was in a similar direction. Relative to melatonin values exhibited by DARK controls during Collection 1 and 2, bright light pulses 2 h in length suppressed melatonin levels at ZT20, but not ZT17. Lastly, 2 h DIM light pulses did not suppress melatonin at ZT20. * $p < 0.05$.

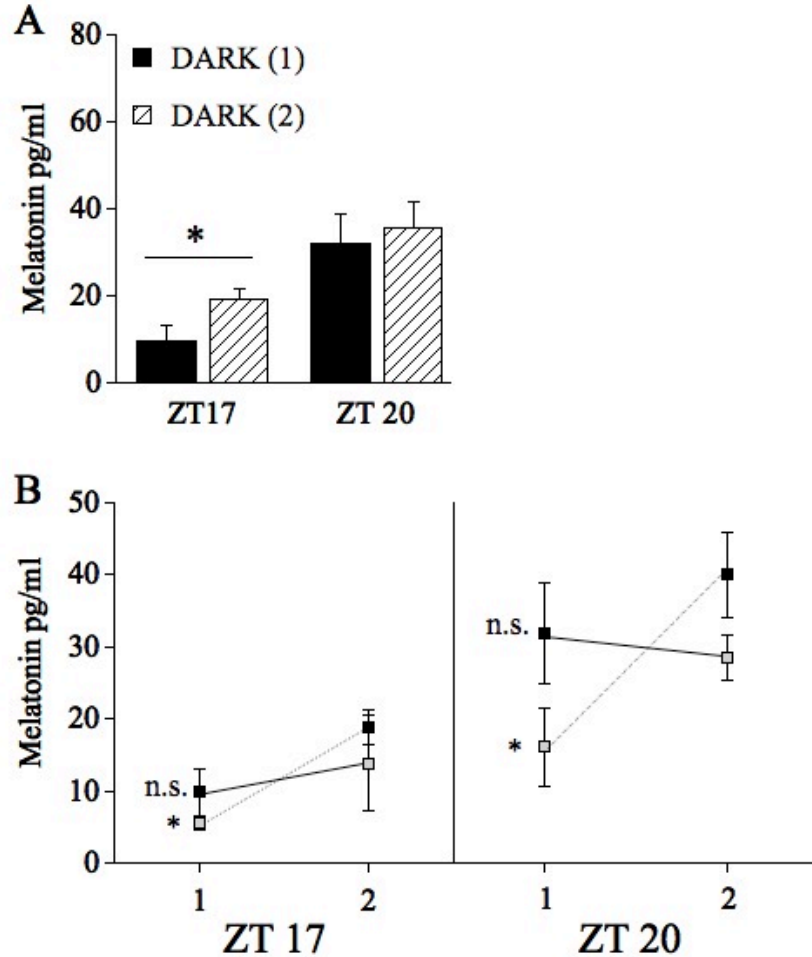


Figure 2.10: Changes in melatonin levels across repeated blood sampling in Experiment 1C. A) Melatonin levels collected from DARK controls at ZT17 during Collection 2 (2) were significantly higher than levels displayed by DARK controls three weeks earlier during Collection 1 (1). B) Within subjects comparisons of melatonin levels sampled at ZT17 or ZT20. Relative to melatonin levels observed at ZT17 during Collection 1, melatonin levels increased under DARK nights during Collection 2, but not under DIM nights. Please note that the color of the symbol at either end of the line do not match in order to represent the counterbalanced nature of the design. * DIM-to-DARK, matched t test, $p < 0.05$ for both ZT17 and ZT20. n.s. DARK-to-DIM, matched t test not significant.

Chapter 3

Experiment 2: The role of dim light in the induction of splitting under LDLD

The present studies examine for the first time the role of dim illumination in LDLD-induced splitting in terms of basic entrainment processes. In an earlier study, male Syrian hamsters with dimly lit scotophases displayed higher wheel running levels than animals with completely dark scotophases (Gorman et al., 2003). Dim light could promote LDLD-induced splitting by increasing the effective dose of the nonphotic stimulus during novelty-induced activity bouts under LDLD. Alternatively, dim illumination may potentiate phase resetting responsiveness to nonphotic cues and/or bright light. Finally, as LDLD-induced split rhythms may reflect changes in the phase relations of coupled oscillators (Gorman et al., 2003), scotopic illumination could exert its influence by altering circadian coupling.

Each of the following experiments characterizes the influence of dim illumination on a factor potentially contributing to the emergence of LDLD-induced split rhythms. Specifically, these studies assessed whether dim illumination operates by modulating 1) novelty-induced activity levels, 2) nonphotic and bright photic phase resetting and 3) re-entrainment under skeleton photoperiods. The first experiment replicates published reports that LDLD-induced splitting is increased with dimly lit scotophases and addresses whether this is produced by increases in wheel running intensity. The second experiment incorporates manipulations designed to mimic the nonphotic and bright photic stimuli under LDLD and examines whether phase

resetting to these stimuli is differentially influenced by dimly lit versus dark free-running conditions. Lastly, the third experiment investigates whether dim illumination affects the emergence of phase jumps elicited by skeleton photoperiods simulating increases in day length.

General methods

Animals and husbandry. Female Syrian hamsters were bred and reared as described previously (c.f., Chapter 2). For each of the following experiments, hamsters (age 10-12 wks) were transferred to individual light-tight housing units (one cage/unit).

Dim illumination. Dim illumination was provided by green light-emitting diodes (LEDs; Arcoelectric, Thousand Palms, CA) mounted in the back wall of each individual housing unit. These LEDs have a peak transmission wavelength of 560 nm with a one half bandwidth of 23 nm as measured by an Ocean Optics PS1000 spectrometer (Dunedin, FL). As measured with an IL1705 Radiometer system (International Light, Newburyport, MA) with the sensor positioned at hamster eye level in the brightest region of the cage floor, dim illumination used currently was 4.2×10^{-3} lux and 7.9×10^{-6} $\mu\text{W}/\text{cm}^2$, equivalent to 2.23×10^9 photons/ cm^2 sec.

Data collection and analyses. Activity rhythms were primarily monitored via home cage running wheels (diameter = 17 cm) located within polypropylene cages (27 x 20 x 15 cm). Entrainment was monitored in wheel-naïve animals via passive infrared (PIR) motion detectors (Coral Plus, Visonic, Bloomfield, CT) positioned ~32 cm

above the cage floor of cylindrical polyethylene cages (26 cm diameter). Half revolutions of home cage wheels or movement under PIR sensors triggered closures of a relay, which were collected and compiled into 6 min bins by DataQuest III or Vital View software (Mini-Mitter, Bend, OR). As in a previous report (Gorman et al., 2003), the scotophase reflecting the phase of the animals' subjective night at the beginning of the experiment is referred to as the "nighttime" scotophase, while the scotophase added during the experiment is designated the "daytime" scotophase. Similarly, the photophases occurring before and after the nighttime scotophase are labeled the "evening" and "morning" photophase, respectively. These conventions are illustrated in Figure 3.1.

Categorical data were analyzed using contingency statistics (Pearson's χ^2). Continuously varying activity and entrainment measures were assessed primarily using parametric statistics. When significant heterogeneity of variance was detected between groups, Kuskall-Wallis nonparametric tests were performed, and these values are reported instead. Statistical tests were conducted with JMP software (SAS Institute, Cary, NC) and values in text and illustrations are expressed as mean \pm SEM.

Experiment 2A: Dim nighttime illumination and nonphotic feedback

Methods and Procedures

Split rhythms were generated in a manner similar to that described previously (Gorman et al., 2003). Seven hours after lights on, wheel-naïve animals were

transferred to individual cages equipped with running wheels. Transfer corresponded to the beginning of the daytime scotophase of the new LDLD cycle (LDLD 7:5:7:5; lights off: 1000; lights on: 1500, lights off: 2200, lights on: 0300 PST). Thereafter, photophase light intensity was 50-75 lux and scotophase illumination depended on group assignment, as detailed below. A cage change was performed two weeks after transfer. During the first 90 min of the daytime scotophase, animals and their wheels were transferred to cages with fresh bedding, water and food under the direction of dim red head lamps (< 1 lux for < 5 minutes/ animal).

At the time of the initial transfer, hamsters were randomly assigned to one of three groups that differed in the intensity of scotophase illumination and the type of wheel provided. One group received scotopic illumination and cages equipped with standard (i.e., unmodified) wheels (DIM-Std Wheel, n=7). For the two remaining groups, scotophases were completely dark, and animals received cages equipped with either standard wheels (DARK-Std Wheel, n=7) or modified wheels (DARK-Mod Wheel, n=8), where the metal rungs were wrapped with a plastic guard to increase wheel-running coordination (c.f. (Mrosovsky et al., 1998).

Data Collection and Analyses

For analytic purposes, this experiment was divided into two 2-week Intervals, beginning with the initial transfer and cage change, respectively. Group differences in split rhythm incidence and novelty-induced activity were analyzed separately for each Interval. Activity rhythms were categorized as split if animals expressed wheel-

running bouts longer than 30 min during both daily scotophases for at least five consecutive days. Consistent with previous experiments (Gorman & Elliott, 2003; Gorman et al., 2003), there was no ambiguity in classifying animals as split or unsplit. Additionally, wheel-running counts across the first three days of each Interval were summed for individual animals in hourly bins. Group differences in total wheel revolutions were assessed for each scotophase and photophase across the first three days of each Interval. NWR was operationally defined as total wheel revolutions expressed during the 5 h daytime scotophase coincident with the initial transfer or cage change.

Results

Emergence of split rhythms. A variety of unsplit and split activity patterns was observed (Figure 3.1). Hamsters that restricted activity to the nighttime scotophase were classified as unsplit (Figure 3.1), while hamsters that displayed activity in each of the two daily scotophases were classified as split, regardless of whether the split rhythm developed during Interval 1 or Interval 2 (Figure 3.1). As illustrated in Figure 3.1, split rhythms emerged in two different patterns: either developing gradually, with daytime scotophase activity accruing on subsequent days (Interval 1), or appearing abruptly, with a robust activity bout appearing in the daytime scotophase (Interval 2). Split rhythms also varied in their stability: either remaining split over both Intervals or consolidating activity into the daytime scotophase (Figure 3.1). The former pattern

was generally characteristic of split rhythms developing after the initial transfer, while the latter pattern was observed in all animals that split after the cage change.

Splitting incidence. The incidence of splitting depended on scotopic illumination (Table 3.1). In Interval 1, DIM-Std Wheel animals tended to exhibit split rhythms more frequently than animals in either DARK group ($\chi^2(2) = 4.59$, $p = 0.08$). During Interval 2, DIM-Std Wheel animals exhibited a significantly higher incidence of splitting than either DARK group, even when previously split animals were excluded from the analysis ($\chi^2(2) = 14.49$, $p < 0.001$). Considering splitting incidence over both Intervals, all DIM-Std Wheel animals exhibited split rhythms, while all but one DARK animal had unsplit rhythms ($\chi^2(2) = 18.22$, $p < 0.001$).

Wheel running in LDLD. Group differences in splitting occurred despite the fact that animals within DIM-Std Wheel and DARK-Mod Wheel animals exhibited comparable NWR levels (Table 3.1; Figure 3.2). Dim light did not significantly increase NWR during Interval 1, but DARK-Mod Wheel animals ran at significantly higher levels than DARK-Std Wheel animals ($F(2, 19) = 4.64$, $p < 0.05$). Similarly, during Interval 2, scotopic illumination did not influence NWR levels of theretofore unsplit animals ($F(2,15) = 1.68$, $p > 0.2$).

For each Interval, all three groups displayed a transient decrease in wheel running after the NWR displayed during the daytime scotophase (Figure 3.2). Activity levels within the subsequent photophase and scotophase did not differ between groups on the first day of Interval 1. Wheel running levels across the first day of Interval 2 were similar, except that the evening photophase activity was reduced, and DIM-Std

Wheel animals were less active than either DARK group ($p < 0.05$). Over the course of the subsequent two days in Intervals 1 and 2, developing split rhythms were evident for the DIM-Std Wheel animals but not for DARK animals.

Experiment 2B: Resetting to photic and nonphotic stimuli simulating LDLD

This experiment assesses whether dim light alters phase resetting induced by the nonphotic and photic stimuli associated with the emergence of split rhythms in LDLD, using procedures specifically designed to mimic conditions experienced under LDLD. Dim light may influence the sensitivity to novelty-induced activity bouts and thereby potentiate nonphotic phase resetting on the first day of each Interval under LDLD. Animals splitting in the two different Intervals of Experiment 2A had different photoperiodic histories. Those animals splitting in Interval 1 had been just previously entrained to LD 14:10, while animals splitting in Interval 2 were previously entrained to LDLD 7:5:7:5, which is technically a skeleton photoperiod of LD 19:5. Thus, LD 14:10 and LD 19:5 were used presently to simulate differences in entrainment prior to Interval 1 and 2, respectively. Lastly, after the initial transfer to LDLD and intense NWR, animals during Interval 1 receive bright light exposure during early subjective night (e.g. former ZT 12-ZT 17). This experiment also assessed whether dim illumination influences phase resetting induced by this compound stimulus (i.e., NWR plus bright light).

Methods and Procedures

Animals were individually housed without running wheels in cylindrical polyethylene cages (35 cm height x 26 cm diameter). For 28 days, animals were entrained to either LD 14:10 (lights on: 0300 PST, lights off: 1700 PST) or LD 19:5 (lights on: 0300 lights off: 2200 PST), during which activity rhythms were monitored with passive infrared (PIR) motion detectors. Photophase and scotophase intensity during entrainment was ~100 and 0 lux, respectively. Midway through this entrainment period, cages were cleaned during the photophase and a handful of soiled bedding was retained in an effort to reduce novelty-induced activity. On one day only, the lights-off transition (zeitgeber time = ZT 12) was advanced by 5 h in order to determine whether activity onset was negatively masked by light during entrainment to LD 14:10 and LD 19:5.

As indicated above, phase shifting conditions were designed to mimic the nonphotic and photic stimulation used during the LDLD-induced splitting paradigm. As illustrated in Figure 3.3, phase shifts were studied under a modified Aschoff Type II design (Aschoff, 1965b), where release into constant conditions coincides with the application of phase shifting manipulations. Seven hours after lights on, animals were transferred from LD 14:10 (transfer at ZT 5) or LD 19:5 (transfer at ZT 0) to cages with modified running wheels (c.f., Experiment 2A). Animals from each photoperiod were transferred to wheel running cages with complete darkness (LD 14:10-DARK; n = 7; LD 19:5-DARK; n = 8) or with dim illumination (LD 14:10-DIM; n = 8; LD 19:5-DIM; n = 8). No attempt was made to control for the intensity or duration of

subsequent wheel running. To determine whether dim light influenced the response to the compound stimulus, two additional groups of LD 14:10 animals received a 7 h light pulse (50-75 lux) after 5 h of NWR in complete darkness (LD 14:10-DARK+L, n = 8) or in dim illumination (LD 14:10-DIM+L, n = 8). After phase shifting manipulations were complete, animals remained in constant conditions for two weeks to calculate phase shift magnitude and free running period length (τ).

Data Collection and Analyses

Using PIR actograms in the Clocklab percentile format, activity onset and offset were determined for each day over the last two weeks under entrained conditions (Week 3 and Week 4), and a regression line was fit to each set of seven points. The average length of activity (α) for each week was derived from the difference between average onset and offset. Average activity onset is expressed as the phase angle of entrainment to the light to dark transition ($\psi_{L/D}$), which is the time difference between the entraining and behavioral event. PIR actograms were visually inspected for activity onset on the day of the dark probe by noting the first 6 min bin after lights off when activity exceeded two counts and was sustained for at least 5 of 8 subsequent bins.

A phase shift was determined for each animal by the displacement between the average activity onset during Week 4 and the time of activity onset predicted for the day of transfer by a regression line fit to visually identified wheel running onsets (7 post-pulse days were used, excluding the first 4 to allow for transients). Pre- and post-

pulse activity rhythms were monitored via different methods (PIR versus wheels), which precludes a precise specification of the absolute size of phase shifts (Aschoff et al., 1973). Phase shifts were determined identically for every group, however, so that DIM versus DARK differences could be assessed. Lastly, the slope of the post-pulse regression line was used to calculate τ and this value was compared between groups free-running in constant dim and dark conditions.

Results

Entrainment to LD 14:10 and LD 19:5. As expected, hamsters displayed photoperiod-dependent differences in entrainment prior to the phase shifting manipulations (Figure 3.3). While under their respective photoperiods, LD 14:10 animals expressed longer active phases than LD 19:5 animals (e.g. Week 4 α : LD 14:10 = 9.88 ± 0.16 h, LD 19:5 = 8.2 ± 0.22 h; Kuskall-Wallis Test; $p < 0.001$) and also initiated activity closer to the light to dark transition (e.g. Week 4 $\psi_{L/D}$: LD 14:10 = 0.28 ± 0.06 h, LD 19:5 = 2.75 ± 0.08 h; Kuskall-Wallis Test; $p < 0.001$).

On the day of the dark probe, more than 85% of animals displayed activity onsets that were advanced by less than 30 min relative to that observed during the preceding week. When the difference between activity onset during Week 3 and on the day of the dark probe was calculated, LD 14:10 and LD 19:5 were not significantly different ($t(53) = 1.04$, $p > 0.3$). Both these observations serve to verify that photoperiod-dependent differences in α and $\psi_{L/D}$ were not a product of negative masking by light.

Wheel running during the first 24 h after transfer. Following transfer, animals within all groups engaged in robust wheel running during the first 5 h after transfer (Figure 3.4, Table 3.2). Wheel running levels tended to taper off and then rise once more several hours later. LD 14:10 and LD 19:5-DARK animals, but not LD 19:5-DIM animals, discontinued wheel running shortly after the initial novelty-induced activity bout (Figure 3.4). Relative to their DARK counterparts, 19:5-DIM animals displayed a long bout of novelty-induced activity after transfer ($p < 0.05$; Figure 3.4) and did not run robustly at a phase consistent with their prior entrainment ($p < 0.05$; Figure 3.4; Table 3.2). After resumption of wheel running, LD 14:10 animals receiving the bright light pulse exhibited wheel running patterns similar to those of LD 14:10-DARK and -DIM groups, with the exception that the former animals exhibited less activity during the 7 h light pulse and a large increase in wheel running during late subjective night (Figure 3.4, Table 3.2). Relative to their DARK cohorts also receiving a light pulse, LD 14:10-DIM+L animals tended to show less wheel running during the pulse ($p = 0.08$; Table 3.2) and a larger increase in subsequent wheel running ($p < 0.05$; Table 3.2).

Phase shifts and τ under constant conditions. The magnitude of phase shifts depended on dim illumination and photoperiodic history, in addition to the type of manipulation provided (Figure 3.5). Nonphotic phase shifts exhibited by LD 14:10-DARK and LD 14:10-DIM animals were negligible, and no difference due to DIM light was evident ($t(13) = 0.57$, $p > 0.5$). In contrast, phase advances exhibited by LD 19:5-DIM animals were ~ 3 h larger than those exhibited by their DARK counterparts

($t(14) = -2.97, p < 0.05$). Additionally, DIM light significantly enhanced the magnitude of phase delays exhibited by LD 14:10 animals receiving NWR followed by a 7 h bright light pulse ($t(14) = 2.12, p = 0.05$). When phase shifts were instead calculated relative to activity onset on the day of the dark probe or to ZT12 (rather than to the Week 4 average activity onset), these results were upheld (data not shown). No significant differences in τ were evident between groups in the two weeks after release into constant conditions. Group means ranged from 23.89-24.07 h.

Experiment 2C: Phase jumping responses under dimly lit and completely dark nights

Ultra long photoperiods (> 16-18 h) challenge circadian entrainment in nocturnal rodents, resulting in the expression of a phase jump if animals are held under skeleton photoperiods simulating increases in day length (Pittendrigh & Daan, 1976b; Sharma et al., 1997; Stephan, 1983). A similar mechanism may contribute to the temporal disassociation of component oscillators under LDLD (Gorman et al., 2003). Experiment 2C was designed to determine whether dim illumination would influence the timing and pattern of phase jumps under skeleton photoperiods. Moreover, this paradigm assesses whether dim light influences photic entrainment when novelty-induced activity is minimized.

Methods and Procedures

Hamsters were held under a series of skeleton photoperiods, where the interval between entraining light pulses was systematically reduced (see Figure 3.6). Under this series of photocycles, scotophases were marked by either complete darkness (DARK, n = 16) or dim illumination (DIM, n = 16). On the first day of the experiment, hamsters were transferred from LD 14:10 to running wheel cages identical to those used in Experiment 2A. Although this transfer occurred during subjective day, the house lights remained on after transfer, and a new light:dark cycle was immediately instated by symmetrically reducing the following scotophase by 3 h (LD 17:7; lights on: 0130 lights out: 1830 PST). LD 17:7 was replaced one week later by an equivalent skeleton photoperiod with two 3 h light pulses (LDLD 3:11:3:7; lights on: 0130, lights off: 0430, lights on: 1530, lights off: 1830 PST). At weekly intervals thereafter, the nighttime scotophase was symmetrically reduced by 30 min. The duration of the daytime scotophase increased equivalently. Cage changes were performed during the evening photophase and a handful of soiled bedding was retained in an effort to reduce novelty-induced activity.

