Computational models of spider activity patterns integrated with behavioral experiments hypothesize adaptive benefits of the circadian clock

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Introduction

Circadian rhythms are internally-generated biological clocks that underlie many important biological processes, including the sleep-wake cycle, basal metabolic rate, and the release of certain hormones (See Box 1 for glossary of termiology; Halberg et al. 2003). These rhythms appear to be nearly ubiquitous across life (Johnson and Kondo 2001), from prokaryotic cyanobacteria (Johnson et al. 1996) to humans (Patrick and Allen 1896), and may have evolved multiple times. Thus, circadian rhythms almost certainly confer an adaptive benefit (Dunlap 1999). Experiments in many diverse taxa, from cyanobacteria (Ouyang et al. 1998) to *Drosophila* (Beaver et al. 2002, 2003) to mammals (DeCoursey et al. 2000) have demonstrated negative effect of clock periods that differ from 24 hours (specifically, the free-running period; see Box 1) on fitness and survival rate of an organism. Despite this ubiquity and fitness cost, the adaptive benefit remains unknown. One hypothesis suggests that circadian rhythms are beneficial because they allow homeostatic regulation corresponding to regular changes in the environment, such as the transition from day to night (reviewed in Young and Kay 2001). For example, these rhythms can cue diurnal prey to seek cover before dusk to avoid nocturnal predators (DeCoursey et al. 2000).

Box 1. Glossary of Circadian Terminology	
Term	**Definition**
Circadian Rhythm	'circa' = around, 'dian' = day Internally generated biological clock with a period of approximately 24 hours that responds to external cues, but can persist in their absence.
Zeitgeber	German for 'time-giver' External time cue (e.g., light, temperature) that can influence the period and phase of the circadian rhythm.
Free-Running Behavior	Occurs when circadian rhythms persist with a regular period, even in the absence of an oscillating zeitgeber.
Free-Running Period (FRP)	Period of circadian rhythm when displaying Free-Running Behavior. Typically differs from 24, with 'typical' defied as within 24±2 hours.
Entrainment	Occurs when circadian system adjusts its period to match that of an oscillating zeitgeber (e.g., day-night cycle).
LD *x*:*y*	External lighting environment with *x* hours of light and *y* hours of dark.
DD	External lighting environment with constant darkness.

The study of circadian rhythms in spiders (Order Araneae), while limited, has revealed some unexpected patterns. Although multiple spider species have typical free-running periods (Table 1), the trashline orb-weaving spider, *Cyclosa turbinata* (Araneidae), has an exceptionally short FRP of 18.74±0.13 h (Moore et al. 2016). This is the shortest known naturally occurring clock. Such a large deviation from 24 hours should have a major negative effect on *C. turbinata*'s fitness, although no such effect has been described (reviewed in Young and Kay 2001). Furthermore, my preliminary experiments with the house spider, *Parasteatoda tepidariorum* (Theridiidae), suggest that light has a strongly inhibitory effect on *P. tepidariorum*'s activity. However, such a strong inhibition of activity by light has not been observed in any other spider species, and the circadian system of no other theridiid spider has been rigorously described.

Table 1. Review of Spider Circadian Rhythms				
Species	**Family**	**Behavior**	**FRP**	**Reference**
Larinioides cornutus	Araneidae	Anti-predator behavior	21.94±0.54 h	(Jones et al. 2011)
Metazygia wittfeldae	Araneidae	Locomitive acvitiy	22.7±0.24 h	(Jones et al. In Review)
Parasteatoda tepidariorum	Theridiidae	Locomitive acvitiy	23.89±3.39	(Wolf 2011)
Frontinella pyramitela	Linyphiidae	Locomitive acvitiy	~24 h	(Suter 1993)
Argyrodes trigonum	Theridiidae	Locomitive acvitiy	~24 h	(Suter 1993)
Lycosa tarantula	Limulidae	Locomitive acvitiy	24.1±0.59 h	(Ortega-Escobar 2002)
Cupiennius salei	Ctenidae	Locomitive acvitiy	24.9±0.31 h	(Seyfarth 1980)
Cave Spiders	Dipluridae and Ctenidae	Locomitive acvitiy	25.18±0.75 h	(Soriano-Morales et al. 2013)

The purpose of this study is to combine behavioral and computational analyses to describe the circadian systems of *C. turbinata* and *P. tepidariorum* in order to hypothesize adaptive benefits of the observed behaviors. In the first chapter, I will present a series of numerical experiments performed on models that recreate *C. turbinata*'s short period circadian clock. The results from these numerical experiments will be used to give us greater understanding about the nature of *C. turbinata*'s molecular oscillators and generate testable hypotheses about the adaptive nature of circadian clocks. In the second chapter, in addition to *P. tepidariorum*, I will also describe the circadian systems of two other theridiid species as a point of comparison: the subsocial spider, *Anelosimus studiosus*; and the southern black widow, *Latrodectus mactans*. The goal of these experiments is to understand the mechanisms underlying the observed variability in spider circadian systems (e.g., FRP, distribution, and affect of light). More generally, understanding how this variability arises can improve mechanistic explanations of adaptive benefits. Examining the free-running behavior, including the FRP and its distribution within a species, of the three Theridiidae spiders will reveal the behavior of their circadian systems in the absence of any external cues, because without the influence of external cues, more endogenous traits about the underlying system become apparent. For example, *C. turbinata* can entrain its circadian period to LD 12:12. It is only when the specimens are removed from light that their 19-hour endogenous period becomes apparent.