Data Collection and Analyses

Phase jumps were identified for individual animals by visually identifying the first day when a wheel running bout at least 18 min long was phased within the daytime scotophase and then repeated on at least 3 of the 4 subsequent cycles. The length of the nighttime scotophase at the time of the phase jump and the number of cycles preceding the phase jump were recorded for each animal and used to compare

DARK and DIM groups. Once a phase jump was initiated, I noted the number of cycles that elapsed before activity was completely realigned into the daytime scotophase.

24-hr histograms were produced for each hamster by averaging wheel revolutions within each 6 min bin across the seven days of each photocycle used in this experiment. Activity onset was defined as the first 6 min bin surpassing the daily mean that was followed by two bins likewise exceeding this threshold. Activity offset was defined as the last time point below the daily mean that was immediately preceded by two bins above threshold. α was calculated as in Experiment 2B, and $\psi_{L/D}$ was derived as the difference between lights off for the nighttime scotophase and activity onset. These measures were then used to compare entrainment of DIM and DARK animals during the first four weeks of the experiment (i.e., before a large number of animals expressed phase jumps). Additionally, α was determined for individual animals during the week before a phase jump and during the final week of the experiment.

Results

Emergence of phase jumps. Dim illumination accelerated the expression of a phase jump, a response that was exhibited ultimately by all animals (Figure 3.6). Four animals with DIM light displayed phase jumps within three weeks of nighttime scotophase reductions, and the remaining DIM animals initiated phase jumps over the

next several weeks. In contrast, DARK animals exhibited phase jumps only after the nighttime scotophase was reduced to 3.5 h. As a result, DIM animals phase jumped while the nighttime scotophase was longer relative to DARK animals (Survival Analysis, $\chi^2(1) = 14.77$; $p < 0.001$) and over a significantly broader range of nighttime scotophases (DIM: 6.5-3.0 h; DARK: 3.5-2.5 h; Kuskall-Wallis test, $\chi^2(1) = 3.72$; $p < 0.001$).

Entrainment before and after the emergence of phase jumps. In addition to the marked effect on the emergence of phase jumps, scotopic illumination affected entrainment early in the study, when animals were transferred from LD 14:10 to LD 17:7. On the week under LD 17:7, activity bouts of DIM animals were shorter relative to their DARK counterparts, (α : DIM = 8.19 ± 0.27 ; DARK = 9.2 ± 0.28 ; $p < 0.05$, LS Means Contrast) and phased closer to the nighttime lights-off transition ($\psi_{L/D}$: DIM = 1.05 ± 0.24 ; DARK = 2.22 ± 0.24 ; $p < 0.05$, LS Means Contrast). In the following week under the matching skeleton photoperiod, however, group differences disappeared ($p > 0.05$, LS Mean Contrasts), and over the next two weeks, DIM and DARK animals continued to entrain to skeleton photoperiods similarly ($p > 0.05$, LS Mean Contrasts). As the majority of DIM animals displayed phase jumps over the subsequent weeks, differences in entrainment were not assessed beyond the fourth week of the experiment.

After the initiation of a phase jump, the phase of wheel running continued to realign into the daytime scotophase, and a phase jump was noted to be complete when no activity remained within the nighttime scotophase. Once a phase jump was

initiated, the latency to realignment was significantly longer under DIM conditions (DIM: 12.67 ± 1.37 days; DARK: 4.88 ± 1.37 days; $t(30) = -4.03$, $p < 0.001$). α for the week preceding the phase jump, however, did not differ between animals in DIM and DARK conditions (5.95 ± 0.41 and 5.93 ± 0.41 , respectively; $t(30) = -0.03$, $p > 0.9$). After phase jump completion, α expanded within the daytime scotophase and at the end of the experiment, DIM animals displayed longer α than their DARK cohorts (DIM: 10.99 ± 0.34 ; DARK: 7.93 ± 0.35 ; $t(30) = -6.33$, $p < 0.001$).

Discussion

As previously reported for male Syrian hamsters (Gorman et al., 2003), dim light facilitated LDLD-induced splitting in females. All animals housed with dimly lit scotophases exhibited split rhythms in Experiment 2A, but this was not a secondary consequence of increases in the amount of nonphotic feedback. DARK animals housed with standard wheels were less active than their DIM counterparts but not significantly so, which could reflect differences in the age or sex of hamsters used in the present study (Gorman et al., 2003). More importantly, provision of modified wheels increased wheel running levels above those of DIM animals but failed to elicit split rhythms in all but one dark-exposed animal. Although dim illumination may augment wheel running, its facilitation of LDLD-induced splitting would not seem to be a mere product of increased activity levels.

If scotopic illumination facilitates LDLD-induced splitting independent of activity levels (c.f., Experiment 2A) and hallmark circadian responses to photic stimuli (c.f., Chapter 2), in what manner could it operate? Experiment 2B examined whether dim light renders animals more responsive to nonphotic and photic resetting. Transfer of wheel-naïve animals to wheel running cages induced large phase advances in LD 19:5-DIM animals only. Since no facilitation of nonphotic phase shifting was observed after entrainment to LD 14:10, the effects of dim light may be limited to animals entrained to photoperiods with short nights. One caveat to the interpretation that dim illumination enhances nonphotic *sensitivity* after LD 19:5 is that relative to their dark cohorts, LD 19:5-DIM animals ran for a longer time after transfer to wheel running cages. However, this may be a consequence, rather than a cause of their larger phase advances. Existing intensity-response curves, collected under admittedly different conditions, saturate at wheel-running levels accomplished by animals in Experiment 2B within the first 5 h after transfer (Bobrzynska & Mrosovsky, 1998; Reeb & Mrosovsky, 1989). Further, the phase of the circadian pacemaker is generally reset within a few hours of exposure to photic and nonphotic zeitgebers (Mead et al., 1992). Thus, the extended activity in LD 19:5-DIM animals may represent continuity between NWR-induced and phase-shifted circadian activity. In support of this point, activity offset on this first day after transfer also appears to be advanced in LD 19:5-DIM animals relative to their DARK cohorts. Dim light also increased the magnitude of phase delays elicited by NWR followed by a long bright light pulse. Because differential phase resetting was not observed in LD 14:10-DIM and -DARK animals

after NWR alone, augmented phase delays after the compound stimulus likely resulted from dim light interacting with the bright light stimulation during early subjective night. Experiment 2C, which focused on photic cues and minimized novelty-induced activity, also indicated that dim light modulates light-induced resetting. Specifically, after the abrupt change from LD 14:10 to LD 17:7, DIM animals displayed a less positive $\psi_{L/D}$ and shorter α relative to DARK animals.

Because photoperiodic compression of α has been implicated in LDLD-induced splitting (Gorman, 2001; Gorman et al., 2003), Experiment 2C primarily investigated whether dim light would influence re-entrainment to skeleton photoperiods simulating increases in day length. Dim nighttime illumination unambiguously accelerated the emergence of a phase jump under these conditions. Phase jump responses observed presently were consistent with previous reports for this species in that activity of all animals advanced into the daytime scotophase (Pittendrigh & Daan, 1976b; Stephan, 1983). However, in the absence of a formal understanding of precisely how phase jumps emerge, it is difficult to specify the mechanisms through which dim illumination accelerates this response. Previous models largely account for phase jumps through an asymmetry in delay and advance regions of the photic PRC (Pittendrigh & Daan, 1976b; Sharma et al., 1997; Stephan, 1983). These early models, however, do not take into account photoperiod-induced changes in the amplitude of the photic PRC, now known to be positively correlated with α (Pittendrigh et al., 1984; Pohl, 1983). During entrainment to ultra long day lengths, like those simulated in Experiment 2C, light-induced phase shifts are

markedly attenuated and thus less clearly able to generate phase jumps. In multi-oscillator models of the circadian pacemaker, the coupling between component oscillators also changes as a function of α (Pittendrigh & Daan, 1976c), and these changes may underlie the expression of phase jumps. Phase jumping under skeleton photoperiods may provide an additional paradigm under which dim light could exert its effect by altering α and the interactions between oscillators. A common mechanism could underlie both phenomena, as a “minimum tolerable night” near 5h characterizes both LDLD-induced splitting (Gorman, unpublished observations) and phase jumping (Pittendrigh & Daan, 1976b; Sharma et al., 1997; Stephan, 1983). If this is indeed the case, then the fact that phase jumping was ultimately observed in all DARK animals would predict that split rhythms will emerge under LDLD cycles with completely dark nights if shorter scotophases are provided.

Considering the results from the present three studies, it is now possible to address the role of dim light in promoting split rhythms under LDLD. The case is perhaps clearest for the animals of Experiment 2A that were unsplit prior to Interval 2. During Interval 1, these animals had activity largely confined to the 5 h nighttime scotophase and were thus effectively entrained to a skeleton LD 19:5, near the threshold for phase jumps for DIM animals in Experiment 2C. The cage change at the beginning of Interval 2 provides a nonphotic zeitgeber similar to that given to LD 19:5 animals in Experiment 2B, which produces little effect unless dim illumination is provided. Thus, animals with scotopic illumination are more responsive to both photic and nonphotic factors operating under LDLD during Interval 2. In contrast, there is

little impetus for dark-exposed animals to alter entrainment, since DARK animals are expected neither to be phase advanced by NWR during the cage change (Experiment 2B) nor rendered susceptible to phase jumps under a skeleton of LD 19:5 (Experiment 2C). These results are consistent with a proposed model of LDLD-induced splitting in which novelty-induced activity induces large phase advances of distinct populations of circadian oscillators (Gorman et al., 2003).

Nonphotic phase resetting, however, does not seem to underlie the dim-enhancement of LDLD splitting during Interval 1. As shown in Experiment 2B, dim illumination and darkness do not differentially influence nonphotic phase resetting in animals previously entrained to LD 14:10. Instead, the critical interaction occurring after the initial transfer to wheel running cages may be that between the dim and bright light exposure. By augmenting photic phase delays, scotopic illumination may enhance α compression under LDLD, similar to its effect after transfer to LD 17:7 in Experiment 2C. Photoperiod-induced compression of subjective night may then increase the likelihood of a phase jump and thereby promote splitting in dim-exposed animals. Dim light interacting with photic phase resetting during Interval 1 and nonphotic phase advances during Interval 2 could provide impetus for the two waves of splitting observed under LDLD (c.f. Figure 3.1C versus Figure 3.1D). There were notable differences between Intervals in the pattern of split rhythm emergence (i.e., gradual versus abrupt), similar to previous reports using male hamsters (Gorman et al., 2003). Additionally, splitting initiated during Interval 2 appeared to be less stable than that emerged during Interval 1. Only further study can determine whether these

patterns derive from the different photoperiodic histories of animals splitting in Interval 1 and 2 or unknown intrinsic differences in the circadian function of these behaviorally distinguished hamster groups.

Dim illumination could influence re-entrainment under the present paradigms by changing the waveform or amplitude of the photic PRC. The modicum of evidence collected thus far indicates that dim light does not interact with bright light in a uniform manner. For example, when the 3 h light pulse scanned subjective night during phase jumping, this did not cause a more rapid realignment of activity rhythms in DIM animals. On the contrary, the transition to the daytime scotophase took significantly longer under dimly lit nights. An interaction between dim and bright light, moreover, is unable to explain the full suite of effects demonstrated thus far. For instance, dim light accelerates re-entrainment in Siberian hamsters transferred from long to short day lengths (Gorman & Elliott, 2004), which is achieved primarily through means other than bright light-induced phase shifts (Gorman et al., 1997). In the Syrian hamster, dim illumination alters circadian waveform under free-running conditions and does not directly potentiate the effects of stronger zeitgebers (c.f., Chapter 2). Thus, dim light can certainly affect both nonphotic and photic resetting but given these interactions appear to be limited to specific conditions (e.g. certain photoperiods); it appears that dim light's fundamental action lies elsewhere.

Collectively, the present data are consistent with the hypothesis that dim illumination alters circadian waveform under LDLD by modulating oscillator interactions. Dimly lit nights facilitate split induction by potentiating two "triggers"

for splitting (i.e., nonphotic advances and phase jumping) in a photoperiod-dependent manner, suggesting that dim illumination is altering interactions between oscillators entrained to ultra long day lengths. As discussed in Chapter 1, the explicit effect on oscillator interactions is difficult to specify because coupling is ill defined. However, it may be speculated that dim illumination produces a global inhibition of oscillator interactions, as conceptualized as a decrease in the amplitude of the coupling response curve for the mammalian pacemaker. By reducing the strength of oscillator interactions, dim light may allow one subgroup of oscillators to “escape” and re-entrain to the daytime scotophase under LDLD. This inhibitory effect may also promote the expansion of α under constant dim illumination, although it is clear that coupling is not completely abolished, since animals under dim light did not become arrhythmic (c.f., Chapter 2). On the other hand, dim light could augment interactions that cause oscillators to repel one another under short nights. By increasing the strength of this response, dim light increases the duration of the “minimal tolerable night,” preventing the compression of subjective night and increasing the duration of α under constant conditions. Under LDLD, rather than “escaping,” this second hypothesis stipulates that oscillators are “re-grouping” into an alternative temporal configuration. In this fashion, the circadian system displays two stable states of organization (i.e., bistability), one in phase and one in antiphase, and it may be that dim illumination has potent effects when the system is in the former but not the latter state. A third hypothesis stipulates that dim illumination does not globally increase or decrease coupling mechanisms, but acts to alter the shape of the coupling response

curve for the mammalian pacemaker. In other words, dim nighttime illumination could increase and decrease the strength of oscillator interactions in a manner that depends on the phase relationships between the underlying oscillators. In this manner, dimly lit nights may act to both accelerate the initiation of a phase jump and slow the consolidation of activity into the alternative scotophase when skeleton photoperiods simulate increasing day lengths. The shape and amplitude of the coupling response curve for the mammalian pacemaker is unknown; making it is difficult to distinguish these hypotheses without further research.

The text of Chapter 3 comprises material published in Behavioral Brain Research: Evans, Jennifer A.; Elliott, Jeffery A.; & Gorman, Michael R. (2005). Circadian entrainment and phase resetting differ markedly under dimly illuminated versus completed dark nights, Behav Brain Res, 161 (1), 116-126. I was the primary investigator and first author of this paper. My co-authors listed in these publications directed and supervised the research that forms the basis for these chapters, and both have granted permission for its use here.

Table 3.1 Splitting incidence and novel wheel running (NWR †) during Experiment 2A

	Interval 1			Interval 2			Interval 1 & 2	
	split	unsplit	NWR	split	unsplit	NWR	split	unsplit
DIM-Std Wheel	3	4	7.4 ± 0.8	4	0	5.3 ± 1.3	7	0
DARK-Mod Wheel	1	7	8.8 ± 0.6	0	7	6.0 ± 1.0	1	7
DARK-Std Wheel	0	7	6.2 ± 0.6*	0	7	3.5 ± 1.0	0	7
	0.1 > p > 0.05			p < 0.05			p < 0.0001	

† wheel running revolutions (in thousands) during the 5 h scotophase coincident with transfer or cage change

* Running levels significantly lower than DARK-Mod Wheel (p < 0.05)

Table 3.2 Wheel running revolutions (in thousands) in Experiment 2B

	h 0-5†	h 5-12	h 12-24	h 0-24
LD 14:10-DIM	4.5 ± 1.1	8.1 ± 1.3	3.3 ± 0.9	16.0 ± 2.9
LD 14:10-DARK	4.7 ± 1.2	9.2 ± 1.4	2.0 ± 0.9	16.0 ± 2.8
LD 14:10-DIM+L	4.5 ± 0.7	3.6 ± 0.8*	9.1 ± 1.0*	15.8 ± 1.4
LD 14:10-DARK+L	4.4 ± 0.7	5.9 ± 0.8	5.6 ± 1.0	15.8 ± 1.5
LD 19:5-DIM	6.8 ± 0.9	7.3 ± 0.9	4.0 ± 0.9*	21.2 ± 2.2
LD 19:5-DARK	6.2 ± 0.9	6.8 ± 0.9	8.2 ± 0.9	18.1 ± 2.2

† hour (h) 0 = time of transfer to wheel running cages

* Levels different from DARK cohort ($p < 0.1$; see text)

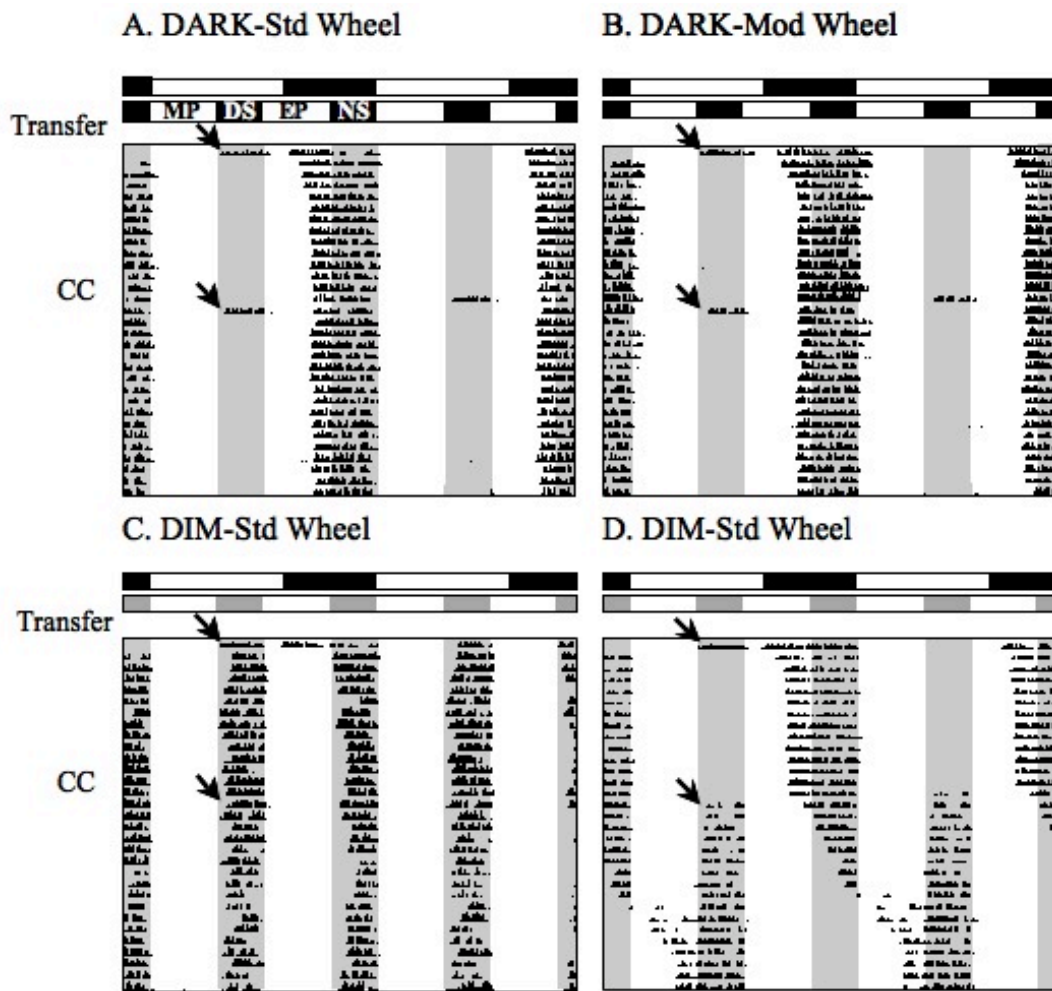


Figure 3.1: Representative double-plotted wheel-running actograms depicting unsplit and split wheel running rhythms exhibited by hamsters during Experiment 2A. Light:dark bars above each actogram represent photoperiods in effect before (top bar) and during the experiment (bottom bar; also internal shading). White rectangles represent photophases, and shaded and black bars represent DIM and DARK scotophases, respectively. MP = Morning Photophase; DS = Daytime Scotophase; EP = Evening Photophase; NS = Nighttime Scotophase. First and second arrows indicate the time of transfer to wheel running cages and cage change, respectively.

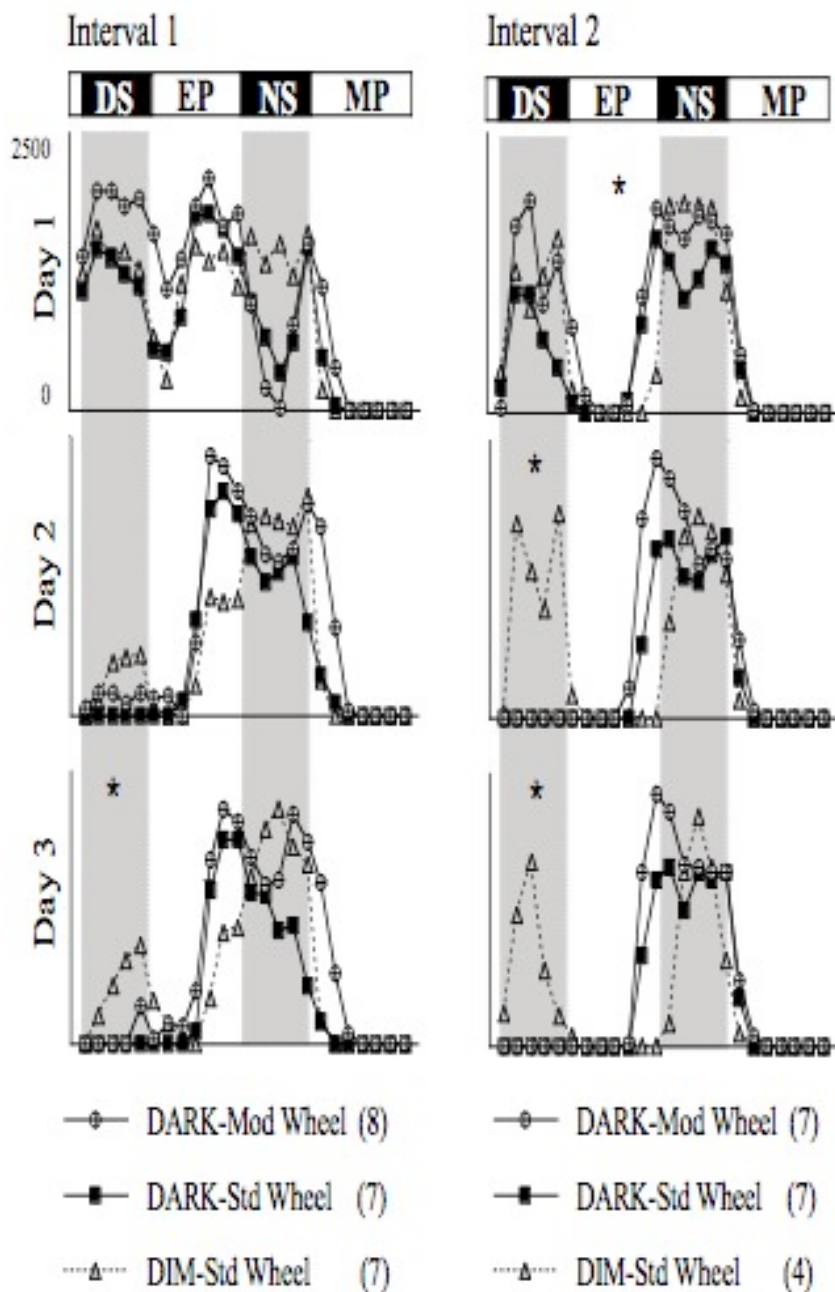


Figure 3.2: Mean (\pm SEM) hourly counts for the first three days of each Interval in Experiment 2A. For figure clarity, standard errors are not shown. Asterisks signify phases of the photocycle (i.e., EP, DS) where the DIM-Std Wheel group displayed activity levels significantly different from the two DARK groups ($p < 0.05$). The number in parentheses is the number of animals per group. Animals that split during Interval 1 were excluded for Interval 2. Abbreviations as in Figure 3.1.

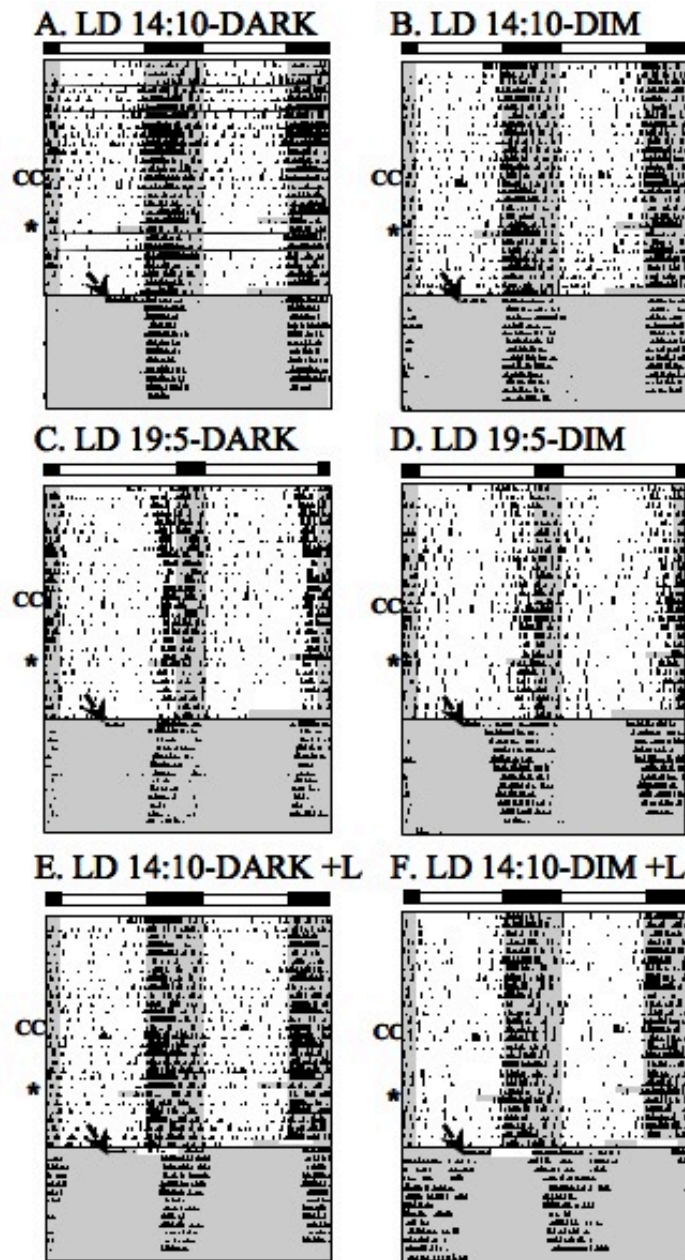


Figure 3.3: Representative double-plotted actograms depicting entrained and free-running activity rhythms exhibited during Experiment 2B. White and black bars above each actogram represent the photocycle in effect while activity was monitored with passive infrared motion detectors (PIR). The change to internal shading marks the day of transfer to wheel running cages (for convenience, shading begins at midnight) and the arrow marks the time of transfer. Entrained PIR rhythms are in Clocklab's percentile format, whereas free-running wheel running rhythms are scaled from 0 to 150 counts. The day of the cage change (CC) and the dark probe (asterisk) are indicated. For the day of the dark probe, the light to dark transition was advanced by 5 h, as represented within each actogram. White boxes on the day of transfer in E) and F) represent 7 h light pulses.

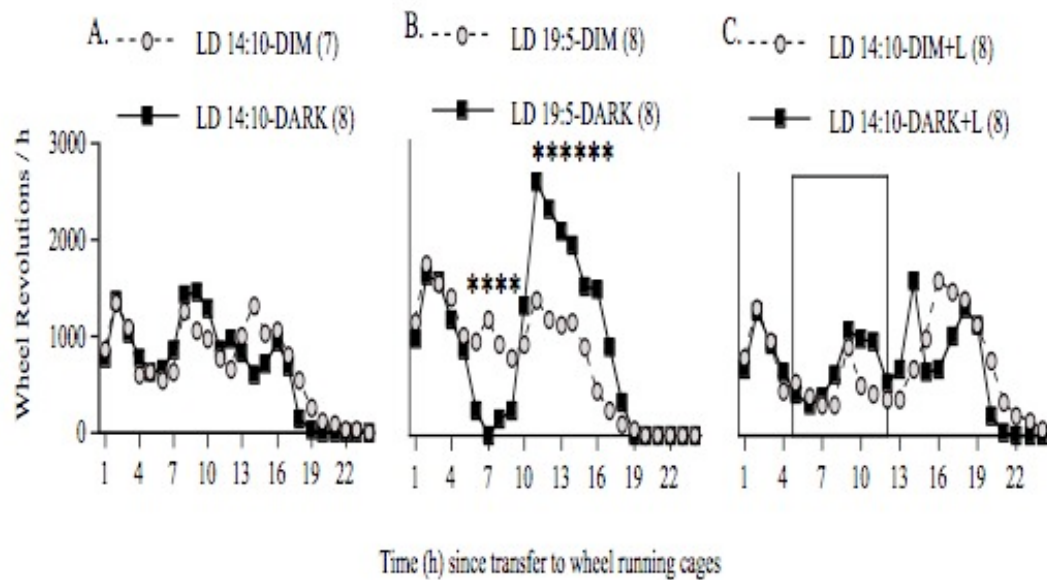


Figure 3.4: Mean (\pm SEM) hourly revolutions across the 24 h after transfer to wheel running cages in Experiment 2B. A) and B) LD 14:10 and LD 19:5 groups were transferred to cages with constant dim illumination (-DIM) or darkness (-DARK). Asterisks signify an hour at which LD 19:5-DARK activity was significantly different from that of LD 19:5-DIM ($p < 0.05$). C) LD 14:10 groups were transferred to cages with constant dim light (-DIM) or darkness (-DARK) and received a 7 h bright light pulse (+L) beginning 5 h after transfer (represented by the internal box). The number in parentheses is the number of animals per group.

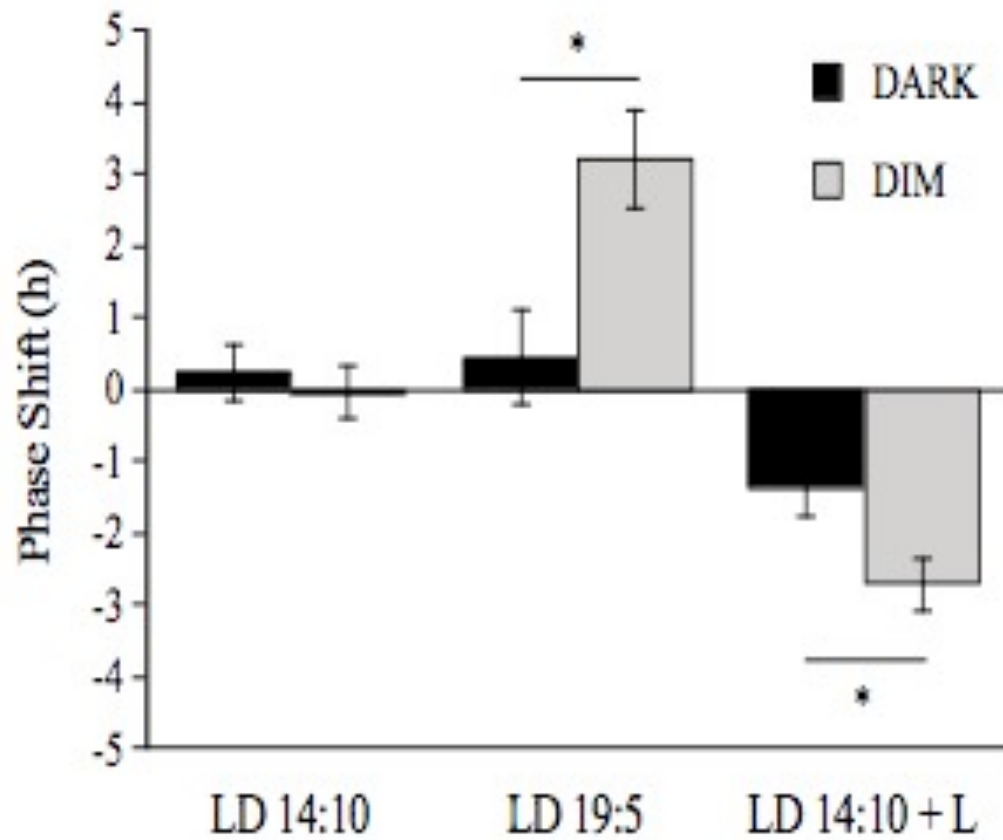


Figure 3.5: Phase shifts (\pm SEM) elicited during Experiment 2B, as determined by the difference in post-transfer wheel running onsets and the PIR onset displayed during entrainment. Phase advances and delays are plotted as positive and negative values, respectively. Asterisks signify significant differences between DIM and DARK groups ($p < 0.05$).

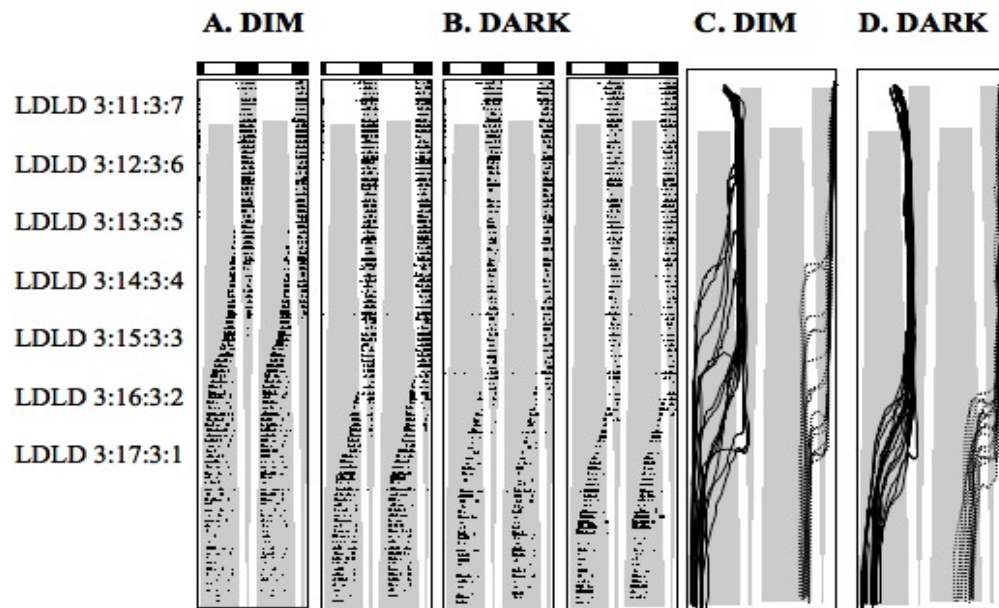


Figure 3.6: Entrainment to the series of skeleton photoperiods simulating increases in day length, where scotophases were marked by dim illumination (DIM) or complete darkness (DARK). A) and B) Representative double-plotted wheel running actograms for two separate animals from DIM and DARK groups, respectively. C) and D) Tracings of activity rhythms exhibited by individual animals within DIM and DARK groups. Each line represents a separate animal. For clarity, onsets and offsets are shown disjointed on separate representations of the double-plotted photocycle. Labels at the far left represent the skeleton photoperiod provided every other week of the experiment, and the last digit indicates the length of the nighttime scotophase.

Chapter 4

Experiment 3: Dim light and the fusion of split rhythms under constant conditions

Split rhythms under LDLD are programmed by two groups of oscillators cycling 12 h out of phase. On the first day after release from LDLD into constant conditions, split hamsters display two distinct activity bouts, one near the entrained phase of each split component. Over the following days under constant conditions, split activity bouts fuse together through a series of transients where one split activity component free-runs shorter than 24 h while the other free-runs longer than 24 h (Gorman, 2001; Gorman & Elliott, 2003). The period and waveform of the activity rhythm typically stabilize within a week, suggesting that the underlying oscillators are free running synchronously thereafter. From this pattern can be inferred that oscillators split under LDLD do not continue to free-run 12 h out of phase with one another or beat in and out of phase, as would be predicted for non-interacting oscillators with identical and different period lengths, respectively. Thus, fusion of split rhythms is mediated by circadian interactions that establish the steady state under constant conditions. This chapter will investigate the nature of the interactions that mediate the fusion of split rhythms after release into constant conditions and will determine whether dim light upon release into constant conditions affects these interactions.

To prevent “beating,” split oscillators with different period lengths could interact and synchronize one another just before they overlap in phase, with no need

for oscillator interactions prior to this point. If this were the case, it would be predicted that the process of fusion would be identical regardless of whether constant conditions begin after the nighttime scotophase (NS) or the daytime scotophase (DS). For instance, the NS activity bout could always adopt a free running period > 24 h and the DS activity bout < 24 h. The free-running periods of each activity component would not be altered by the phase of release, and the latency to fuse would be largely determined by the difference in the initial phase of split oscillators and the difference in their inherent free-running periods. However, in Siberian and Syrian hamsters, the pattern of fusion after release from LDLD into constant conditions does depend on whether the system is released from the DS or NS (Gorman & Elliott, 2003); Gorman and Elliott, personal observations). Release from different phases of the LDLD cycle does not result in the same pattern of fusion, suggesting that oscillators are interacting shortly after release from LDLD.

As split oscillators are joining, are the underlying interactions between oscillators mutual and of equal strength? If split oscillators were interacting in this manner and also identical in their free-running periods, then release from LDLD coincident with each scotophase would be predicted to result in the fusion of split rhythms in two similar patterns ~ 12 h out of phase with one another. In this case, the phase of the fused rhythm would depend on which scotophase was coincident with release. Release from the daytime and nighttime scotophase does not produce similar patterns of fusion displaced by 12 h (Gorman & Elliott, 2003); Gorman and Elliott, personal observations). Thus, oscillators split under LDLD do not appear to be

interchangeable in terms of how they contribute to the fused activity rhythm. One caveat to this interpretation is that the split state is not always symmetrical under LDLD, in that split activity components may not be exactly 12 h apart and activity levels may not be equally distributed into each of the two daily scotophases (Gorman, 2001; Gorman & Elliott, 2003). It has never been determined whether asymmetries in the split state account for differences in fusion after NS versus DS release.

The present studies examine the fusion of split rhythms after release from LDLD coincident with either the NS or DS to assess whether dim nighttime illumination influences oscillator interactions underlying this process. As discussed in Chapter 3, dim illumination facilitates the induction of split rhythms under LDLD by altering coupling mechanisms under short nights; however, the specific nature of this effect is difficult to specify. Dim illumination could inhibit oscillator interactions, which would serve to allow a subgroup of oscillators to re-entrain to the new daytime scotophase of LDLD. In this case, the fusion of split rhythms is predicted to be slowed when released into constant dimly lit versus completely dark conditions. On the other hand, dim light could strengthen an interaction that resists the compression of subjective night under ultra long days by inducing a phase jump response. According to this second hypothesis, constant dim illumination may not alter the rate of fusion after release into constant conditions, but dim light would alter the period and waveform of the fused rhythm (c.f., Experiment 1A). The present data support both hypotheses, revealing that dim illumination increases the latency to fusion and alters the state to which the system returns.

Methods and Procedures

Male Syrian hamsters were bred and raised as described previously (c.f., Chapter 2). At 10-11 weeks of age, animals were transferred to individual polypropylene cages (48 x 27 x 20 cm) located inside light-tight environmental chambers (11-12 cages/chamber) and equipped with running wheels (diameter 17 cm) wrapped with plastic guards to prevent injuries. Transfer coincided with the “daytime” scotophase (DS) of a LDLD 8:4:8:4 cycle (c.f., Figure 4.1, lights on: 0230 PST, lights off: 1030 PST, lights on: 1430 PST, lights off: 2230 PST). DIM nighttime illumination (< 0.1 lux) was provided to all animals by narrow bandwidth green light-emitting diodes (LEDs, peak $\lambda = 560 \text{ nm} \pm 23 \text{ nm}$ half bandwidth) mounted externally and facing the back wall of each cage. One week after transfer to LDLD, cages were changed during the first 90 minutes of the DS, and each animal was relocated to a new cage with fresh food and water under the direction of a dim red headlamp (< 2 min exposure/ animal). Cages were changed at an identical phase two weeks later.

After five weeks under LDLD, the first group of hamsters (N = 24) was released at the beginning of the daytime scotophase (DS) into either constant dim illumination (DIM) or darkness (DARK). Animals remained undisturbed in constant conditions for two weeks. Four months later, a separate group of hamsters (N = 22) was transferred to LDLD for five weeks and then released at the beginning of the “nighttime” scotophase (NS) into constant DIM or DARK conditions for two weeks.

Data Collection and Analyses

Split entrainment. Entrainment under LDLD was quantified to assess whether fusion under constant conditions is influenced by asymmetry in the split state prior to release (Figure 4.2). Bout analyses (Clocklab) were used to characterize each split activity component during the last week under LDLD. Starting three hours before lights-off for each scotophase, onset for that activity bout was identified as the first 6 min bin above a threshold value of 15 counts, preceded by at least one hour of inactivity and followed immediately by two consecutive bins above threshold. Offset for each activity bout was determined by a similar but opposite rule. For each split activity component, total wheel revolutions were summed (NS_{cnts} , DS_{cnts}), the bout length was calculated from the temporal difference between activity offset and onset (NS_{BL} , DS_{BL}), and the phase angle of entrainment was quantified from the temporal difference between activity onset and the light-to-dark transition ($NS\psi_{L/D}$, $DS\psi_{L/D}$).

To assess whether activity under LDLD was symmetrically distributed between split activity components, the ratio of the DS and NS bout length (DS_{BL}/NS_{BL}), and the ratio of DS and NS wheel revolutions (DS_{cnts}/NS_{cnts}) was calculated. These measures will be 1 if LDLD-induced split rhythms are symmetrical and >1 if the DS is the more robust activity component. Additionally, the number of hours between the average onset of the DS and that for the NS (ψ_{DS-NS}) was determined. This latter measure will be 12 h if split activity components are in antiphase under LDLD, and less than 12 if the DS phase leads the NS. Split entrainment measures were correlated with measures of fusion after each phase of release to determine whether properties of

the split rhythm were related to individual differences in the fusion of split rhythms under constant conditions.