Adaptive Benefit of Circadian Rhythms

As previously mentioned, circadian rhythms can be found in many diverse taxa across life (Johnson and Kondo 2001). The study of circadian rhythms and their genetic underpinnings was greatly aided with the advent of genomics and transcriptomics, which expanded to many different organisms. Surprisingly, despite the similarities of circadian rhythms on a behavioral level (e.g., free-running behavior, entrainability) across life, phylogenetic analysis of the underlying genetic mechanisms (so-called clock genes) across a wide variety of organisms suggests that circadian clocks may have evolved multiple times. Whole genome searches of *Synechocystis*, a cyanobacteria, failed to return homologs of any of eukaryotic clock genes (Golden et al. 1998). Furthermore, whole genome searches of both *Drosophila* and humans have failed to find orthologs of *Neurospora* clock genes (reviewed in Young and Kay 2001). Conversely, orthologs of *Drosophila* clock genes have been identified in many other animal species, including several arthropod species and humans (Young and Kay 2001). These results combined suggest that molecular clocks have arisen independently several times in the evolution of life. The similarities among the mechanisms of these clocks on the behavioral level would then be a result of convergent evolution. However, it is also possible that because these clocks are under divergent evolutionary pressures so that homology cannot be detected across kingdoms (Dunlap 1996).

The fact that circadian clocks appear to have independent origins across diverse taxa suggests the circadian clocks confer a fitness benefit to species, thus allowing their propagation. Experimental evidence supports this claim across many diverse taxa. In prokaryotes, Ouyang et al. (1998) examined competition between strains of *Synechococcus* sp. with varying rhythms, induced by mutation of their clock genes, under different light-dark cycles (e.g., 11:11, 12:12, 15:15 LD cycles). They found that strains with FRP that most closely resonating with the period of the lighting cycles outcompeted other strains. For example, they found that under an 11:11 LD cycle, a strain with a 23-hour free-running period, SP22, outcompeted a strain with a 28-hour free-running period, P28. However, when the same strains were placed under a 15:15 LD cycle, the P28 strain outcompeted the SP22 strain.

Emerson et al. (2008) tested the effect of nonresonant LD cycles on the fitness of pitcher-plant mosquitos, *Wyeomyia smithii*. Resonance in LD cycles occurs when the total period of the LD cycle matches the organisms FRP (or some integer multiple of the FRP). For example, both LD 12:12 and 24:24 would resonate an individual with a 24-hour clock, whereas it would be nonresonant for an individual with a 19-hour free-running period. Rather than mutate clock genes, they raised cohorts of wild-type individuals in different LD cycles, some of which were resonant (LD 10:14, 10:36, 18:06) and and some of which were not resonant with *W. smithii*'s free-running period (LD 10:25). The FRP was estimated to be approximately 21 hours based on experimental data. They found that cohorts raised in nonresonant LD cycles experienced a significant decrease in fecundity, which is a measure of fitness. In *Drosophila*, loss-of-function mutations in either *period*, *timeless*, *cycle*, or *clock* resulted in both reduced sperm quality in males and reduced production of oocytes in females, both of which resulted in decreased fertility, again a measure of fitness (Beaver et al. 2002, 2003).

These studies were all performed under laboratory conditions. DeCoursey et al. (2000) examined how abolishing circadian rhythms affected the survival rate of eastern chipmunks, *Tamias striatus*, in their natural habitat. In order to abolish circadian rhythmicity, DeCoursey et al. surgically (2000) lesioned the suprachiasmatic nucleus, a region of the brain responsible for controlling circadian rhythms. These chipmunks still retained the ability to entrain to LD cycles, but are arrhythmic under DD conditions. The chipmunks were then released into their natural habitat, tagged with a radio-tracking device. The study found that lesioned individuals had significantly higher mortality rates than individuals who received either sham surgeries or no surgeries at all. This higher mortality was hypothesized to be due to higher nighttime activity in lesioned individuals because they lacked the internal cues to decrease activity at night, though this hypothesis was never measured. They reasoned that the increased activity of the lesioned individuals then alerted nocturnal predators to their location.

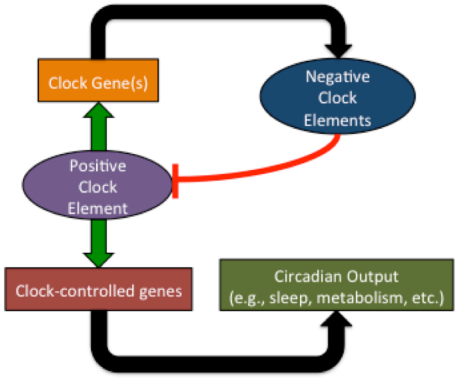

Figure 1. Schematic of General Circadian System.
Adapted from Dunlap 1996.
Green arrow-tipped lines represent stimulator effects.
Red blunted arrows represent inhibitory effects.