Fusion under constant conditions. To quantify the process of split fusion, I first determined the phase of the LDLD cycle from which the fused rhythm derived. Period (τ) of the fused rhythm was measured by the slope of a regression line fit to 5-7 consecutive activity onsets, excluding the first four days after release into constant conditions (Figure 4.2). Activity onset was identified as the bin above a threshold of 15 counts that was preceded and following by at least two bins below and above threshold, respectively. Using the phase and slope of this regression line, activity onset was projected backwards to the last day under LDLD, and the retrojected phase of activity onset was recorded for each individual animal. The LDLD phase of retrojected activity onsets is plotted in angular degrees, with 0° being the midpoint of the NS.

While it can be difficult to identify visually the exact cycle at which fusion is complete, the latency to the fused state may be operationalized by the day on which circadian period and waveform assume steady state levels. Day-to-day measures of circadian period and waveform were calculated for each cycle during the two weeks after release into constant conditions (Figure 4.2), with the expectation that fusion is complete when these measures stabilize. On the first few cycles after release into constant conditions, it is ambiguous which of the two activity bouts corresponds to activity onset and which to activity offset; however, once the two activity components have fused together, activity onset and offset are unambiguous. Thus, day-to-day measures of circadian period and waveform were based on the characteristics of the

fused activity rhythm. Starting with the last full cycle under constant conditions and working backwards, activity onset was identified on each cycle using the threshold criteria described above and temporal continuity with the fused activity rhythm. Activity offset was identified on each cycle as the last bin above threshold before the next activity onset. For each cycle, α was calculated from the difference between activity offset and onset. The duration of the longest inactive period (ρ) was also determined because this measure was unambiguous, even when two activity bouts were evident on the first few cycles immediately after release from LDLD. Lastly, the day-to-day period of activity onset was calculated by the number of hours separating two consecutive activity onsets, and the day-to-day period of activity offset was determined using two consecutive activity offsets. Variance in the period for each phase marker was then calculated with a three-day moving window.

Statistical analyses. Most statistical tests were conducted with JMP software (SAS Institute, Cary, NC). Split entrainment measures (DS_{BL}/NS_{BL} , DS_{cnts}/NS_{cnts} , ψ_{DS-NS}) were analyzed with the Goodness of Fit test to determine whether average activity ratios were different from 1 and whether the average ψ_{DS-NS} was different from 12 h. The Raleigh test was used to test for randomness of the distribution of activity onsets projected back to the last day under LDLD (Batschelet, 1981). Continuously varying circadian measures under free-running conditions (day-to-day α , ρ , and variance of period for activity onset and offset) were assessed using repeated measures ANOVA with Bonferroni tests to control for multiple comparisons. Comparisons where $p < 0.05$ are also reported. Split entrainment measures (i.e., $NS\psi_{L/D}$, $DS\psi_{L/D}$, DS_{BL}/NS_{BL} ,

DS_{cnts}/NS_{cnts} , ψ_{DS-NS}) were correlated with measures of fusion to determine if the fusion of split rhythms is related to individual differences in entrainment prior to release.

Results

LDLD-induced splitting. All animals exhibited split activity rhythms shortly after transfer to LDLD with 4 h dimly lit nights, with the majority of animals splitting under LDLD after the first or second cage change (Table 4.1). Split rhythms under LDLD 8:4:8:4 were relatively stable, with no more than 9% of animals rejoining (Table 4.1). On average, split activity components were not symmetric in terms of activity ratios or ψ_{DS-NS} (Table 4.1; Goodness of fit test, $p < 0.001$). In general, the DS activity bout was longer and more robust than the NS, but phase lagged behind the NS since ψ_{DS-NS} was greater than 12 h.

Fusion under constant conditions. DIM and DARK groups released from the same scotophase did not differ in the retrojected phase of activity onset on the last day of LDLD (Figure 4.3). For animals released from the NS, the retrojected phase of activity onsets were significantly clustered within the NS (Raleigh test, $p < 0.001$ for both DIM and DARK groups), and groups of animals released into DIM and DARK did not differ in average phase under LDLD ($t(20) = 0.33$, $p = 0.75$). Relative to NS release, there was more group variability in the phase of retrojected activity onsets after DS release (post hoc, Levene test, $p < 0.001$), but DS groups were still significantly clustered (Raleigh test, $p < 0.001$ for both DIM and DARK groups). The

average activity onset after DS release was ~ 8 h after the end of the NS, and again scotopic condition was not a significant factor ($t(18) = 1.82$, $p = 0.09$). The average phase of activity onset for the DS release did not project back to the same phase as that observed after NS release (post hoc, χ^2 test, $p < 0.001$), nor to a phase 12 h later than the average activity onset for NS release (post hoc, χ^2 test, $p < 0.001$).

Variation in the period of activity onset and offset was negligible within five days after release into constant conditions, and DIM and DARK groups differed in the latency to adopt steady state levels in a manner that depended on the phase of release from LDLD (Figure 4.4). After NS release, day-to-day variation in the period of activity onset was immediately low in both DIM and DARK groups, but variation in the period of activity offset was smaller in animals released into DARK rather than DIM (LS Means Contrasts, $p < 0.05$). After DS release, variation in the period of activity onset was high in the first few days immediately after release and smaller if animals were transferred to DARK instead of DIM (LS Means Contrasts, $p < 0.05$). Variation in the period of activity offset did not differ between DIM and DARK groups released from the DS.

Phase of release and scotopic condition also influenced day-to-day changes in circadian waveform after release in constant conditions (Figure 4.5). Over the first three days after release, all groups displayed increases in the duration of the inactive phase (ρ) and decreases in the duration of the active phase (α). After NS release, both DIM and DARK groups increased ρ to ~ 14 h, but DARK-NS animals showed longer ρ than DIM cohorts on the first few days after release (LS Means Contrasts, $p < 0.05$).

Relative to NS release, DIM-DS animals adopted a similar steady state level ($\rho \sim 14$ h), but DARK-DS animals showed a larger increase in the inactive phase, with $\rho \sim 16$ h for the first week after release (LS Means Contrasts, $p < 0.05$). Measures of α complement results obtained with ρ , and DARK animals had shorter α than DIM animals after both NS and DS release (LS Means Contrasts, $p < 0.05$).

Fusion after NS and DS release is illustrated by tracings of activity onsets and offsets for individual animals within each group (Figure 4.6). The most striking differences in these tracings are those produced by NS and DS release, although scotopic differences in circadian waveform are also evident in groups released from the same scotophase.

Circadian period and activity duration after fusion. After one week under constant conditions, τ increased under DIM regardless of which scotophase coincided with release (Table 4.2; SC: $p < 0.05$, Release, SC*Release: $p > 0.25$). Thirteen days after release, DIM-NS animals increased α , such that they had longer α than DARK-NS animals at the end of the experiment (Figure 4.5). Additionally, DARK-DS animals increased α approximately thirteen days after release and were no different from DIM-DS animals at the end of the experiment (Figure 4.5).

Split entrainment. Correlations between split entrainment and measures of fusion are compiled in Table 4.3. After DS release, split entrainment measures were largely unable to account for individual differences in the fusion of split rhythms, with the exception that animals with a DS component phased closer to the light-to-dark transition (i.e., less negative $DS\psi_{L/D}$) exhibited less variation in the period of activity

offset on the first few cycles after release into constant condition (test on slope, $F(20) = 5.99$, $p < 0.05$). In contrast, several parameters of split entrainment correlated with fusion after NS release, which was the group with the least variation between animals. After NS release, animals with activity ratios > 1 (i.e., larger DS component) displayed a retrojected activity onset that was phased later in the NS (test on slope, $F(18) > 4.62$, $p < 0.05$ in both cases). Furthermore, the variation in the period of activity onset on the first few cycles after NS release was lower in animals that displayed 1) a NS activity onset phased closer to the light-to-dark transition (i.e., less negative $NS\psi_{L/D}$), 2) a DS activity onset phased later relative to the light-to-dark transition (i.e., more negative $DS\psi_{L/D}$), and 3) split rhythms that were symmetrical (test on slope, $F(18) > 5.95$, $p < 0.05$ in all cases).

Discussion

The present studies are the first to quantify the fusion of split rhythms after release from LDLD. Fusion of split rhythms after release into constant conditions reflects interactions between functionally distinct oscillators, which can be studied under conditions lacking the confounding influence of other cues (e.g., bright light). Furthermore, the present studies test whether the interactions underlying the fusion of split rhythms are sensitive to dim illumination. Dim illumination modulated the fusion of split rhythms in two different ways: constant dim light increased the latency to fusion and altered the properties of the fused rhythm. The present results provide

insights into differences between oscillators split under LDLD and the manner in which dim light influences circadian coupling.

In many cases, the emergence of split rhythms under LDLD is inconsistent with a strict dual oscillator model postulating that one of two indivisible oscillators is re-entrained to the daytime scotophase (Evans & Gorman, 2002; Gorman, 2001; Gorman & Lee, 2001). In previous studies, using admittedly different splitting protocols, the daytime activity component first emerges close to the lights-on transition of the DS and advances over successive days as its bout length increases. Simultaneously, the activity onset of the NS activity component delays and its bout length decreases. Progressive changes in the daytime activity component and the nighttime activity component are highly correlated, which suggests that this pattern of splitting represents the re-entrainment of successive cohorts of oscillators into the DS. This gradual pattern of split emergence was also displayed by animals that split before a cage change in Experiment 2A (c.f., Chapter 2). In the present study, split rhythms induced under LDLD with dimly lit nights were not symmetrical or 12 h apart prior to release into constant conditions in a manner consistent with the results of previous studies (Gorman, 2001; Gorman & Elliott, 2003; Gorman & Steele, 2006). The DS activity bout was longer and more robust than the NS activity bout (DS_{BL}/NS_{BL} and $DS_{cnts}/NS_{cnts} > 1$), in addition to lagging behind the phase of the NS activity bout ($\psi_{DS-NS} > \psi_{NS-DS}$). This asymmetry suggests that a larger group of oscillators are programming the DS than that programming the NS, and it could be that the fused rhythm will always derive from the scotophase with the most oscillators (i.e., the DS)

regardless of the phase of release. The present study does not support this view, and the derived phase of the fused rhythm was not strictly determined by asymmetries in the split state prior to release. Instead, the fused rhythm was more likely to derive from a phase within a half cycle of the NS (i.e., 95% of the retrojected activity onsets were between 0 and 180 degrees, c.f., Figure 4.3).

NS and DS release do not produce identical or mirrored patterns of split fusion, indicating that the group of oscillators programming the NS and DS activity bouts are functionally distinct. While DS and NS release were conducted here four months apart using two separate groups of animals, the present results confirm those from a previous study that simultaneously released two groups of split Syrian hamsters from either the DS or NS (Gorman and Elliott, personal observations). Consistent with this earlier study, retrojected activity onsets after NS and DS release produced highly clustered and variable patterns, respectively. Similarities between the retrojected phases of activity onset for the DIM and DARK groups provide an internal replication for each release phase. Functional distinctions between the oscillators programming the NS and DS activity bouts and the timing of the last bright light exposure likely influence the interactions between oscillators that mediate the fusion of split rhythms after release into constant conditions.

Differences in the variation of circadian period after NS and DS release reflect disparities in the pattern of fusion after each phase of release from LDLD. In the first few days after DS release, it was quite common for two activity components to appear near the prior phase of each scotophase (c.f., Figure 4.1). In these records, the activity

bout closest to the phase of the NS delayed over 2-3 cycles before it disappeared, which coincided with systematic advances of a larger activity bout tracing back to the phase of the DS. Large advances of this “DS” component produced variation of the period of activity onset immediately after DS release. In general, this pattern was much more common in animals that had a fused activity rhythm derived from a phase closer to the NS. In contrast, it was much harder to distinguish two distinct bouts on the cycles immediately after NS release (c.f., Figure 4.1). Activity onset of the fused rhythm always coincided with a phase within the NS, whereas activity offset largely emerged from a phase close to the DS. Variation in the period of activity offset did not assume steady state levels until a few days after release since activity offset advanced over the first few days following NS release. Thus, similar to that observed after DS release, the phase marker coinciding with the DS activity component showed systematic advances and large variation in period over the first few days after NS release. This correspondence suggests that oscillators formerly programming the DS activity component were shifting closer to the phase of the NS activity component after both NS and DS release.

However, the interactions between split oscillators released into constant conditions were not completely one-sided, since the NS activity component was also affected by the properties of DS activity component. After NS release, animals that displayed a fused rhythm deriving from a phase late within the NS exhibited delaying transients in activity onset over the first few days immediately after release (c.f., Figure 4.1). Since the derived phase of the fused rhythm was later in animals that had

a longer and more robust DS component, this suggests that the DS component is able to shift the phase of the NS activity component when it is larger than the NS component. Additionally, there were marked changes in the phase and duration of the NS activity component immediately following DS release, as described above. Furthermore, the variation in the period of activity offset was smaller when animals had a DS activity component phased closer to the light-to-dark transition, again suggesting that entrained properties of the DS modulate its influence over the NS activity component. The present pattern of results suggests that the rate and pattern of split fusion is influenced by differences in the degree to which the NS and DS oscillators are able to affect one another. Future studies incorporating more than two phases of release may lend insight into the nature of these interactions and how each split activity component differs in their contribution.

Dim illumination did not alter the derived phase of the fused rhythm, or the general pattern of rejoining displayed by DIM and DARK groups released from the same phase (e.g., NS or DS). Relative to complete darkness, constant dim illumination nevertheless modulated the latency to reaching a steady state in a manner dependent on the phase of release. DIM increased variability in period of activity onset over the first few days after DS release, and increased variability in period of activity offset over the first few days after NS release. Higher variation in period expressed by DIM-DS and DIM-NS animals reflects large systematic changes in activity onset and offset, respectively, which manifested in the first few days after release. DARK cohorts tended to show a more instantaneous change in the phase of each marker.

Furthermore, DIM increased the latency to steady state ρ and α , with larger effects when released from the DS. While DIM-NS, DIM-DS, and DARK-NS groups appeared to reach a similar steady state level within five days of release from LDLD, DARK-NS animals displayed an initial state with a larger ρ and smaller α . Thus, the largest effects of DIM were not on the process of fusion per se, but the fused state to which the split system readily returned after DS release.

After release from LDLD coincident with each of the two daily scotophases, DIM increased the latency to steady state period and waveform by approximately one day, which is consistent with a transient inhibition of the interactions that promote rejoining of split oscillators under free-running conditions. Conditions of dim illumination also increased circadian period of the fused rhythm after both NS and DS release and promoted a long α steady state after DS release. This latter effect is consistent with the hypothesis that dim illumination strengthens a response that causes underlying oscillators to interact in such a way to resist the compression of subjective night. It remains unclear why this latter effect is dependent on the phase of release from LDLD, but this potentially arises from an interaction between dim light and the timing of the last bright light exposure. Rather than supporting the hypothesis that dim illumination globally inhibits or strengthens oscillator interactions, the present study is consistent with the hypothesis that dim illumination alters the shape of the coupling response curve for oscillators within the mammalian pacemaker.

Under LDLD, the split rhythm is maintained by the long bright light photophases that inhibit interactions between underlying oscillators that would

otherwise cause the system to rejoin. Previous studies that deleted or reduced individual photophases on a single cycle found the DS activity onset advanced more than the NS activity onset (Gorman & Steele, 2006). The present studies are consistent with the results of this earlier study in that the DS activity component was more labile than the NS under conditions where bright light stimulation is minimized. This indicates that oscillator interactions that produce the fusion of split rhythms are attenuated by bright light during the daily photophases under LDLD. Dim nighttime illumination may interact with bright light during daily photophases to maintain the split rhythm under entrained conditions. Determining whether dimly lit nights promote the maintenance of split rhythms under LDLD will provide insight into the mechanisms through which dim nighttime illumination alters coupling under LDLD.

Table 4.1 Split entrainment in Experiment 3

Release	% Split	Split at Transfer	Rejoined	DS_{BL} / NS_{BL}	DScnts / NS cnts	ψ DS-NS
DS	100%	46%	8%	1.28 ± 0.05	1.24 ± 0.05 h	12.62 ± 0.1 h
NS	100%	32%	9%	1.20 ± 0.05	1.14 ± 0.04 h	12.5 ± 0.1 h

BL: Bout Length

ψ DS-NS: Phase angle between the DS and NS

cnts: total wheel revolutions within that scotophase

DS: Daytime Scotophase

NS: Nighttime Scotophase

Table 4.2 Circadian period (h) after release from LDLD

Release	DIM	DARK	p value
DS	24.22 ± 0.03	23.96 ± 0.03	< 0.05
NS	24.15 ± 0.02	24.06 ± 0.03	< 0.05

Table 4.3 Correlations (r) between measures of split entrainment and fusion of split rhythms

DS Release	NS ψ /D	DS ψ /D	ψ DS-NS	DS _{RL} /NS _{RL}	DSents/NS ents
LDLD phase	0.01	-0.13	-0.05	-0.15	-0.08
α Day 1	0.07	-0.04	-0.07	-0.01	0.10
ρ Day 1	-0.25	-0.08	0.20	0.09	0.17
Var Per-onset Day 1-3	-0.14	-0.26	0.04	-0.01	0.10
Var Per-offset Day 1-3	0.13	-0.48*	-0.04	0.05	0.08
α Day 15	0.11	0.16	-0.05	-0.08	-0.08

NS Release	NS ψ /D	DS ψ /D	ψ DS-NS	DS _{RL} /NS _{RL}	DSents/NS ents
LDLD phase	-0.33	0.34	0.40†	0.45*	0.51*
α Day 1	0.42†	0.10	-0.27	-0.29	-0.34
ρ Day 1	-0.39†	-0.16	0.22	0.27	0.29
Var Per-onset Day 1-3	-0.57*	0.50*	0.65*	0.62*	0.67*
Var Per-offset Day 1-3	0.03	-0.18	-0.10	-0.08	-0.03
α Day 15	0.44†	0.15	-0.26	-0.29	-0.23

* $p < 0.05$ † $0.05 < p < 0.1$

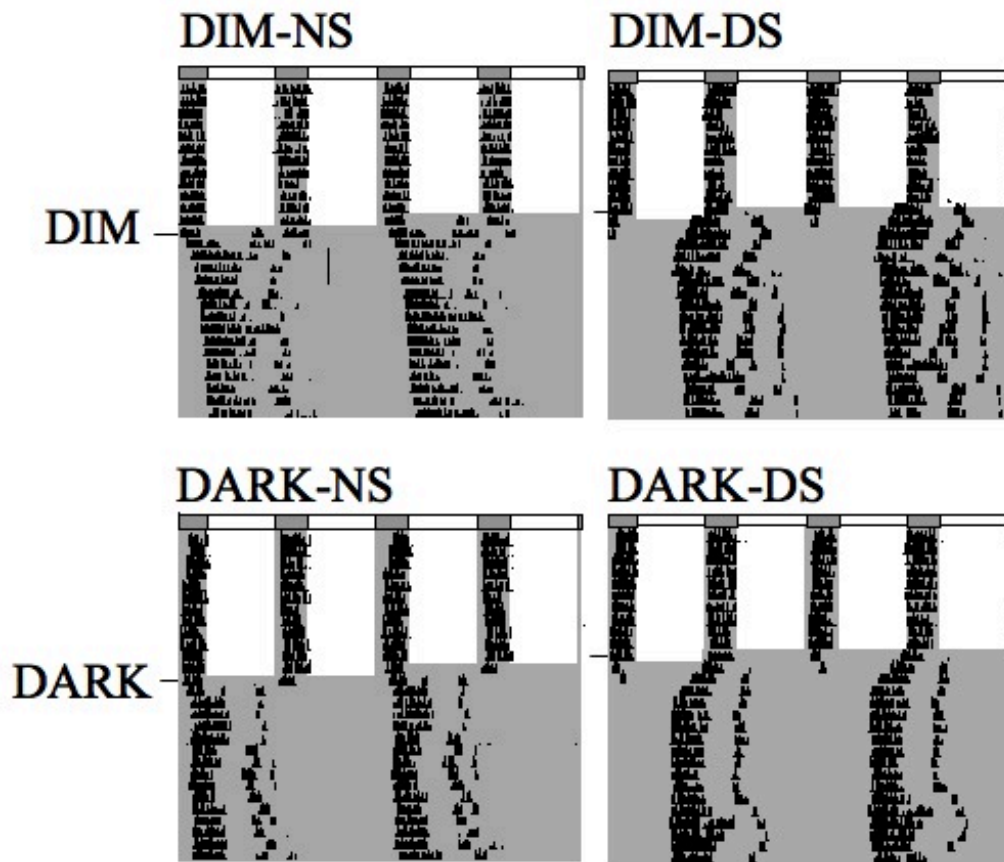


Figure 4.1: Representative double-plotted actograms of wheel-running rhythms of animals split under LDLD with dimly lit nights, then released from LDLD into constant DIM (top) or DARK (bottom) conditions coincident with either the Nighttime Scotophase (NS, left) or the Daytime Scotophase (DS, right).

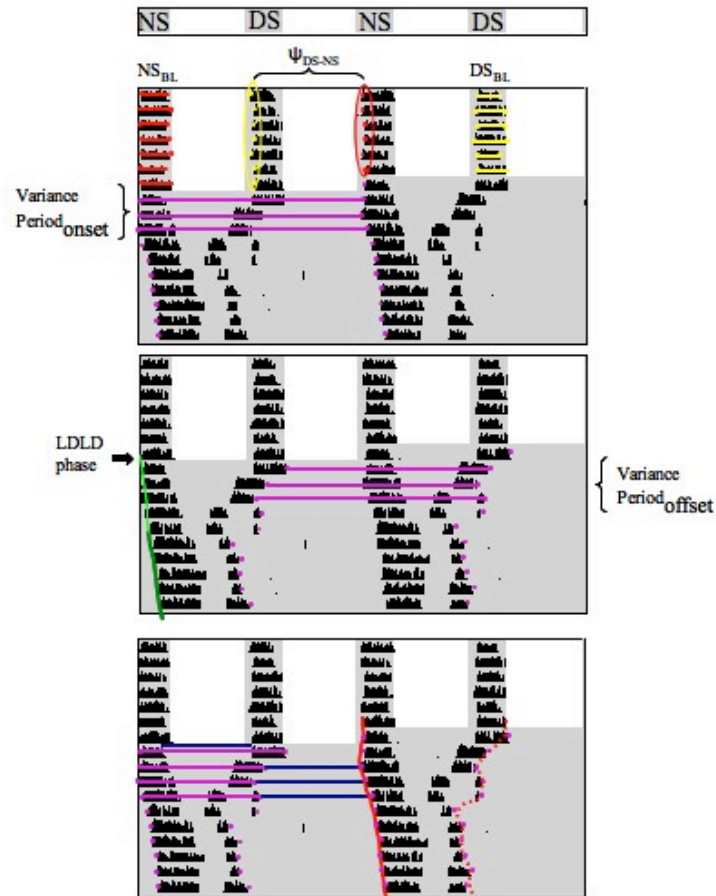


Figure 4.2: Measures of split entrainment and fusion of split rhythms after release from LDLD. Top: Activity onset and offset for the nighttime activity component (NS, red points) and the daytime activity component (DS, yellow points) was used to calculate the average bout length (BL) of each activity component (NS_{BL} , DS_{BL}) during the last week under LDLD. The phase difference between the DS and NS activity components (Ψ_{DS-NS}) is the number of hours separating their respective average onsets (yellow and red circles). After release into constant conditions, the day-to-day period of activity onset was determined using the difference in time between two consecutive onsets (purple lines) and variance was derived using a three-day moving window. Middle: The variance in the period of activity offset was likewise derived using two consecutive offsets (purple lines). Also, a linear regression line was fit to 5-7 consecutive activity onsets, excluding the first four days after release (dark green line). This line was then retrojected back to the last day under LDLD (light green line) to find the phase of the LDLD cycle from which the fused rhythm derived (LDLD phase). Bottom: Using the phase of activity onset on each day after release, the duration of the active phase was determined. The daily duration of the longest inactive phase was also recorded (blue lines). Illustrated to the right is a tracing of the “pattern” of fusion after release into constant conditions (solid and dashed red lines represent activity onset and offset, respectively).

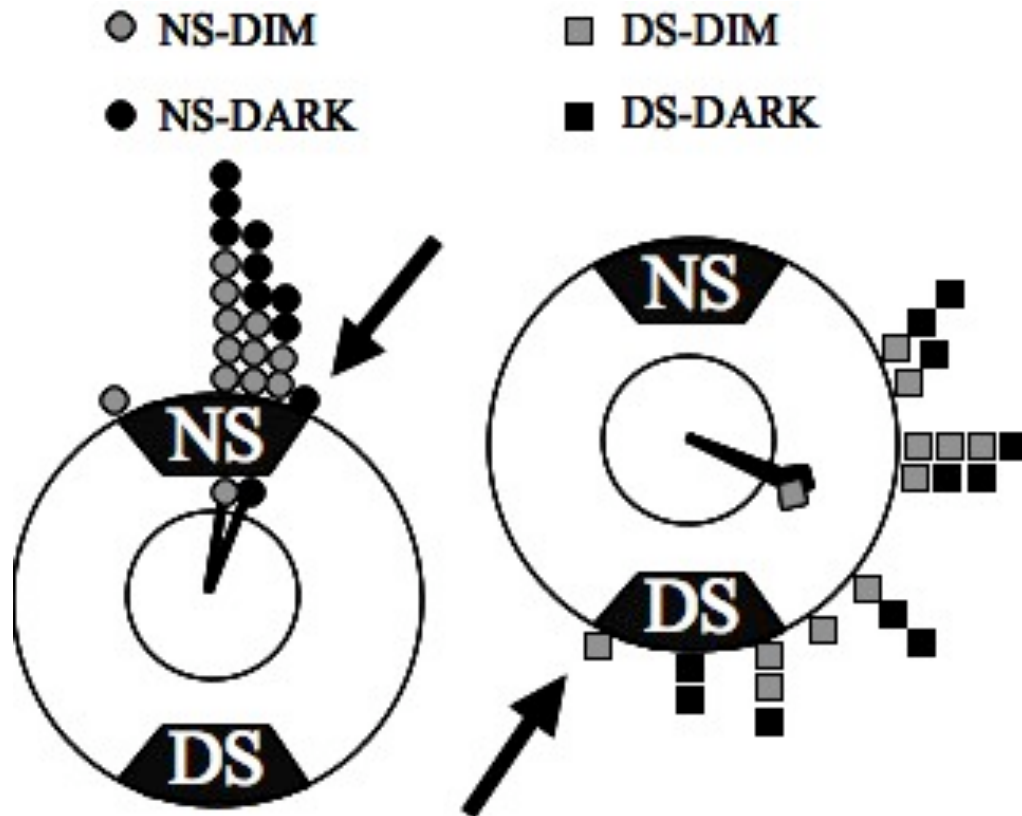


Figure 4.3: Phase distributions of activity onsets projected back to the first cycle after release from LDLD into constant DIM or DARK conditions coincident with the Nighttime Scotophase (NS, left) or the Daytime Scotophase (DS, right). Phase of activity onset is plotted in angular degrees, with the midpoint of the NS as 0 degrees. Each symbol on the circumference of the circle is the activity onset for a single animal. Phase on the first cycle was interpolated using the free-running period and average phase of activity onset calculated for days 4-9 post-release. Shaded sections within circle indicate the prior phase of the NS and DS, and the large arrow outside each circle represents the phase of release. Lines extending from the center of the circle represent mean angular vectors, and those extending outside the inner circle indicate significant clustering for that group as determined by the Raleigh test ($p < 0.05$).

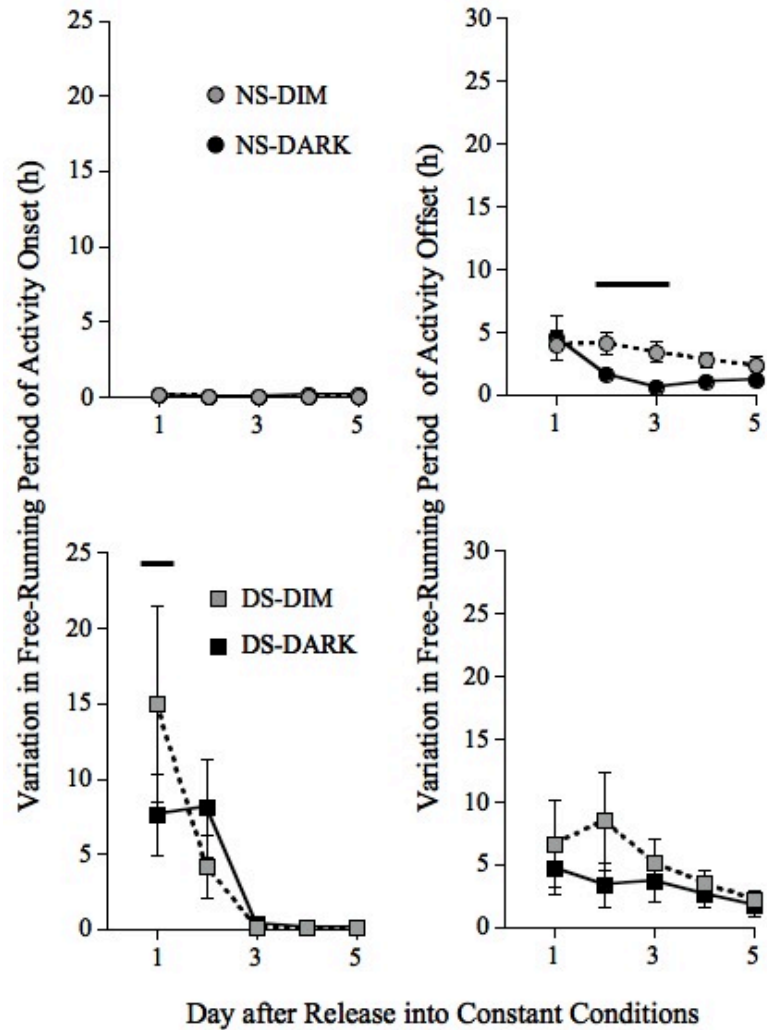


Figure 4.4: Variation in period of activity onset and offset after release from the NS (top) or DS (bottom) into constant DIM (gray symbols) or DARK conditions (black symbols). Left: Variation in day-to-day period of activity onset after release. Right: Variation in day-to-day period of activity offset after release. Variance in period is measured by a 3-day moving window, and the day since release into constant conditions represents the first day in this window. ---- $p < 0.05$, uncorrected for multiple comparisons.

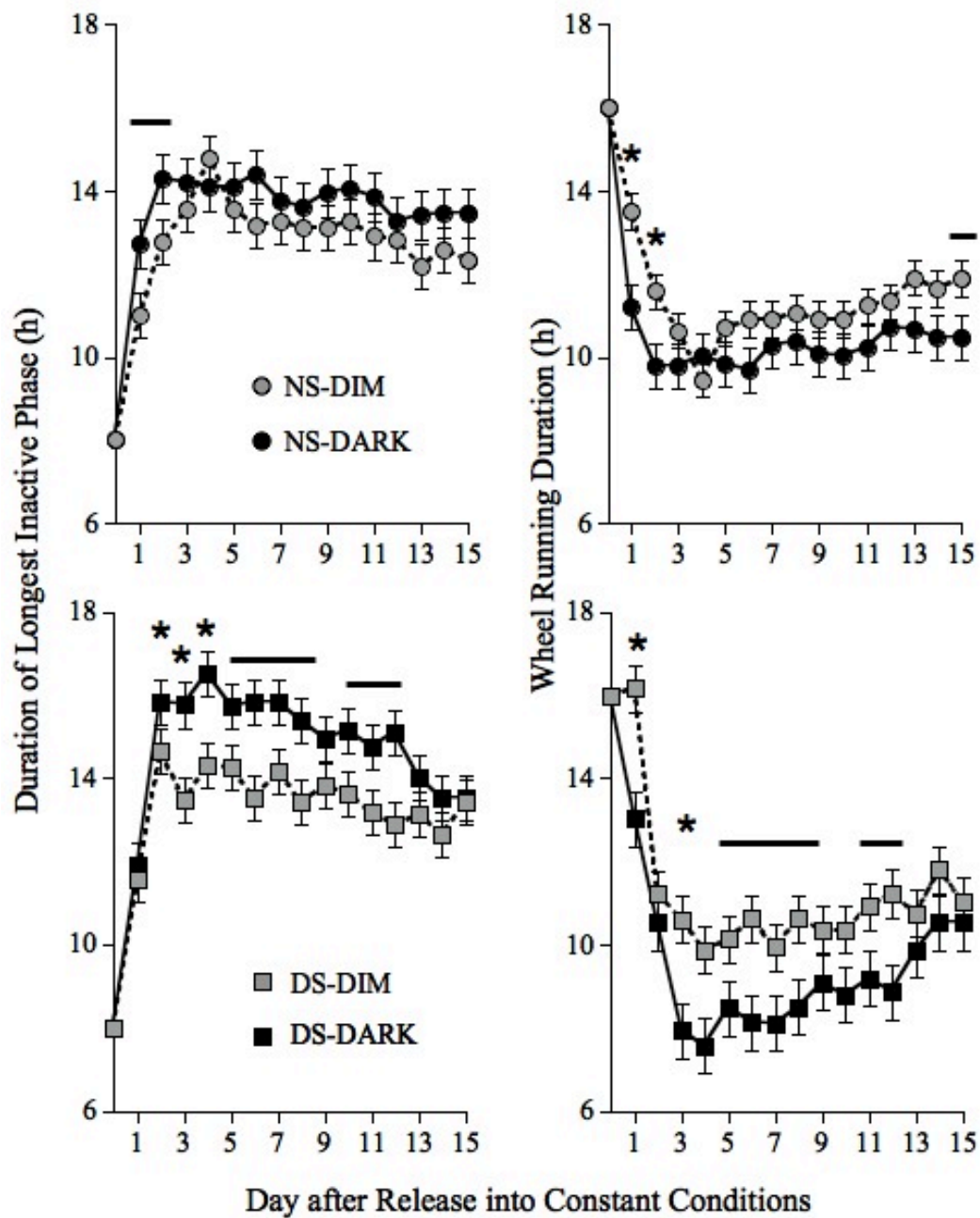


Figure 4.5: Changes in circadian waveform after release from the NS (top) or DS (bottom) into constant DIM or DARK conditions. Left: Day-to-day duration of the longest inactive phase during the first two weeks after release. Right: Day-to-day duration of the active phase during the first two weeks after release. DIM versus DARK: * $p < 0.003$ according to the Bonferroni correction for multiple comparisons, ---- $p < 0.05$ uncorrected for multiple comparisons.

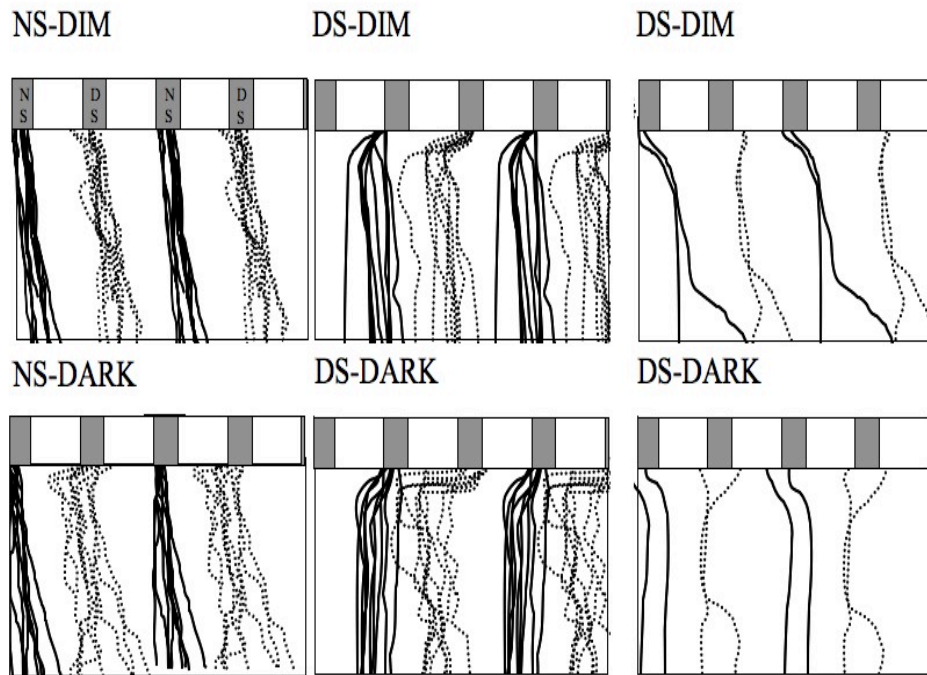


Figure 4.6: Double-plotted tracings of wheel-running rhythms after release from LDLD into constant DIM (top) or DARK (bottom). Lines tracking changes in activity onset (solid lines) and activity offset (dashed lines) were based on the phase of rejoined rhythms. Please note, two DIM and two DARK animals fused after DS release in a manner that differed from the majority of animals, and are plotted separately for clarity.

Chapter 5

Experiment 4: Dim light and the maintenance of split rhythms under LDLD

Dim illumination facilitates the induction of split rhythms under LDLD and modulates fusion after release into constant conditions. This demonstrates that dim light not only affects oscillator interactions when the system is entrained to short nights, but can also influence coupling after the circadian pacemaker is reorganized under LDLD. However, split rhythms rapidly rejoined after release into both constant dim and dark conditions, and the strongest factor influencing fusion under constant conditions was the phase of release. Bright light during the daily photophases under LDLD is critical for preventing fusion. Once split under LDLD, split oscillators may be entrained via a nonparametric action of bright light (i.e., resetting by light pulses at the beginning and end of subjective night) or a parametric action of bright light throughout the daily photophases. Nonparametric actions of light appear sufficient to entrain split rhythms in the hamster since split rhythms in Syrian and Siberian hamsters can be entrained to “skeleton” LDLD cycles (c.f., Figure 5.1), where two short light pulses simulate each of the two daily photophases (Gorman & Elliott, 2003). In contrast, split rhythms readily rejoin in split mice held under skeleton LDLD cycles, establishing that parametric actions of light during the “full” photophases are necessary to maintain split rhythms in the mouse (Gorman & Elliott, 2003; Rosenthal et al., 2005).

Dim nighttime illumination is critical for LDLD-induced splitting in the hamster but it may not be necessary for maintaining the split state. Dimly lit nights facilitate two “triggers” for splitting under LDLD (i.e., nonphotic phase advances and phase jumps, c.f., Chapter 3). Through the latter mechanism, dim nighttime illumination increases the duration of the “minimal tolerable night” and causes a subgroup of oscillators to re-entrain to the daytime scotophase. Once the system is split, bright light during the daily photophases may be sufficient to prevent split activity components from rejoining. If dim light has only a modest effect on the interactions between oscillators that promote rejoining, then extinguishing dim nighttime illumination would have very little effect on the maintenance of the split rhythm. However, if dim light interacts with bright light under entrained conditions to inhibit interactions between oscillators that promote the integrated state, then extinguishing dim nighttime illumination would cause the system to rejoin under LDLD.

Dim light is able to interact with bright light in a manner that depends on the entrained state of the pacemaker. Relative to complete darkness, background conditions of dim illumination do not augment the amplitude of the phase response curve to 5 min 100 lux light pulses (c.f., Chapter 2, Experiment 1B), indicating that dim light does not directly potentiate the resetting actions of brighter light. When animals were transferred from LD 14:10 to LD 17:7 in Experiment 2C, however, re-entrainment with dimly lit nights occurred faster than with completely dark nights. Subsequent entrainment under skeleton photoperiods simulating increasing day

lengths with 1 h bright light pulses did not differ between animals held under dimly lit and completely dark nights until the former group started to phase jump into the alternative scotophase (c.f., Chapter 3, Experiment 2C). Furthermore, dim light augmented phase resetting after novel wheel running was followed by a 7 h bright light pulse, but not when novel wheel running was provided alone (c.f., Chapter 3, Experiment 2B). The present body of data suggests that dim light is able to interact with bright light pulses that are longer than 1 h in length. This may reflect an interaction between dim nighttime illumination and a nonparametric or parametric response to bright light.

The present studies address whether dim nighttime illumination facilitates the maintenance of split rhythms under LDLD, and whether this is due to an interaction with a parametric response to bright light during the two daily photophases. Animals were split under LDLD with dimly lit scotophases, and after several weeks, dim nighttime illumination was either retained or extinguished. If dim light acts to maintain split rhythms under LDLD, extinguishing dim nighttime illumination should cause split activity components to rejoin. Further, if dim light maintains the split state by interacting with a parametric response to bright light, then dim nighttime illumination should not promote the maintenance of split rhythms under “skeleton” LDLD cycles. In the first study investigating these hypotheses, dimly lit nights facilitated the maintenance of split rhythms under LDLD cycles with “full” photophases, and subsequent manipulations were designed to test the effects of dimly lit nights under skeleton LDLD cycles. In this first study, I found marked differences

in the influence of dimly lit nights under each of the LDLD conditions; however, the sequential nature of the design limited the ability to make strong conclusions since the skeleton LDLD was imposed after a greater number of weeks under LDLD. Two further experiments were designed with this in mind, and all three experiments are presented here.

Methods and Procedures

Male and female Syrian hamsters were bred and reared as described previously (c.f., Chapter 2). Equal numbers of males and females were used in each of the experiments below, but there were no sex differences in the response to experimental manipulations. Hamsters (starting age: 6-8 wks) were transferred to a LDLD 7:5:7:5 cycle (photophase: 300 lux, lights on: 0300 PST, lights off: 1000 PST, lights on: 1500 PST, lights off: 2200 PST) coincident with the start of the daytime scotophase (DS). DIM nighttime illumination (< 0.1 lux) was provided to all animals by narrow bandwidth green light-emitting diodes (LEDs, peak $\lambda = 560$ nm with a half band width of 23 nm) mounted externally and facing the back wall of each cage. Unless indicated otherwise, cages were changed during the first 90 minutes of the DS one week after transfer to LDLD. Each animal was relocated to a new cage with fresh food and water under the direction of a dim red headlamp (< 2 min exposure/ animal). Cages were changed at an identical phase once every two weeks thereafter.