Molecular Mechanisms of Circadian Rhythms

Molecular work across many taxa, from Cyanobacteria (blue-green algae); the breadmold, *Neurospora* (Sordariaceae); and fruit flies, *Drosophila* (Drosophilidae; Dunlap 1999), has revealed that the endogenous nature of circadian rhythms arises from the interactions of a complex gene network that form a negative feedback loop, rather than the influence of a single gene. Furthermore, not only are circadian rhythms similar at the behavioral level across taxa,

they are remarkably similar at the molecular level. These networks appear to follow a general schema (Figure 1). First, positive elements promote the transcription of clock genes and clock-controlled genes (*ccgs*). Clock genes are the genes that are directly involved in the circadian cycle, whereas *ccgs* are genes that are merely regulated by circadian genes but do not participate in the cycle, such as those involved in the sleep-wake cycle or certain hormones. Negative elements, which can be the products of the clock genes themselves or elements stimulated by the clock genes, inhibit the activity of positive elements. Once the negative elements degrade (either naturally or enzymatically), the positive elements are reactivated, and the cycle begins again (Dunlap 1999). Positive and negative elements of model organisms are included in Table 2 (reviewed in Dunlap 1996).

Table 2. Overview of Positive and Negative Circadian Elements (Adapted from Dunlap, 1996)

Organism	Positive Element	Negative Element
Cyanobacteria	kaiA	kaiC
Neurospora	WHITE COLLAR 1 & WC-2	FREQUENCY (FRQ)
Drosophila	CLOCK & CYCLE	PERIOD & TIMELESS
Mammals	CLOCK & BAML1 (MOP3)	PER1, PER2, PER3

Because of the homology among animal clock genes, the most relevant model organism to my study of the circadian mechanisms of *C. turbinata* is *Drosophila*. In *Drosophila*, using a non-specific mutagenesis screen, Konopka and Benzer (1971) identified a mutant strain that had abnormal eclosion times, a behavior which had been demonstrated to have circadian rhythmicity (Chandrashekaran, 1967). Males with this mutation were then bred with attached-X females with wild-type circadian rhythms, which have two X-chromosomes that share a common centromere along with a Y-chromosome. So the only viable progeny can either (1) inherit the attached X-chromosome from the mother and the Y-chromosome from the father, in which case the offspring would be female, or (2) inherit the Y-chromosome from the mother and the X-chromosome from the father, in which case the offspring would be male. They found that only the male progeny of this breeding showed similarly aberrant circadian rhythms. This inheritance pattern implies that the mutation occurred on the X chromosome, because progeny males could have only received an X-chromosome from the mutated father. The mutated gene was named *period*. Studies using similar mutagenesis screens found that mutations in *timeless*, *clock*, and *cycle* affected circadian rhythmicity in locomotive activity or eclosion activity, two common behavioral markers for circadian rhythms (Konopka and Benzer 1971; Sehgal et al. 1995; Rutila et al. 1998).

Furthermore, using BLAST in the Drosophila Expressed Sequence Tag Database, Emery et al. (1998) identified a protein, later named cryptochrome (CRY), belonging to the photolyase family, which was hypothesized to be involved in the light sensitivity of circadian rhythms. Northern Blot analysis of samples taken throughout a LD cycle showed that *cry* transcripts are expressed cyclically under normal LD and DD conditions, though DD had a lower amplitude. Furthermore, in mutants with non-functional *per*, *timeless*, *clock*, or *cycle*, the cyclic expression of *cry* transcripts was partially or completely abolished. And finally, using Western Blot analysis of samples taken under different lighting conditions, cycling of CRY protein levels was found to be dependent on light. Under LD conditions, CRY cycles normally, while under DD conditions, cycling was completely abolished. So in a wild-type individual, under both LD and DD, *cry* transcripts cycle normally, whereas CRY proteins only cycle under normal LD conditions.

Next, when analyzing the interactions among these genes, qPCR analysis by Sehgal et al.

(1995) found that *timeless* mRNA cycled in an almost identical manner to *period* under 12:12 LD conditions, suggesting that the two products work in tandem. Similarly, Western Blot analysis found evidence that CLOCK and CYCLE interact to form a complex (Rutila et al. 1998). Furthermore, Rutila et al. (1998) also found that cyclic expression of *period* and *timeless* was abolished in mutants with either non-functional CLOCK or CYCLE, implying that both proteins are necessary for cyclic expression. Thus, they concluded that CLOCK and CYCLE form a complex that promotes the transcription of *period* and *timeless*.