As described below, Experiment 4 was conducted as three successive rounds (4A, 4B, 4C), which were largely replications with slight differences in protocol and

sample size. All experimental manipulations are illustrated in Figure 5.1. In each experiment, dim nighttime illumination was either extinguished or retained (DARK- and DIM-) under LDLD with “full” 7 h photophases (fLDLD, same as the initial LDLD cycle to which animals were first transferred) or a “skeleton” LDLD cycle (sLDLD), where daily photophases were simulated using 1 h light pulses bracketing each of the daily scotophases. Note that sLDLD is essentially a LD 1:5 cycle in which each 1 h light pulse is followed by 5 h of relative darkness.

In Experiments 4A, hamsters (N = 38, 19/sex) were transferred to individual wheel-running cages (27 x 20 x 15 cm) inside light-tight environmental chambers (1 cage/chamber). Scotopic manipulations under fLDLD and sLDLD occurred five and nine weeks after the start of the experiment, respectively (Figure 5.1 and Figure 5.2), and were always implemented two days before a cage change. Five weeks after transfer to LDLD, LEDs were extinguished in a subset of animals (DARK-fLDLD) during the photophase immediately before the nighttime scotophase (NS). Dim nighttime illumination was retained for the remaining animals (DIM-fLDLD). Four weeks later, a subset of DIM-fLDLD animals were provided with DARK nights coincident with the start of a “skeleton” LDLD cycle (DARK-sLDLD), beginning immediately after the NS. The remaining animals were provided sLDLD and dimly lit nights (DIM-sLDLD). Animals remained under these latter conditions for four weeks.

The results of Experiment 4A suggested that the effects of dim nighttime illumination were not the same under fLDLD and sLDLD, however, these results were confounded by a discrepancy in the number of weeks under LDLD. Thus, Experiment

4B was conducted to assess whether dimly lit nights influence the maintenance of split rhythms after nine weeks under LDLD, which coincides with the sLDLD manipulation in Experiment 4A (Figure 5.1). As in Experiment 4A, hamsters (N = 38, 19/sex) were transferred to wheel-running cages coincident with the DS of a LDLD 7:5:7:5 cycle. After nine weeks under LDLD, dim nighttime illumination was either extinguished (DARK-fLDLD) immediately before the NS or retained (DIM-fLDLD). Animals remained under these conditions for four weeks (Figure 5.3). No sLDLD manipulation occurred in Experiment 4B.

In Experiment 4C, hamsters (N = 60, 30/sex) were transferred to wheel-running cages (48 x 27 x 20 cm) inside light-tight environmental chambers (12 cages/chamber) coincident with the DS of a LDLD 7:5:7:5 cycle. Different housing conditions were used in this final experiment to increase the initial sample size and permit simultaneous fLDLD and sLDLD manipulations (Figure 5.1). The first cage change did not occur until two weeks after transfer to LDLD and cages were changed once every two weeks thereafter. After five weeks under fLDLD, hamsters were divided into four groups, which differed by 1) whether the daily dark periods were DIM- or DARK- and 2) whether the LDLD cycle was fLDLD or sLDLD (Figure 5.4 and Figure 5.5). Beginning with the NS, daily dark periods were either DIM or DARK and followed by either full 7 h photophases or skeleton photophases. Animals remained under these conditions for five weeks.

Data Collection and Analyses

Split entrainment was quantified during the fifth week under LDLD using bout analyses (c.f., Chapter 4, Experiment 3). In Experiment 4B, bout analyses were also conducted on split rhythms during the ninth week under LDLD since scotopic manipulations were performed later in this study. Using data for each split activity component, split symmetry measures (i.e., ψ_{DS-NS} , DS_{BL}/NS_{BL} , DS_{cnts}/NS_{cnts}) were calculated and tested as in Experiment 3 (c.f., Chapter 4).

To determine the effects of dimly lit nights and completely dark nights on the maintenance of split rhythms under fLDLD and sLDLD, qualitative analyses categorized animals as split or rejoined during the four weeks subsequent to each experimental manipulation. Consistent with previous experiments, there was no ambiguity in classifying animals. Activity rhythms were categorized as split if animals displayed wheel-running bouts longer than 30 min during each of the two daily dark periods for at least five consecutive days. Activity rhythms were categorized as rejoined if formerly split animals confined activity to one of the two daily dark periods for at least five consecutive days. If an animal displayed a rejoined rhythm it was possible for the split rhythm to re-emerge. An unsplit animal was noted to re-split if the split activity rhythm reappeared and persisted for at least five consecutive days. Effects of dimly lit nights versus completely dark nights on the incidence of re-splitting were also tested as means of replicating earlier results demonstrating that dim nighttime illumination facilitates the induction of split rhythms under LDLD. Data from Experiments 4A, 4B, and 4C were tested separately but are plotted together.

Statistical analyses. Most statistical tests were conducted with JMP software (SAS Institute, Cary, NC). Categorical data were analyzed using contingency statistics (Pearson's χ^2). To assess split symmetry, entrainment measures (DS_{BL}/NS_{BL} , DS_{cnts}/NS_{cnts} , ψ_{DS-NS}) were tested to determine whether activity ratios were equal to 1 and whether ψ_{DS-NS} was equal to 12 h (Goodness of Fit test).

Results

In each experiment, the majority of animals exhibited split activity rhythms under LDLD with dimly lit nights (Table 5.1). In Experiment 4A, approximately half of the animals that split did so in the week immediately following the initial transfer to LDLD; whereas in Experiment 4B, a much smaller percentage of animals split within this Interval (Table 5.1). In Experiment 4C, the majority of animals split in the two weeks immediately following the initial transfer to LDLD, which was before the first cage change in this study (Table 5.1). The timing of split induction influenced whether split rhythms would rejoin spontaneously, with animals that split after a cage change exhibiting a larger incidence of rejoining during the first five weeks under LDLD (Figure 5.6).

After transfer to LDLD in Experiment 4A, 89% of animals split (34/38), and 45% (15/34) of these split animals rejoined within five weeks after transfer to LDLD. Of animals that remained stably split during the fifth week of LDLD, split rhythms were asymmetrical, with unequal activity bouts not exactly 12 h apart (Table 5.1; Goodness of fit test, $p < 0.05$). On average, the DS activity bout was longer and more

robust than the NS, but phase lagged behind the NS, since ψ_{DS-NS} was greater than 12 h. When the dim nighttime illumination was extinguished five weeks after transfer to LDLD, all of the split DARK-fLDLD animals rejoined their split rhythms during the following four weeks (Figure 5.7). Fewer animals maintained under fLDLD with dimly lit nights rejoined their split rhythms during this time ($\chi^2(21) = 14.83$, $p < 0.0005$). Moreover, among animals displaying unsplit rhythms during the fifth week of LDLD and those animals that rejoined thereafter, dimly lit nights increased the incidence of re-splitting relative to completely dark nights (Figure 5.7, $\chi^2(22) = 10.97$, $p < 0.005$). When sLDLD cycles were introduced, nearly all animals under both dimly lit and completely dark nights rejoined within four weeks, and only two animals under each scotopic condition remained split throughout the four weeks under sLDLD. Many animals rejoined within one week of sLDLD, and groups of animals held under dimly lit and completely dark nights did not differ in the incidence of rejoining during the first week under sLDLD (Figure 5.8, $p > 0.3$). Moreover, groups under sLDLD with dimly lit and completely dark nights did not differ in the incidence of re-splitting over the four weeks under sLDLD (Figure 5.8, $p > 0.3$).

The effect of dimly lit nights under fLDLD but not sLDLD in Experiment 4A is consistent with the hypothesis that dim nighttime illumination maintains split rhythms by interacting with parametric light exposure; however, fLDLD and sLDLD manipulations did not occur after the same number of weeks under LDLD. The absence of an effect of dimly lit nights under sLDLD might reflect a decrease in the sensitivity to dim light after longer entrainment to LDLD. Thus, Experiment 4B was

conducted to assess whether dim nighttime illumination influences the maintenance of split rhythms under fLDLD nine weeks after transfer to LDLD.

In Experiment 4B, 95% of animals split during the first nine weeks under LDLD (36/38), but 86% (31/36) of these split animals rejoined spontaneously. Of animals that were split in the fifth week of LDLD, split rhythms were asymmetrical, with unequal activity bouts not exactly 12 h apart (Table 5.1; Goodness of fit test, $p < 0.05$). Similar to that observed in Experiment 4A, the DS activity bout was longer and more robust than the NS and phase lagged behind the NS, since ψ_{DS-NS} was greater than 12 h. Of animals split during the ninth week of LDLD, however, split rhythms were statistically symmetrical (Table 5.1, Goodness of fit test, $p > 0.5$). This pattern was also evident in animals that remained split throughout the first nine weeks under LDLD ($n = 4$), with asymmetrical rhythms in the former Interval and symmetrical split rhythms in the latter Interval, thus split entrainment was changing over time under LDLD even in animals that never rejoined. Extinguishing dim nighttime illumination after nine weeks under LDLD caused the majority of split DARK-fLDLD animals to rejoin within the following four weeks, whereas fewer split DIM-fLDLD animals rejoined (Figure 5.7, $\chi^2(12) = 4.07$, $p < 0.05$), thus confirming the results of Experiment 4A at this later time point. However, dim nighttime illumination did not affect the incidence of re-splitting relative to animals under completely dark nights (Figure 5.8, $p > 0.8$).

In Experiment 4C, fLDLD and sLDLD manipulations were performed simultaneously to test directly whether dim nighttime illumination influences the

maintenance of split rhythms under LDLD through an interaction with parametric actions of bright light. After transfer to LDLD in Experiment 4C, 97% of animals split (58/60), and 41% (24/58) of these split animals rejoined within five weeks after transfer to LDLD. Animals that were split during the fifth week under LDLD displayed split rhythms that were asymmetrical in a manner consistent with that observed during Experiments 4A and 4B (Table 5.1; Goodness of fit test, $p < 0.05$).

In Experiment 4C, the stability of the split state under fLDLD depended on whether the daily scotophases were dimly lit or completely dark. 5/10 split animals rejoined within four weeks under DARK-fLDLD, whereas only 1/10 split animals rejoined under DIM-fLDLD (Figure 5.7, $\chi^2(18) = 3.81$, $p < 0.05$). Additionally, a smaller percentage of animals re-split their rhythms over the following four weeks if provided fLDLD with DARK rather than DIM nights (Figure 5.7), but these two groups were not significantly different from one another ($p > 0.2$).

In Experiment 4C, very few animals released into sLDLD retained their split rhythms over the subsequent four weeks. The two animals that remained split throughout sLDLD were both provided with DARK nights. Moreover, during the first week under sLDLD, the majority of split sLDLD-DIM animals rejoined, whereas most sLDLD-DARK cohorts remained split during this time (Figure 5.8). Thus, post hoc analyses were conducted to determine whether dimly lit nights accelerated the rejoining of split rhythms under sLDLD. The number of days until rejoining was determined by counting the number of cycles until activity appeared in a dark period other than the NS or DS. As illustrated in Figure 5.8, split DARK animals retained

their split rhythms under sLDLD much longer than split DIM animals (DIM: 4.6 ± 2.5 days, DARK: 12.6 ± 2.6 days, $t(17) = 2.3$, $p < 0.05$). As illustrated by individual activity profiles for the first week under sLDLD (Figure 5.9), the majority of DARK-sLDLD animals displayed activity only during the NS and DS of sLDLD. In contrast, the majority of DIM-sLDLD animals displayed additional bouts of activity that most frequently appeared during the dark period separating the NS and DS. One DIM-sLDLD animal displayed a bout of activity in each of the four daily dark periods (Figure 5.9, bottom DIM-sLDLD animal).

Some animals under sLDLD with either DARK ($n = 4$) or DIM ($n = 3$) nights were able to re-split their activity rhythms (Figure 5.8). Re-splitting under sLDLD was interesting in that during split emergence, very little activity was evident in the intervening dark period (Figure 5.4, upper right actogram).

Discussion

The present studies demonstrate that dimly lit nights not only facilitate the induction of split rhythms, but also serve to maintain the split state under LDLD. Across three separate studies, extinguishing dim nighttime illumination led to an increase in the incidence of rejoining under fLDLD, with significant effects of dim light present after both five and nine weeks under LDLD. In contrast, the vast majority of animals under sLDLD rejoined regardless of whether the daily dark periods were dimly lit or completely dark. This suggests that dim nighttime illumination acts to

maintain the split rhythm under fLDLD by interacting with a parametric response to bright light.

Fusion after release into constant conditions (c.f., Chapter 4) and rejoining under entrained conditions reflects interactions between split oscillators that promote the integrated state. After release into constant conditions, these interactions produce shifts in the phase of one or both oscillators until the system is consolidated. Likewise, rejoining under entrained conditions is mediated by oscillator interactions that cause one oscillator to shift to the phase of the other. Since various features of entrainment are influenced by the phase difference between split activity components, this also indicates that split oscillators entrained to the LDLD cycle are able to interact (Gorman & Steele, 2006). Thus, each oscillator is not only influenced by light falling immediately before and after each scotophase, but is also affected by the other oscillator. If these interactions are inhibited, then the split rhythm will be stably entrained by the actions of bright light. Consistent with the hypothesis that dim light inhibits the interactions between split oscillators that promote the integrated state, the present studies reveal that dim nighttime illumination facilitates the maintenance of the split state.

Additionally, dim nighttime illumination influenced the incidence of re-splitting under LDLD. Both the initial induction of splitting and re-splitting reflects the re-entrainment of an oscillator subgroup through either a nonphotic advance or a phase jump (c.f., Chapter 3). In Experiment 4A, the incidence of re-splitting was reduced when dim nighttime illumination was extinguished after five weeks under

LDLD. The return to the split state is likely prevented because animals under completely dark nights do not exhibit significant nonphotic phase shifts and are characterized by a shorter “minimal tolerable night.” Thus, unsplit animals under dark nights lack the impetus to establish the split rhythm once more. In Experiment 4B, re-splitting after nine weeks under fLDLD was not different between animals held under dimly lit and completely dark nights, which may suggest that the effect of dim light on re-splitting diminishes over time in LDLD. However, this particular group of animals displayed several odd characteristics that may preclude this interpretation. For instance, a much larger percentage of animals in Experiment 4B split after a cage change and thus rejoined at a much higher rate than animals in the two other experiments.

Dimly lit nights facilitated the maintenance of split rhythms under fLDLD, but did not act to maintain the split rhythm under sLDLD. Differential effects of dim nighttime illumination on the maintenance of split rhythms under fLDLD and sLDLD occurred regardless of whether these manipulations were imposed after five or nine weeks under LDLD. Since in Experiment 4C, the only difference between animals exposed to fLDLD and sLDLD was the nature of the photophase, this is strong evidence that dimly lit nights specifically interact with a parametric response to bright light. Split rhythms rapidly rejoined after the abrupt transfer to sLDLD cycles in the present studies, which is inconsistent with the relative stability of the split state observed under previous studies using more gradual transitions to sLDLD with 1 h light pulses (Gorman & Elliott, 2003). In this previous study, 6/7 Syrian hamsters

maintained split entrainment when each 7 h photophase was first replaced by a LDL 3:1:3 for two weeks, then by a LDL 2:3:2 for two weeks, before the final sLDLD cycle (LD 1:5) was imposed. This is a much higher proportion of animals maintaining the split state under sLDLD than that observed in the present studies (4/22 animals in 4A, 2/22 animals in 4C). This discrepancy is likely due to the manner in which the sLDLD cycle was imposed. In Siberian hamsters, the percentage of animals maintaining the split state under sLDLD cycles is increased when the sLDLD cycle is introduced in a gradual rather than abrupt fashion (Gorman & Elliott, 2003; Rosenthal et al., 2005).

After sLDLD cycles were imposed, the two split activity components did not immediately consolidate into one activity bout. Instead, it was quite common for an additional activity bout to emerge in one of the two intervening dark periods. In Experiment 4C, the additional activity commonly appeared in the dark period juxtaposed between the NS and the DS (c.f., Figure 5.9). Since fusion and rejoining likely reflect related coupling responses, it is of interest to note that when split rhythms are released into constant conditions, the rhythm fuses such that activity onset is phased between the former NS and DS, and not within the other half of the cycle (c.f., Chapter 4). Furthermore, when one of the two daily photophases is deleted, the activity onset of the DS component is more labile relative to the NS component (Gorman & Steele, 2006). Again, the data suggest that the NS is able to influence the phase of the DS more than vice versa.

While sLDLD animals provided with dimly lit and completely dark nights did not differ in rejoining during Experiment 4A, the latency to rejoin was actually longer in DARK-sLDLD animals in Experiment 4C. If the actions of dimly lit nights under LDLD only serve to inhibit interactions between split oscillators that cause them to rejoin, then it would not be expected that animals under dimly lit and completely dark nights would differ in the latency to rejoin under sLDLD. Instead, this unexpected pattern of results suggests that after the system is split, dim light and bright light continue to interact to facilitate phase jumps of oscillators. This would accelerate the rejoining of split rhythms under sLDLD based on the definitions employed here, but may be more accurately described as dimly lit nights promoting the further division of oscillators into more than two groups. While multi-modal rhythms were not stable under the sLDLD cycles used presently, LD 3:3 and LD 5:1 cycles can support multi-modal entrainment when the daily dark periods are dimly lit, but not when daily dark periods are completely dark (Evans, personal observations). Again, the present data do not support the hypotheses that dim nighttime illumination globally inhibits or strengthens the interactions between oscillators, since it is able to do both depending on the context. These results are most consistent with the hypothesis that dim light acts to alter the shape of coupling relations within the mammalian pacemaker.

Before experimental manipulations, animals in the present studies exhibited a very high rate of spontaneous rejoining, especially in animals that split following a cage change. This was a large obstacle to conducting simultaneous fLDLD and sLDLD manipulations; however, this is not the first time that such a pattern has

emerged. As noted in Experiment 2A, it is quite common to observe two different waves of splitting in studies using LDLD, and these subgroups of behaviorally distinguished animals often display differences in the maintenance of split rhythms (c.f., Chapter 3). Animals splitting before a cage change display a gradual pattern of splitting and more stable split rhythms, whereas animals that split after a cage change display an abrupt pattern of splitting and relatively less stable split rhythms. The induction of splitting in these two different waves is likely produced by different mechanisms (c.f., Chapter 3). In animals that split prior to a cage change, subgroups of oscillators are phase jumping into the daytime scotophase, and in animals that split in response to a cage change, oscillators are phase advanced into the daytime scotophase due to the resetting actions of novelty-induced wheel running.

But why do these two subgroups of animals differ in the maintenance of split rhythms? One clue may lie in the fact that dimly lit nights not only accelerate phase jumping responses, but also increase the variability between animals (c.f., Chapter 3). Animals that split before the cage change may be those with a “minimal tolerable night” of 5 h or longer, which prevents consolidation of the activity rhythms since each scotophase alone does not allow for stable unsplit entrainment. In contrast, when the minimal tolerable night is less than 5 h, animals may be induced to split by a cage change but interactions between the split oscillators permit consolidation of the activity rhythm. This predicts that rejoining will be prevented in the vast majority of animals when LDLD cycles with dimly lit nights shorter than 5 h are used, which is

consistent with the very low rates of spontaneous rejoining under LDLD with 4 h nights in Experiment 3 (c.f., Chapter 4).

If this is an accurate explanation of why these two waves of splitting emerge, then this suggests that in the vast majority of animals, activity rhythms initially split under LDLD may not be as stable as they seem. In Experiment 4B, there was evidence that the split rhythms were changing over time under LDLD, even in animals that did not rejoin their split rhythms. This is consistent with visual inspection of the actograms for many animals, in that after the initial emergence of the split rhythm, the nighttime scotophase continues to recede and the daytime scotophase continues to grow larger. In some animals, this pattern is checked and the split rhythm does not rejoin (e.g., Figure 5.3, lower left actogram). In other animals, however, the nighttime activity component fully disappears, only to return during the following weeks if dim nighttime illumination is retained (e.g., Figure 5.3, upper right actogram). This pattern is frequently observed, raising the possibility that subgroups of oscillators continue to reorganize under dimly lit nights, even after the split rhythm is initially established. Recent studies investigating the reorganization of the SCN have also suggested that complete manifestation of the split state may take several weeks under LDLD (Gorman, unpublished observations).