Finally, constitutive overexpression of *period* due to a transfected expression vector reduced its own mRNA cycling, suggesting that the circadian clock operates with a negative transcriptional feedback loop (Zeng et al. 1994). However, because PERIOD protein lacks DNA interaction sites (Hao et al. 1997), it was hypothesized that it must interact with a mediator to affect its own transcription. This hypothesis was confirmed by Darlington et al. (1998), who found that the activity of CLOCK significantly decreased in cells that were cotransfected with both *per* and *tim* cDNA expressed with a Drosophila 5C-actin promoter, which causes constitutive overexpression. However, using Western Blot, CLOCK protein levels were unaffected in the cotransfected cells. This implies that PERIOD-TIMELESS affect only the functionality of CLOCK rather than its expression or stability in the cell. Later that year, Lee et al. (1998) found that when performing immuneprecipitation of CLOCK-CYCLE complexes, PERIOD-TIMELESS complexes also copurified, implying that the two complexes are physically interacting in order to cause the affect demonstrated by Darlington et al. (1998). Thus CLOCK-CYCLE complexes act as positive elements, while PERIOD-TIMELESS act as negative elements. This negative feedback loop is illustrated in Figure 2.

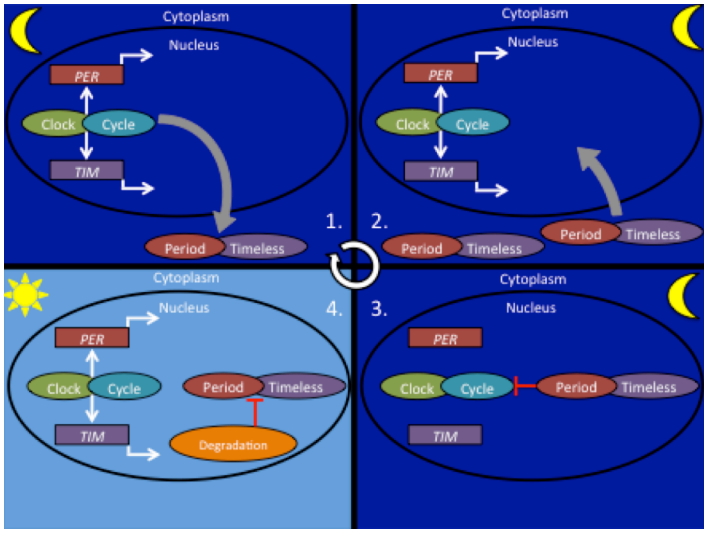

Figure 2. General Schematic of Drosophila Circadian Oscillatory System.
Adapted from (Rosbash et al. 2003)
Degradation in 4. includes both enzymatic degradation by CRY (in LD) and natural degradation (DD)

Computational Modeling of Circadian Rhythms

While molecular analysis has traditionally provided invaluable insight into the underlying mechanisms of circadian clocks, computational modeling of circadian systems has also played a significant role in the study of circadian clocks. For example, one of the most extensively studied mammalian circadian models is the tau hamster, which has a mutation that shortens its free-running period to 20 hours (Ralph and Menaker 1988). This mutation, named the tau mutation, was identified as missense mutation in the substrate-recognition site of CKIε, an enzyme implicated in the phosphorylation of PERIOD and in blocking its transportation into the nucleus (Eide et al. 2002; Lowrey et al. 2000). Homologs of the CKIε gene have been identified in *Drosophila* (Kloss et al. 1998) and the horse shoe crab, *Limulus polyphemus* (Limulidae), which belongs to the same subphylum as spiders, Chelicerata (Chesmore et al. 2016). How the tau mutation specifically affected the functionality of CKIε, however, was unknown.

Early molecular work suggested that the tau mutation decreased CKIε catalytic activity (Preuss et al. 2004). Thus, the mutation was proposed to speed up the period of the circadian oscillation by either (i) decreasing the phosphorylation of PERIOD, or (ii) decreasing the inhibition of nuclear transport of PERIOD (Gallego et al. 2006). However, Gallego et al. (2006), using a computational model developed by Forger and Peskin (2003), simulated decreased CKIε catalytic activity by decreasing the rate of steps that CKIε was known to play a role. They found that this actually increased the length of the period, rather than shortened it, as the molecular hypothesis would predict. This result suggested that the mutation was actually a gain-of-function mutation that *increased* phosphorylation of PERIOD, contrary to the proposed molecular mechanism. This claim was verified by comparing the degradation rate of PERIOD in cell-based assay containing either the tau-mutated CKIε or kinase-inactive CKIε. Tau-mutated CKIε was found to significantly decrease PERIOD concentration, while kinase-inactive CKIε had no affect on PERIOD concentration, which suggests that the tau-mutation of CKIε is in fact a gain-of-function mutation (Gallego et al. 2006).