Table 5.1 Split entrainment in Experiment 4

Expt	% Split	Split at Transfer	Rejoined	Expt Wk	DS BL/ NS BL	DSents / NSents	ψ DS-NS
4A	89%	45%	45%	5	1.69 \pm 0.1	1.42 \pm 0.1 h	13.94 \pm 0.3 h
4B	82%	13%	91%	5	1.51 \pm 0.3	1.44 \pm 0.2 h	12.93 \pm 0.6 h
4B				9	0.96 \pm 0.1	0.99 \pm 0.1 h	11.49 \pm 0.4 h
4C	97%	67%	41%	5	1.44 \pm 0.1	1.26 \pm 0.1 h	13.05 \pm 0.2 h

BL: Bout Length

ψ DS-NS: Phase angle between the DS and NS

ents: total wheel revolutions within that scotophase

DS: Daytime Scotophase

NS: Nighttime Scotophase

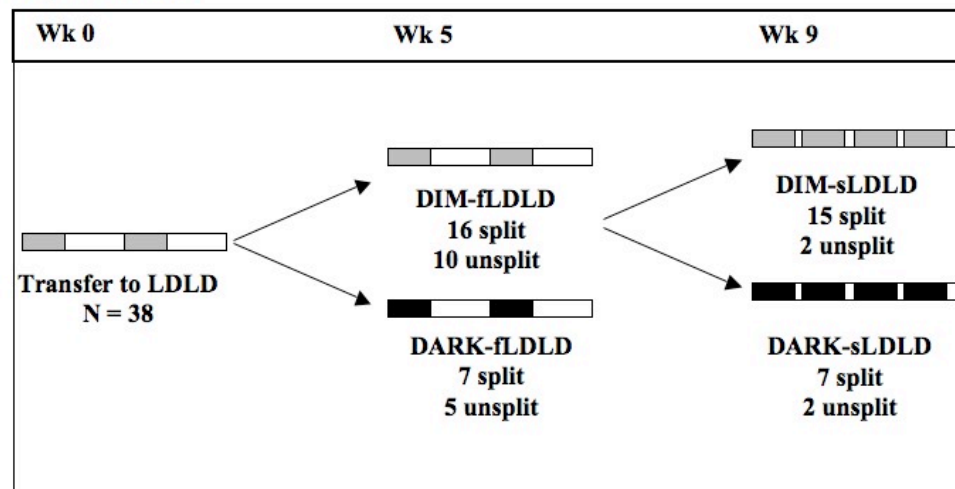
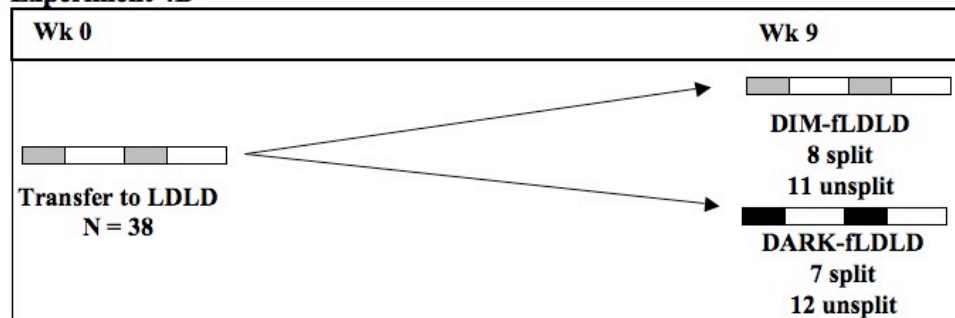
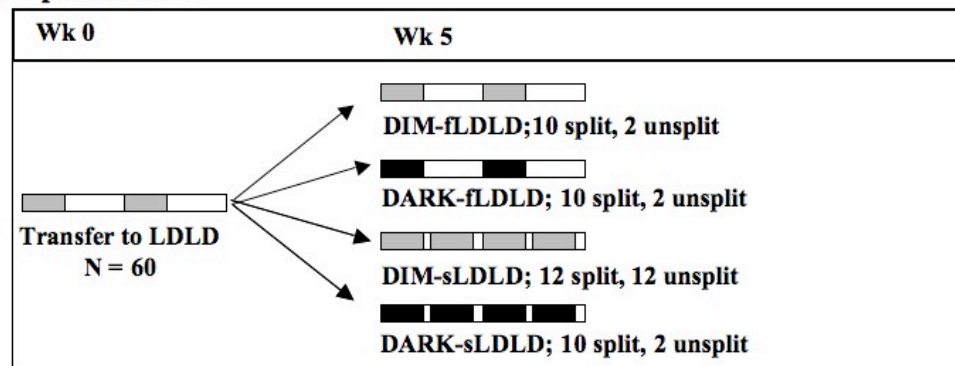
Experiment 4A**Experiment 4B****Experiment 4C**

Figure 5.1: Schematic of experimental protocols used in Experiment 4A, 4B, and 4C. Light:dark bars represent the photoperiods animals were exposed at the start of the week indicated. Split and unsplit animals were transferred to LDLD cycles with “full” 7 h photophases (fLDLD) or “skeleton” photophases (sLDLD), where the nights were either dimly lit (DIM-) or completely dark (DARK-). The sample sizes of each group at the beginning of these experimental manipulations are indicated immediately below each light:dark bar.

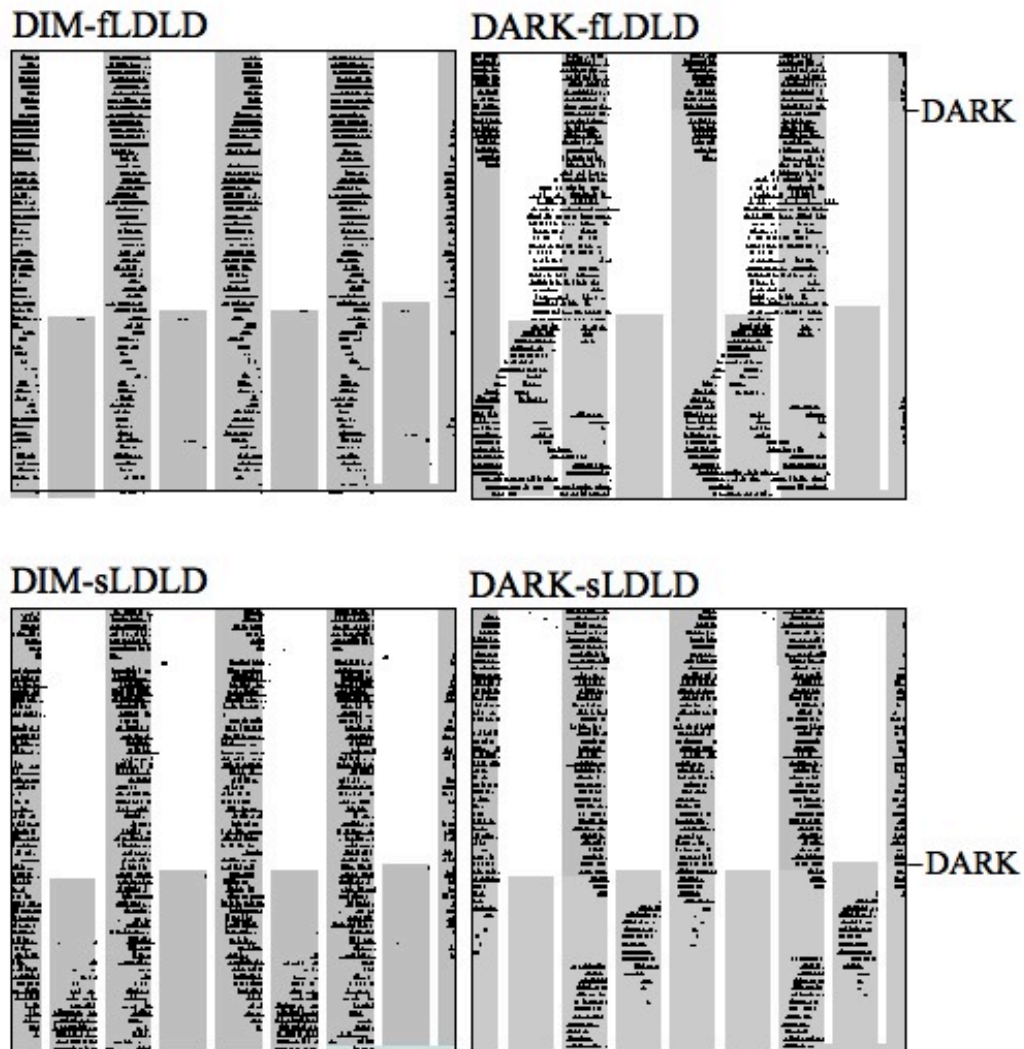


Figure 5.2: Representative double-plotted actograms of wheel-running rhythms from animals split under LDLD with dimly lit nights in Experiment 4A. Please note that the first three weeks under LDLD are not depicted. After five weeks under LDLD, animals were either retained under LDLD cycles with dimly lit nights (DIM-fLDLD) or transferred to fLDLD with completely dark nights (DARK-fLDLD). After nine weeks under LDLD, animals were re-entrained to skeleton LDLD cycles (sLDLD) either with dimly lit nights (DIM-sLDLD) or completely dark nights (DARK-sLDLD). The day on which dim nighttime illumination was extinguished for DARK- animals is indicated to the right of these actograms.

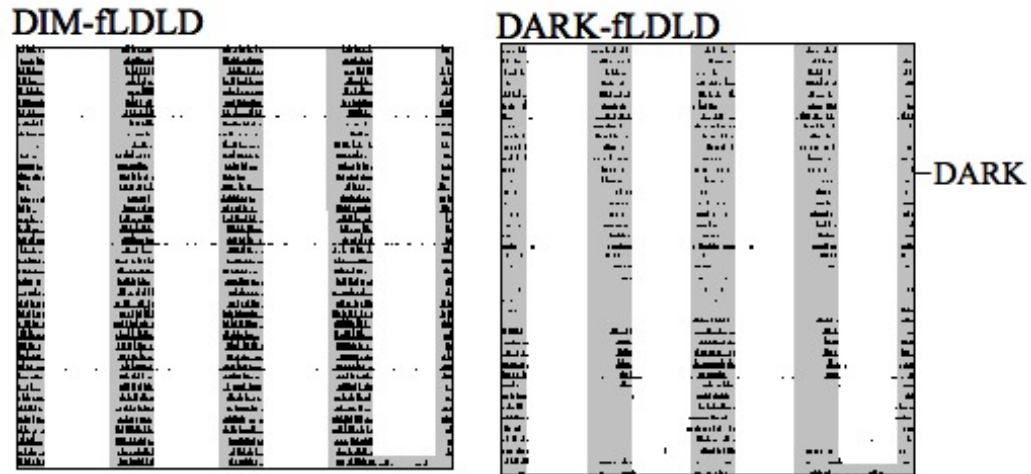


Figure 5.3: Representative double-plotted actograms of wheel-running rhythms from animals split under LDLD with dimly lit nights in Experiment 4B. Please note that the first seven weeks under LDLD are not depicted. After nine weeks under LDLD, animals were either retained under LDLD cycles with dimly lit nights (DIM-fLDLD) or transferred to fLDLD with completely dark nights (DARK-fLDLD). The day on which dim nighttime illumination was extinguished for the DARK- animal is indicated to the right of the actogram.

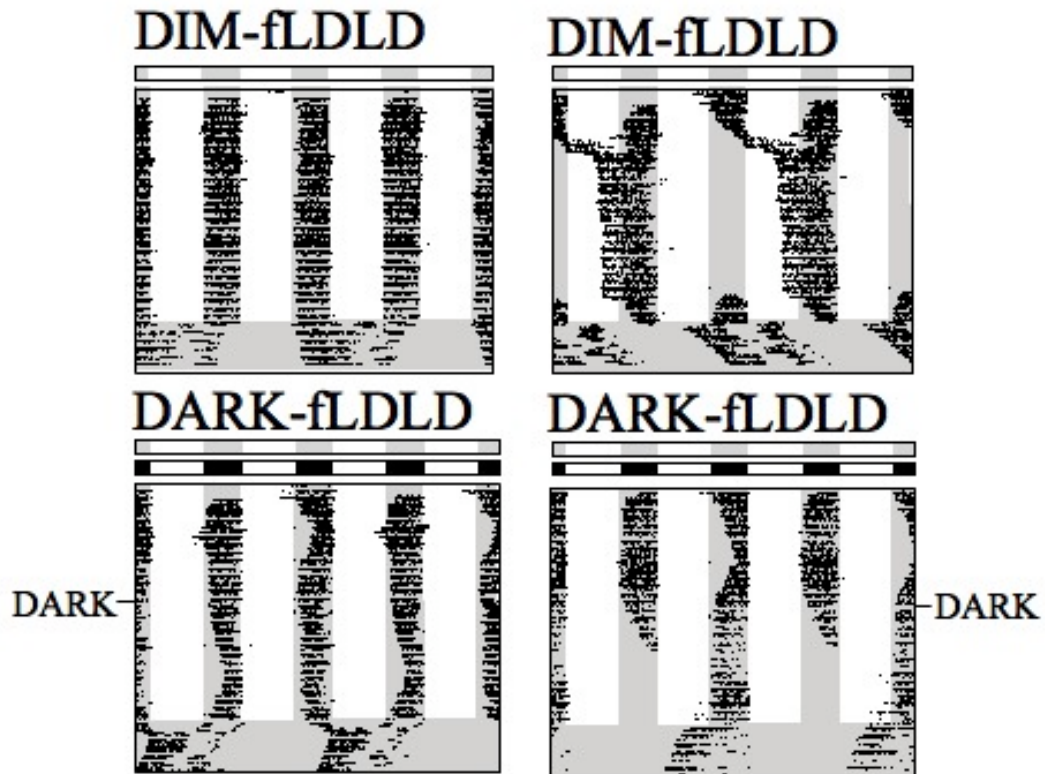


Figure 5.4: Representative double-plotted actograms of wheel-running rhythms from animals split under LDLD with dimly lit nights in Experiment 4C, then either retained under LDLD cycles with dimly lit nights (DIM-fLDLD, top) or transferred to fLDLD with completely dark nights (DARK-fLDLD, bottom). The day on which dim nighttime illumination was extinguished for DARK- animals is indicated to the right of these actograms. Please note that every day of the experiment is shown.

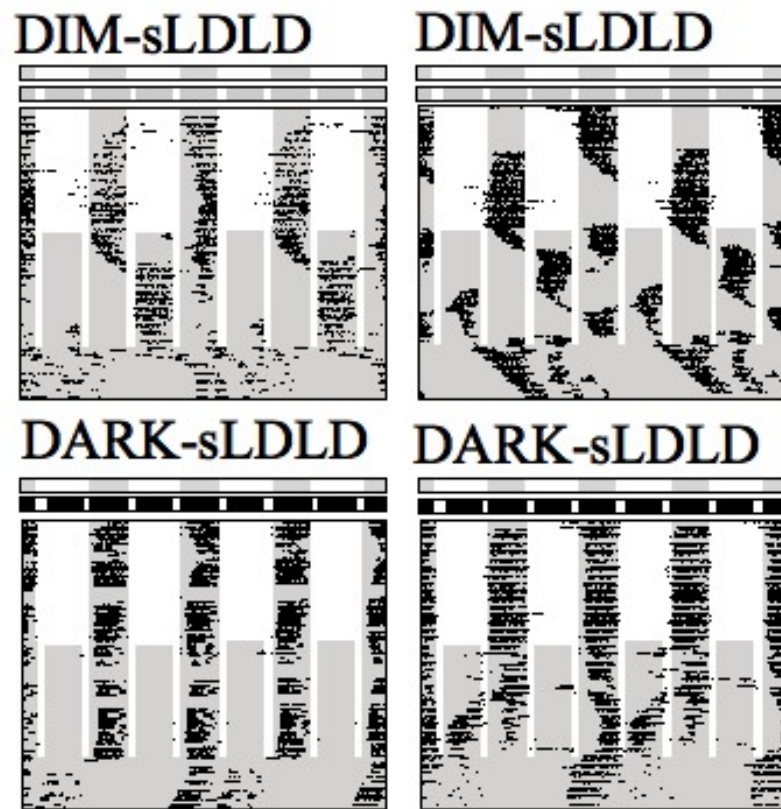


Figure 5.5: Representative double-plotted actograms of wheel-running rhythms displayed by animals split under LDLD with dimly lit nights in Experiment 4C, then transferred to a “skeleton” LDLD cycle with either dimly lit (DIM-sLDLD, top) or completely dark nights (DARK-sLDLD, bottom). The change in the internal shading represents the start of sLDLD, and the start of dark nights in animals in the DARK-sLDLD condition. Please note that missing data on the lower left actogram is due to a mechanical failure.

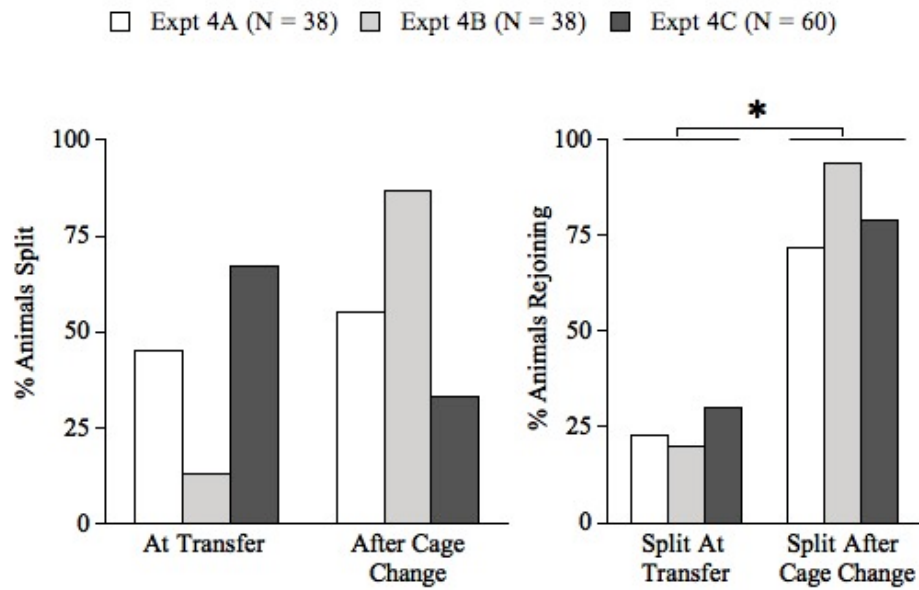


Figure 5.6: Timing of split induction influenced whether animals spontaneously rejoined. Some animals split during the first week under LDLD, prior to the first cage change (Split At Transfer), whereas other animals required a cage change before splitting under LDLD (Split After Cage Change). Animals that split at transfer were less likely to spontaneously rejoin their split rhythms. * $p < 0.05$

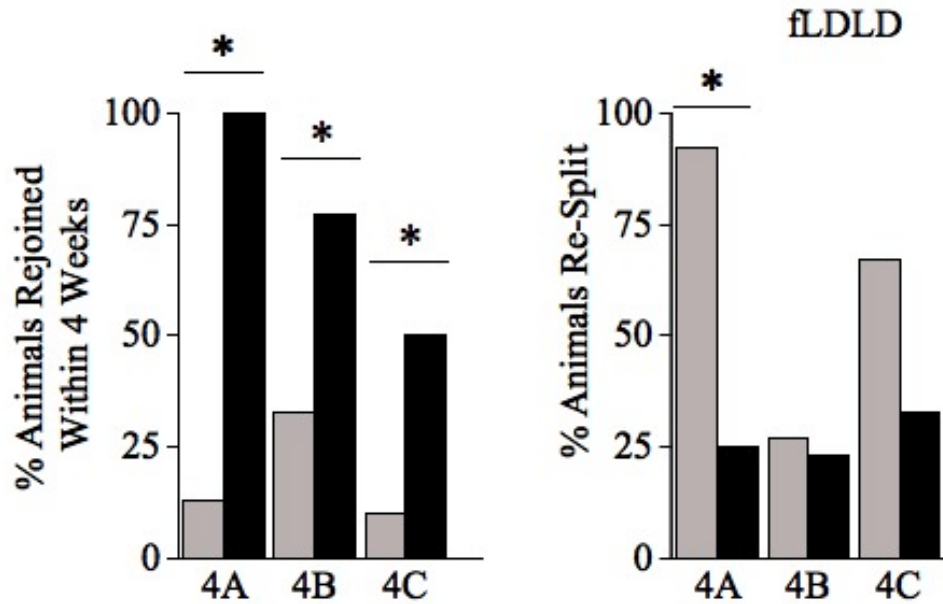


Figure 5.7: Fusion of LDLD-induced split rhythms under LDLD with 7 h “full” photophases (fLDLD). Incidence of rejoining and re-splitting during the four weeks under fLDLD with DIM or DARK nights. Each gray and black bar grouped together represents the DIM-fLDLD and DARK-fLDLD group, respectively, for a single experiment. Please note that scotopic manipulations were conducted after 5 weeks under fLDLD in Experiments 4A and 4C, but after 9 weeks in Experiment 4B. * $p < 0.05$

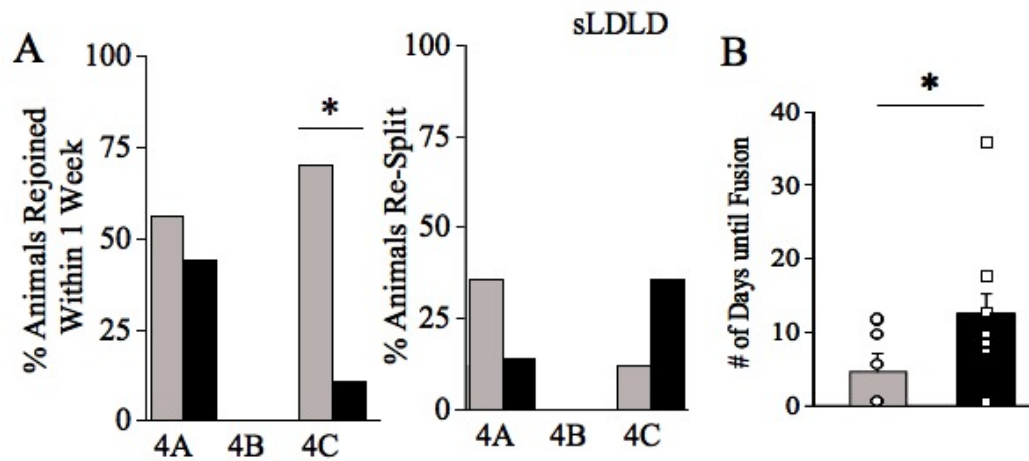


Figure 5.8: Fusion of LDLD-induced split rhythms under “skeleton” LDLD (sLDLD) where each of the two daily photophases were simulated with two 1 h light pulses. A) Incidence of rejoining (during first week) and re-splitting (during four weeks) under sLDLD with DIM or DARK nights during Experiments 4A and 4C. Please note in Experiment 4A, the sLDLD manipulation occurred nine weeks after the initial transfer to LDLD, whereas in Experiment 4C, the sLDLD manipulation occurred five weeks after transfer to LDLD. No sLDLD manipulation occurred in Experiment 4B. B) Number of days until split animals rejoined under sLDLD with DIM or DARK nights in Experiment 4C. Each symbol represents an individual animal. *p < 0.05

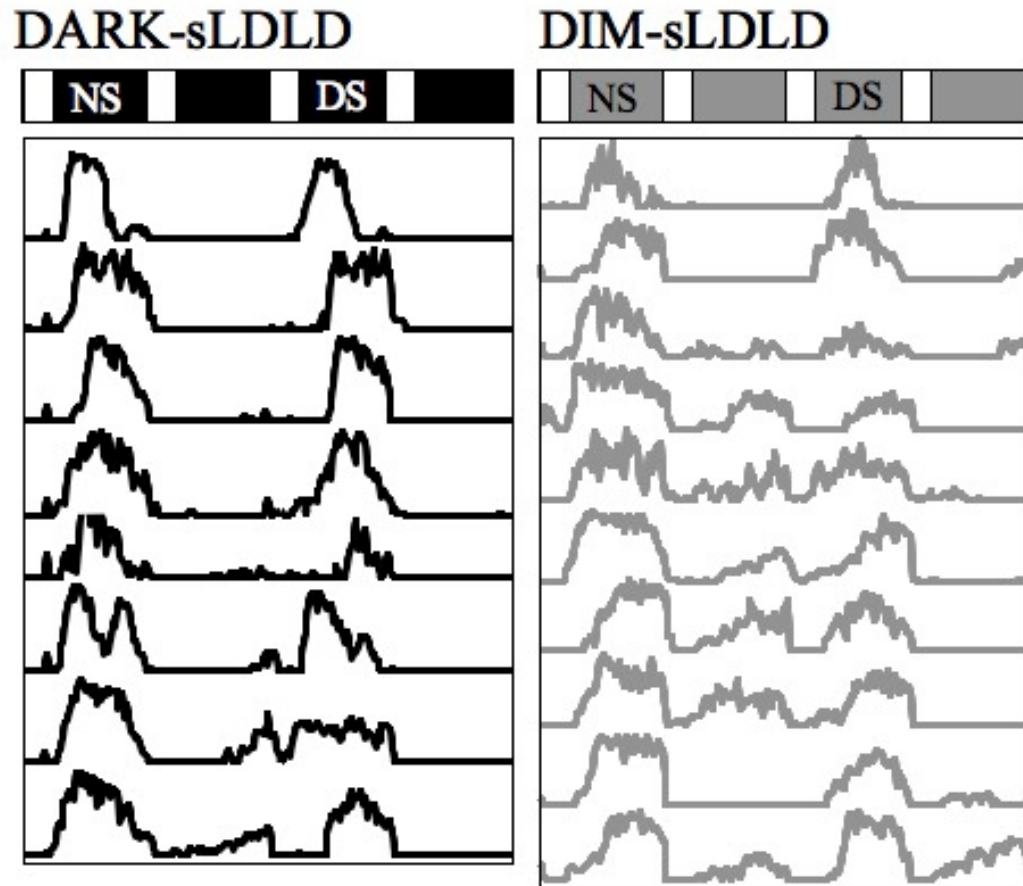


Figure 5.9: Average activity profiles of individual animals during the first week of sLDLD with DIM or DARK nights. Activity profiles are arranged in order of how much activity is expressed in the dark period between the nighttime scotophase (NS) and daytime scotophase (DS). The animal represented by the last line in the DIM group displayed four activity components during the first week under sLDLD.

Chapter 6

Conclusions

The findings of the present studies may be summarized as follows:

1) Dim illumination is not a strong zeitgeber when provided on its own, consistent with previously published fluence response curves. Furthermore, dim illumination can mask behavioral activity and melatonin rhythms, but these effects do not account for the potency of dim nighttime illumination under entrained conditions.

2) Dim illumination does not potentiate circadian responses to bright light and nonphotic stimuli under free-running conditions. Dim light can potentiate resetting to photic and nonphotic stimuli after entrainment to ultra long day photoperiods, but not more standard photoperiods (e.g., LD 14:10). This suggests that dim light does not potentiate phase resetting by directly augmenting the strength of circadian input pathways. Instead, the ability of dim light to modulate phase resetting depends on the state of central pacemaker itself.

3) Dim illumination alters circadian parameters that are intrinsic to the central pacemaker itself. Constant dim light lengthens circadian period in a manner consistent with Aschoff's first rule for nocturnal rodents; however, the 0.3 h increase in period is insufficient to account for dim light induced changes in circadian entrainment. In violation of Aschoff's second rule for nocturnal rodents, dim illumination increases the duration of subjective night by ~3 h through a redistribution of the active phase.

The basic change in circadian waveform produced by exposure to dim illumination may explain effects of dimly lit nights under non 24 h T-cycles and short day photoperiods, but not those under 24 h LDLD cycles. The change in circadian waveform under free-running conditions is consistent with an effect of dim light on the interactions between central oscillators, which may account for the potent effects on entrained and free-running rhythms.

4) By studying how dim light promotes the induction of splitting under LDLD, I sought to test the hypothesis that dim illumination alters circadian coupling. First, I found that dim light does not facilitate splitting under LDLD by merely increasing activity levels and novelty-induced wheel running. Instead, dim nighttime illumination facilitates the induction of split rhythms under LDLD by altering nonphotic resetting and phase-jumping responses after entrainment to ultra long day photoperiods. The formal similarities between phase jumping responses and LDLD-induced splitting suggest that these two phenomena are mechanistically related.

6) Furthermore, dim nighttime illumination influences the way in which split oscillators interact under entrained and free-running conditions. In contrast to the potent effects of dim light under entrained conditions with full photophases, the relatively modest effects of dim illumination under conditions lacking long bright light exposure indicate that dim illumination interacts with bright light exposure under skeleton ultra long day photoperiods (i.e., LDLD) to promote stable split entrainment.

Collectively, the present results are consistent with the hypothesis that dim illumination alters the coupling of circadian oscillators within the central pacemaker.

As illustrated in Figure 6.1, dim nighttime illumination alters the induction, maintenance, and fusion of split rhythms by changing the nature of coupling relations within the mammalian pacemaker. The induction of split rhythms under LDLD represents interactions between oscillators entrained to short nights that cause a subgroup of oscillators to re-entrain to the daytime scotophase through either phase jumps or nonphotic advances. Dimly lit nights reduce the “minimal tolerable night” and thereby accelerate phase jumps and augment nonphotic advances after entrainment to ultra long day lengths (c.f., Chapter 3). After oscillators are reorganized under LDLD, bright light during the daily photophases attenuates interactions between oscillators that would otherwise cause them to rejoin. Dim nighttime illumination interacts with a parametric response to bright light during the daily photophases to prevent rejoining under entrained conditions (c.f., Chapter 5). Lastly, dim illumination had relatively modest effects on the fusion of split rhythms after release into constant conditions, which is consistent with only a weak inhibition of the interactions that promote the unsplit state in the absence of bright light (c.f., Chapter 4). The present pattern of results is consistent with the hypothesis that dim nighttime illumination alters the shape of coupling response curves for interactions between circadian oscillators.

The question of oscillator synchronization in mammals is an important one, since cellular and physiological analyses of the SCN indicate that it is a population of neural oscillators with different inherent periods (Herzog et al., 2004; Welsh et al., 1995). Studies conducted at the formal level in both invertebrates and mammals make

it clear that a complete analysis of the circadian pacemaker will require an understanding of mechanisms by which oscillators interact as an ensemble. Elucidating the bases of circadian coupling in invertebrates relied on the identification of distinct pacemakers. Similar progress in mammalian physiology has been hindered by the complexity of the mammalian brain and by the lack of analytical paradigms well suited for studying disassociated oscillators in mammals. Combining elaborated conceptual models for circadian coupling, behavioral assays indicative of coupled oscillators, and modern neuroscience techniques may provide the necessary framework for further defining the nature and bases of oscillator interactions within the central pacemaker.

Since dim nighttime illumination has potent effects under “coupling” paradigms, dim light may operate as an environmental stimulus that influences oscillator interactions within the SCN. Testing the assumption that different coupling paradigms are mediated by a common mechanism, I found in the Siberian hamster that the response to SD photoperiods, LDLD, and constant dim illumination is related. Relative to SD-NonResponders, SD-Responders displayed greater plasticity in circadian waveform under each context (Evans, personal observations). Thus, these three behavioral paradigms are co-modulated by both a common intrinsic factor and a common extrinsic factor, namely dim illumination. Changes in the function of neurons within the SCN using dimly lit versus completely dark conditions may provide invaluable insights into how the circadian pacemaker is organized under a variety of behavioral paradigms.

Dim illumination lower than traditionally reported photic thresholds for the circadian visual system has pronounced effects on circadian waveform and pacemaker function, revealing that the circadian visual system is more sensitive than is perhaps currently appreciated. Other photic stimuli previously assumed to be relatively inconsequential have also been reported to alter free-running and/or entrained circadian rhythms in mammals (Boulos et al., 2002; Erkert et al., 1976; Hofstetter et al., 2005; Kavanau, 1967, 1968; Klante & Steinlechner, 1995; Meijer et al., 1990). Together with the present results, these data reveal new insight into the capacity and organization of the circadian visual system. Notably, the present studies reveal a novel action of light on the plasticity of circadian waveform, which is interpreted in the context of the multi-oscillator model of the mammalian pacemaker. In this context it is interesting to note that fruit flies display changes in their bimodal activity rhythms when housed under conditions incorporating dim nighttime illumination comparable to moonlight levels (Bachleitner et al., 2007). Dim illumination is a relatively weak zeitgeber in terms of changes in phase, melatonin secretion, and τ , but this same stimulus is able to alter circadian waveform in a marked fashion. Ongoing studies assessing the light dependence of photic effects on circadian waveform will aid in determining whether this novel visual response reflects physiological mechanisms categorically distinct from those mediating phase shifting and melatonin suppression.

Physiological mechanisms responsible for conveying dim light stimuli may or may not be distinct from those involving hallmark circadian responses to light.

Melanopsin-containing photoreceptors possess several unique features that enable

detection of ambient light levels, such as photon integration, large dendritic processes, and diffuse retinal organization (Berson, 2003). While the intrinsic visual responses of melanopsin-containing retinal ganglion cells display high fluence thresholds relative to rods and cones, the ability to integrate photons may enable these cells to respond to light pulses longer than those possible to provide in culture (~20 min). However, many photic responses of the SCN (e.g., electrical activity and *c-fos* induction) have high irradiance thresholds like those described in vivo (Kornhauser et al., 1990; Meijer et al., 1986; Meijer & Schwartz, 2003). Some molecular responses within the SCN are sensitive to lower light levels (Lin et al., 1997), complementing suggestions that rod- and cone-mediated input do influence circadian function (Aggelopoulos & Meissl, 2000; Dacey et al., 2005; Dkhissi-Benyahya et al., 2006; Mrosovsky, 2003). Rod and cone input may be mediated directly through the retina via bipolar cells that synapse on melanopsin cells or via non-melanopsin containing retinal ganglion cells within the RHT. An indirect source of rod and cone information may arise from visual structures that are afferent to the SCN, such as the IGL within the LGN of the thalamus, since the IGL is characterized by lower thresholds for light-induced gene expression (Muscat & Morin, 2006). Further investigations into the formal and physiological mechanisms underlying the effects of dim illumination may provide novel insights into both the circadian visual system and the circadian pacemaker itself.

Living by the Clock in Modern Times

Future studies should also assess whether dim nighttime illumination has therapeutic applications for circadian rhythmicity and re-entrainment in humans. As is true for other animals, human life is characterized by robust daily rhythms in behavior and physiology, including but not limited to: 24 h cycles of sleep and wakefulness, cognitive performance, hormone secretion, metabolism, cell division, and protein production (Aschoff, 1965a; Aschoff & Daan, 1997; Moore-Ede et al., 1982). In modern industrialized societies, technological advances and practices can produce misalignments between internal rhythmicity and outside environmental schedules, producing negative consequences for both the affected individual and the community at large. As discussed in detail below, maladaptive syndromes caused by the mismatch between internal and external time include the malaise experienced after transmeridian flight (i.e., jet lag) and after work during the late nocturnal phase of the circadian cycle, or the “graveyard” shift (i.e., shift lag).

Jet lag is experienced by a large percentage of the population after transmeridian travel. People who travel across three or more time zones frequently report symptoms of sleepiness and poor attention during the day, inability to sleep properly at night, and gastrointestinal problems related to eating at times when the digestive system is not properly prepared for food intake. Frequently, the malaise associated with jet lag reduces worker productivity or enjoyment while on vacation. While most symptoms subside as the circadian clock is shifted to the new time zone, reentrainment can be a long and tedious process. Many studies find the human circadian pacemaker can only be shifted by 1 h per day (Aschoff et al., 1975), and

consequently, many days may be required to recover from travel across several time zones. Recovery from jet lag can be further prolonged if the shift to the new time zone is in the antidromic direction, where reentrainment may not be fully complete even two weeks after arrival (Takahashi et al., 1999; Takahashi et al., 2001).

Shift lag is becoming a more frequent problem as artificial lighting and globalization are producing an increasing trend towards a 24/7 society with work conducted at all hours of the day and night. Approximately 15% of the labor force work irregular hours (U.S. Bureau of Labor Statistics, 2004). Shift workers, such as nurses, police, and factory workers, often perform key services at a non-optimal phase of their circadian cycle, since reentrainment to the night shift is rare, even in permanent shift workers reporting a high degree of satisfaction (Benhaberou-Brun et al., 1999; Dumont et al., 2001; Roden et al., 1993; Weibel et al., 1997). Myriad health and occupational problems may be caused by the conflict between biological time and the imposed work schedule. Shift workers frequently lodge complaints of impaired sleep, irritability, psychoneuroses, cancer, diabetes, and cardiovascular, gastrointestinal, and fertility problems (Colquhoun et al., 1996; Stevens, 2005; Xu et al., 1994). Negative social and economic consequences are also prevalent, including higher reports of falling asleep on the job, absenteeism, and work-related accidents (Colquhoun et al., 1996). Indeed, several well-known events, such as the Three Mile Island scare, Exxon Valdez oil spill, and Chernobyl nuclear accident, have been attributed to human error occurring during the night shift. Some experts, concluding that the majority of humans can not tolerate inverted work schedules, instead

recommend the use of rapidly rotating work schedules, which do not eliminate misalignment and may impose problems of their own.

Most currently applied treatments for circadian rhythm disorders apply knowledge of human phase resetting responses to light and melatonin in an attempt to shift a person's internal clock to the new schedule as quickly as possible (Revell & Eastman, 2005). Most treatments designed to alleviate jet lag and shift lag require precise timing of chronobiotics (e.g., light, melatonin, exercise) relative to the often-unknown phase of the circadian clock. The time-dependent actions of these chronobiotics and the difficulty of completely shielding people from sunlight, oftentimes make it difficult to produce practical results (Reid & Zee, 2004). Consequently, much confusion exists concerning the optimal procedures for reducing jet lag in the everyday world. A different approach may be to find ways to make the human circadian system more flexible; however, few studies are designed with this goal in mind.

Since controlled studies of human circadian rhythmicity can be difficult and costly, studying the circadian systems of model animal species in the laboratory is useful and practical. Studies examining the circadian organization and entrainment of commonly used nocturnal rodent species, like the hamster, have established basic principles that have later proven to be applicable to most mammal species, including humans. In many ways, studies of rodents under ecological and unconventional conditions can reveal aspects of circadian function that have both empirical and

medical benefits. Thus, methods for increasing the circadian plasticity in rodents may be useful for developing novel techniques to enhance circadian plasticity in humans.

Future studies should determine whether dim nighttime illumination has therapeutic applications for circadian disorders in humans. Foremost, it should be determined whether dim nighttime illumination has detrimental effects on the health and physiology of nocturnal rodents. Since reproduction is especially sensitive to stress and sickness, this may be a good assay for investigating whether dim illumination produces negative consequences in rodents. Under conditions incorporating dimly lit nights, reproduction and offspring viability are not compromised relative to that observed under conditions with completely dark nights (Evans, personal observations). Thus, there is no evidence so far that dim nighttime illumination compromises the health of rodents, although further studies should assay more direct measures of immune function and stress.

Additionally, future studies should assess whether dim nighttime illumination increases circadian plasticity under behavioral paradigms that humans commonly experience (e.g., jet lag paradigm). Ongoing studies investigating whether dimly lit nights can accelerate re-entrainment after a shift in the light:dark cycle are providing very promising results. Siberian hamsters provided with completely dark nights take 2-3 weeks to re-entrain to a 4 h advance of the light:dark cycle; however, re-entrainment occurs within 2-4 days in the majority of animals provided with dimly lit nights (Evans, unpublished observations). Further research is needed to determine

whether this result can be generalized to other species, especially diurnal species, which display differences in light sampling behaviors and photic thresholds.

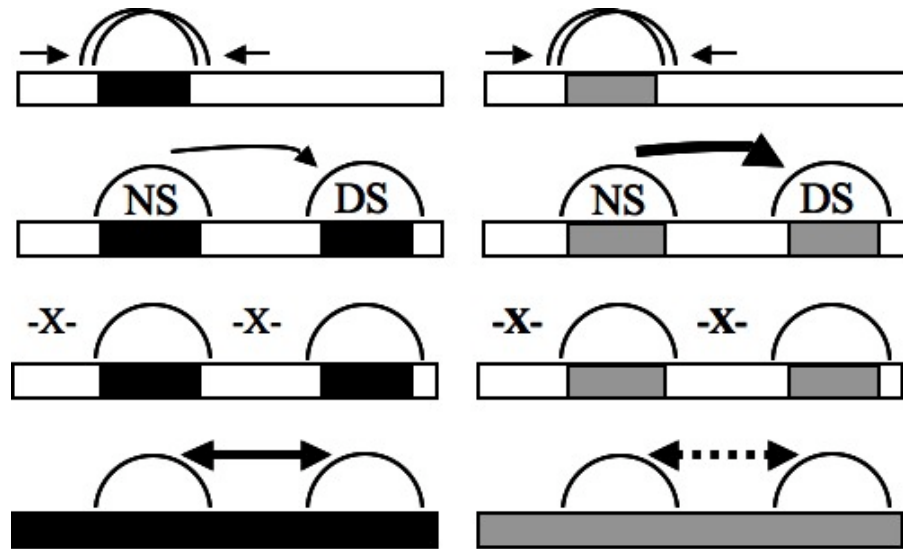


Figure 5.10: Conceptual model for understanding how dim nighttime illumination alters the interactions between oscillators split under LDLD. Entrainment to ultra long day photoperiods (ULD) compresses the duration of subjective night by reducing the phase angle between underlying oscillators. Exposure to 24 h light:dark:light:dark (LDLD) cycles causes a subset of oscillators to re-entrain to the daytime scotophase (DS), and a remainder of oscillators remains entrained to the nighttime scotophase (NS). Dim nighttime illumination facilitates splitting (bold arrow) by increasing the amplitude of nonphotic phase resetting under short nights and increasing the “minimal tolerable night” required for phase jumps. The split state is maintained under LDLD via an inhibition of interactions that would otherwise cause the system to rejoin (Xs). Dim nighttime illumination maintains the stability of the split state under LDLD by interacting with bright light exposure throughout the daily photophases (bold Xs) to inhibit rejoining under entrained conditions. After release into constant conditions, split activity bouts fuse through a series of transients lasting 2-7 cycles. Dim illumination modulates this response in two ways, transiently increasing the latency to rejoin and altering the steady state. The effects of dim light on fusion are modest and largely depend on the phase of release from LDLD, and thus, the arrow here is dashed rather than depicted as considerably smaller.

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