In the context of my investigation of the circadian systems of spiders, I will integrate data from behavioral experiments into my circadian model in order to recreate their circadian activity. I will run numerical experiments that can, for example, create different lighting environments to test the response of the activity. The results of these numerical experiments will be used to generate testable hypotheses for later experimental work. My primary model will be a model developed by Smolen et al. (2002). This model is fitted to *Drosophila* data, and recreates cycling in PERIOD and CLOCK (Appendix 1 for discussion of model). Using the default parameters, the proteins cycle with a period of ~24 hours (Figure 3), though its parameters can be adjusted to recreate an 18 hour period (Smolen et al. 2003). And although this model is highly simplified (only two differential equations, as opposed to more complex models, which can reach up to 30 equations), it can still successfully recreate behavioral data.

Chapter 1.

Simplified *Drosophila* circadian model fails to recreate *Cyclosa turbinata* circadian oscillator

1.1. Introduction

Circadian rhythms are internally-generated biological clocks that control many important biological processes, such as activity levels, basal metabolic rate, and the sleep-wake cycle (Halberg 1960). The driving force behind these rhythms can be linked to daily oscillations of several so-called 'clock' proteins, most notably CLOCK and Period (reviewed in Dunlap 1999). These molecular oscillators, and their associated biological rhythms, are able to persist in the absence of external time cues, such as light and temperature, in a process known as free-running behavior. However, the molecular oscillators are not completely independent from the external world, and are able to respond ('entrain') to time cues.

Circadian rhythms appear across nearly all life, from single-celled cyanobacteria to humans. And within animals, there are high levels of conservation on both the behavioral and molecular levels (Johnson and Kondo 2001). The near ubiquity of circadian rhythms suggests that there is an evolutionary benefit associated with circadian rhythms. This claim has been supported by experimental evidence that there is a significant decrease in survival rate of chipmunks whose circadian rhythms have been ablated and released in the wild (DeCoursey et al. 2000). In addition, there is a significant cost to fitness when an organism's circadian rhythms are out of resonance with the environment in *Neurospora*, *Drosophila*, and the pitcher plant mosquito (Ouyang et al. 1998; Beaver et al. 2002, 2003; Emerson et al. 2008). However, Moore et al. (2016) found that locomotor activity in the trashline orb-weaving spider, *Cyclosa turbinata*, has an exceptionally short free-running period of 18.74±0.13 h. *C. turbinata*'s clock represents the shortest known naturally-occurring clock, on par with the 19-hour per^S Drosophila mutant (Hamblen-Coyle et al. 1989) or the 18-hour tau hamster (Ralph and Menaker 1988).

Given the fitness costs associated with non-resonant circadian clocks, there should be a significant cost associated with *C. turbinata*'s short period clock. However, no such cost has been observed. Thus, we predict that there is an adaptive benefit associated with *C. turbinata*'s short clock that is able to offset the cost of being out of resonance with the environment. The purpose of this study is to use computational models to describe the molecular oscillators underlying *C. turbinata*'s short clock. Our models will then be used in numerical experiments in order to generate testable hypotheses about potential adaptive benefits. Computational models have been powerful tools in previous circadian studies, for example in determining the role of the mutation that led to the tau hamster's short period (Gallego et al. 2006). Using similar methods, computational models are used to narrow down the field of possibilities for experimental verification. And in organisms like spiders, where circadian research is limited on the behavioral level, and non-existent on the molecular level, our computational models will provide a first-step analysis of their molecular oscillator to generate hypotheses that can guide further molecular analyses

1.2. Methods

Model

We used a modified version of Smolen et al.'s (2002) reduced circadian model fitted to *Drosophila* data. Because of the high conservation of circadian mechanisms across animals, a *Drosophila* model could accurately recreate spider circadian oscillators (Young and Kay 2001). Furthermore, we chose a reduced model because there is little known about the molecular

mechanisms underlying spider circadian rhythms and more complex models make assumptions about the system that we do not necessarily know are true in spider systems (e.g., the nature of post-translational modifications). This model uses a system of two differential equations to describe the concentrations of two clock proteins, PER (Period) and dCLK (*Drosophila* CLOCK) proteins as a function of time:

$$\frac{d[\mathrm{PER}]}{dt} = v_{sP} R_{sP} - k_{dP}[\mathrm{PER}]$$
$$\frac{d[\mathrm{CLOCK}]}{dt} = v_{sC} R_{sC} - k_{dC}[\mathrm{CLOCK}]$$

where R_{sP} is a time-delayed Hill Function, with time delay τ_1, that describes CLOCK stimulation of PER transcription. Similarly, R_{sC} is a time-delayed Hill Function, with time delay τ_2, that describes the self-inhibition of CLOCK transcription. The model's parameters were adjusted to generate stable oscillations with unperturbed (free-running) periods of 18 and 23 h (Figure 1). Parameters for the two simulations can be found in Table 1 (asterisks denote parameters that differ between the two simulations). A more detailed description of the model can be found in Appendix 1.

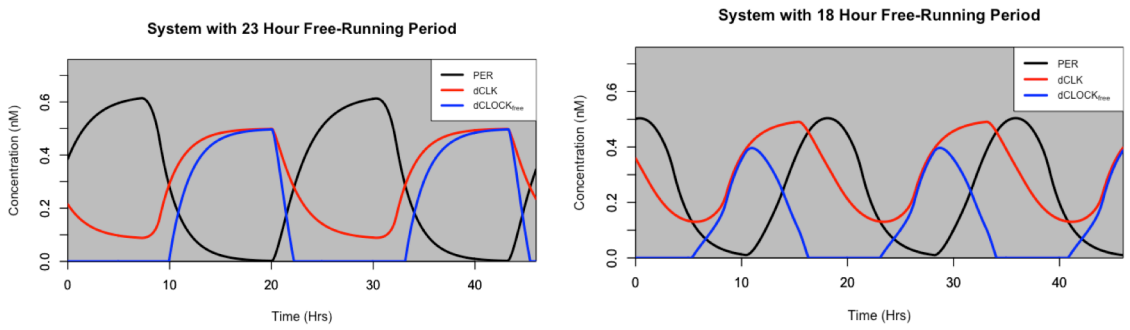

Figure 1. Smolen et al. (2002) model is able to recreate stable oscillations with free-running periods of 23 and 18 h. Shading represents simulated dark conditions.

Table 1. Parameters in Smolen et al. (2002) Model

Name	Interpretation	Period = 23 h	Period = 17 h
τ_1^*	Time delay in PERIOD production	10	5
τ_2	Time delay in CLOCK production	10	10
v_{sp}	Production rate of PERIOD	0.5	0.5
v_{sc}	Production rate of CLOCK	0.25	0.25
k_{dp}	Degradation rate of PERIOD	0.5	0.5
k_{dc}	Degradation rate of CLOCK	0.5	0.5
K_1	Hill Coefficient for Production of PERIOD	0.3	0.3
K_2	Hill Coefficient for Production of CLOCK	0.1	0.1

Numerical Simulations

We focused on the effects of different lighting conditions on the oscillatory behaviors of specifically the 18 h and 23 h systems. These two systems were then run under differing lighting conditions in order to determine their window of entrainment. The effect of light was simulated as a step-function increase in the degradation of PER (k_{dp}). The following lighting conditions were simulated in three different categories: Equal Length Light and Dark (LD) phases, Longer Dark-Phase, and Longer Light-Phase (Table 2). For each simulation, we examined the general behavior of the PER oscillators (e.g., number and location of peaks, amplitude of peaks).

Table 2. Light-Dark Simulations		
Equal Length LD	**Longer Dark-Phase**	**Longer Light-Phase**
12:12	11:13	13:11
13:13	10:14	14:10
14:14	9:15	15:9
15:15		

1.3. Results

Entrainment of different period clocks

Our model predicts that shorter period clocks can entrain to a wider range of light-dark cycles compared to the 23-hour clock (Figure 2). While both systems can entrain to LD 12:12 – 14:14, in LD 15:15, the short period clock maintains a stable oscillation, while the typical period clock becomes erratic. Thus, the model predicts that shorter period clocks can entrain to a wider variety of light-dark cycles. While the 23 hour system is still technically 'entrained' and the behavior repeats with a four-day cycle, such unstable oscillations would be detrimental to an organism. Furthermore, the model also predicts that the short-period clock shows two peaks in PER concentration in a single day under LD 13:13, 14:14, and 15:15. We next tested the emergence of a second peak in short period clocks under more physically realistic lighting conditions, in which the length of lights-on and lights-off add up to 24 hours.

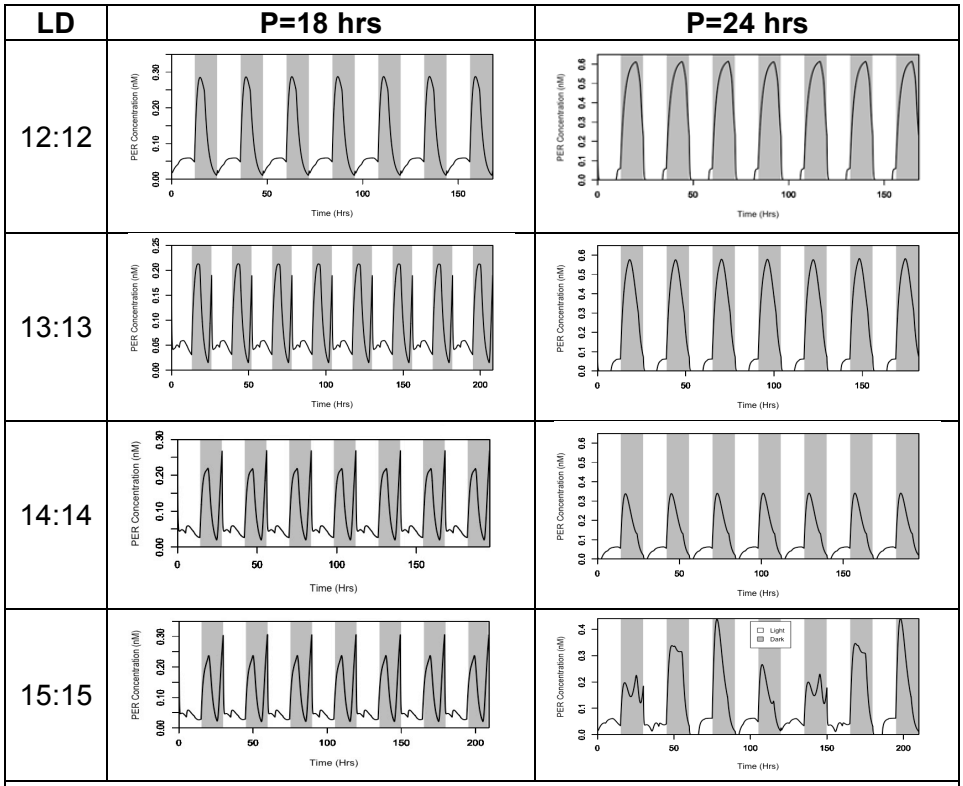

Figure 2. Entrainment profiles for 18 h vs. 23 h free-running period for a range of equal length light-dark cycles (LD 12:12 – 15:15). Shaded region represents dark conditions

Emergence of second PER peak in short period clocks under physically realistic light-dark cycles

The model predicts that short period clocks also display a second peak in PER concentration in light-dark cycles in which lights-on is shorter than lights-off, i.e., LD 11:13, 10:14, and 9:15 (Figure 3). As the length in lights off became shorter, the second peak became more pronounced, as in Figure 1 in the equal-length light-dark cycles. However, under light-dark cycles in which lights-on is longer than lights-off, i.e., LD 13:11, 14:10, 15:9, the model does not predict a second peak in PER concentration (Figure 3).

1.4. Discussion

Our results suggest that the simplified Smolen et al. (2002) model does not accurately reflect *C. turbinata*'s molecular oscillator, both behaviorally and ecologically. Actograms of *C. turbinata* do show two peaks of activity in a single night, which would correspond to two peaks in PER concentration (Moore et al. 2016). However, the second peak appeared even during LD 12:12, which our model does not predict. Furthermore, our model's predictions are not ecologically relevant. The second peak only appears when the night is longer than the day, which occurs during winter, when *C. turbinata* is either inactive or dead. So it is unlikely that a secondary peak as predicted by our model would hold any adaptive benefit.

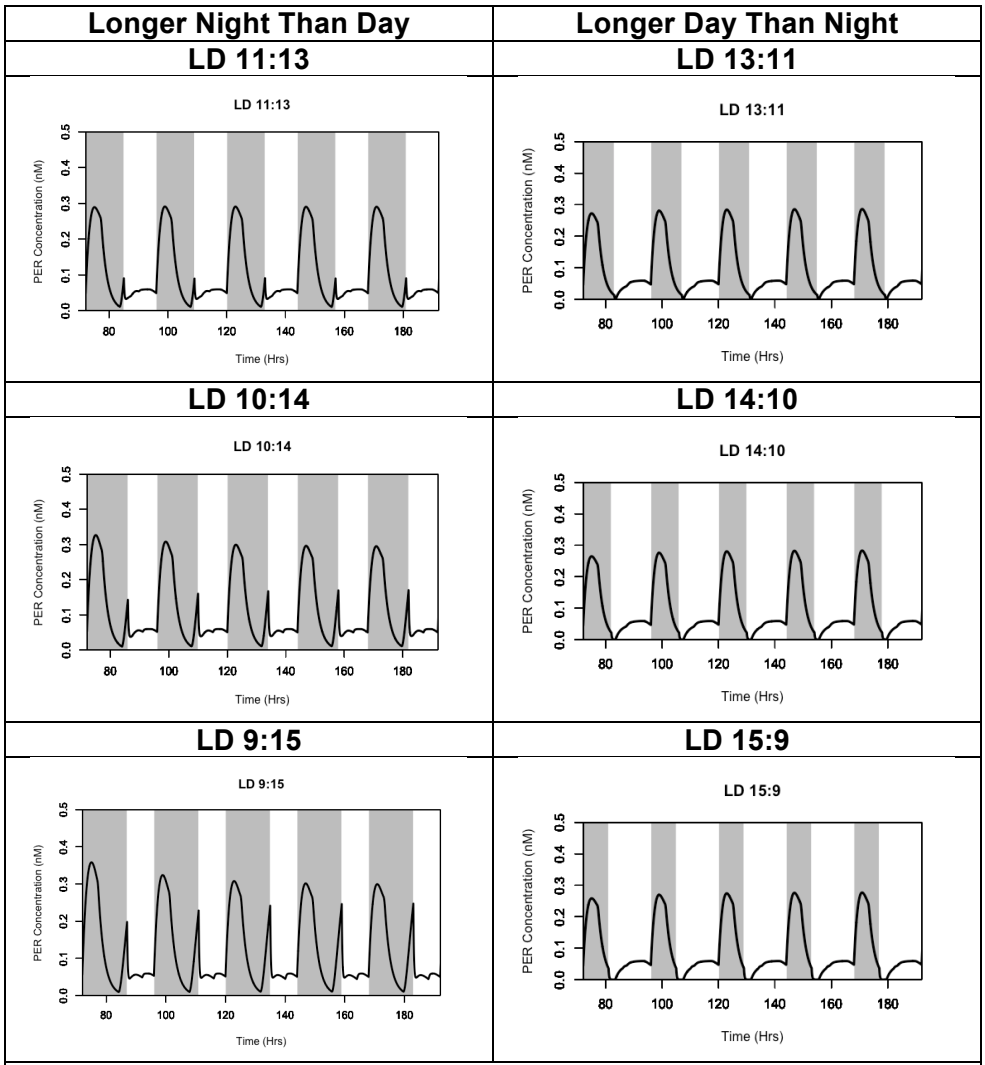

Figure 3. Entrainment profile for 18 h free-running period under physically realistic light-dark conditions

The discrepancies in the conditions that give rise to a second peak may have arisen from our modeling of the effects of light on the system. In our model, as per the original Smolen et al. (2002), we modeled light as a tenfold stepwise increase in PER degradation rate, k_{dp}. And while this may be a valid technique when modeling a *Drosophila* system, my behavioral analysis of three spiders from the family Theridiidae (Chapter II), suggest that spiders have a more robust response to light. In terms of modeling, this would mean that our stepwise increase in PER

degradation does not accurately reflect the effect of light on *C. turbinata*. Alternatives that might better model the effect of light on *C. turbinata* could include a non-linear response, or a graded, rather than stepwise, increase.

However, our model does agree with behavioral data in the appearance and timing of a second peak, despite the differences in conditions that would give rise to a second peak. Behaviorally, *C. turbinata*, which shows two peaks, first in aggressiveness and second in anti-predator behavior over the course of a single night (Watts et al. 2014), which are under circadian control in another orb-weaving spider species, *Larinioides cornutus* (Jones et al. 2011). From an ecological standpoint, we hypothesize that the first of these peaks, shortly after sundown, corresponds to an increase in aggressiveness to prompt prey-capture behavior. Then the second peak, which occurs right before sunrise, corresponds to a cue to repair their web and increase anti-predator behavior in preparation for the day. This could provide an adaptive benefit for a species like *C. turbinata*, which must balance both aggression as a predator and passivity as a prey.

Chapter 2.

High variability in free-running behavior among three theridiid species suggests relaxed selection on spider circadian rhythms

2.1. Introduction

Circadian rhythms are biological clocks that appear nearly ubiquitous across life. These rhythms underlie the timing of many important biological processes, ranging from the sleep-wake cycle to the release of hormones like prolactin and cortisol (reviewed in Halberg et al. 2003). Even in the absence of external time cues, such as light or temperature, circadian rhythms persist with a regular period, known as the free-running period. These rhythms, however, are not independent of the external world, and can still adjust ('entrain') their periods to time cues. At their most fundamental level, circadian rhythmicity is driven by oscillations of so-called 'clock' proteins over the course of the day (reviewed in Dunlap 1996).

This ability to entrain to the external environment appears to be a crucial feature of circadian rhythms. Previous studies have found that there is a significant negative cost to fitness when the period of an organism's circadian clock does not closely match the period of the external time cues, or when circadian clocks cease to function altogether (Ouyang et al. 1998; DeCoursey et al. 2000; Beaver et al. 2002, 2003; Emerson et al. 2008). This, combined with the ubiquity of circadian rhythms across life, suggest that there is an adaptive benefit associated with circadian rhythmicity with a period that closely matches that of the external world (~24 hours). The prevailing hypothesis is that circadian rhythms allow organisms to anticipate regular changes in the environment, such as the transition from night to day, rather than simply react (reviewed in Young and Kay 2001).

Despite the apparent pressure towards a circadian rhythm with a 24-hour period, Moore et al. (2016) found that the trashline orb-weaving spider, *Cyclosa turbinata* (Family: Araneidae), has a free-running period (FRP) of 18.74±0.13 h. This represents the shortest known, naturally-occurring clock described thus far, on par with lab-generated short-period strains, including the tau hamster (FRP ~ 20 h; Ralph and Menaker 1988) or the *per*^{S} *Drosophila* strain (FRP ~ 19 h; Konopka and Benzer 1971). Whether *C. turbinata's* short period represents an outlier among spiders, however, is unknown. Work in spider circadian rhythms has been limited. Behaviorally, circadian rhythmicity has been described in locomotor activity in eight species from seven families (Table 1). Each of these studies found 'typical' free-running periods (24±2 h). Furthermore, on the molecular level, no work has been done.

The purpose of this study is to describe circadian rhythmicity in locomotor activity of three additional species in the family Theridiidae: the subsocial spider, *Anelosimus studiosus*; the common house spider, *Parasteatoda tepidariorum*; and the southern black widow, *Latrodectus mactans*. Our results almost double the number of described circadian rhythms in spider locomotor activity. Furthermore, because our three study species are all from the same family, we can also understand how circadian rhythms vary within families. In addition to understanding the circadian rhythms of our three species on a behavioral level, we will also use computational models in order to understand the results from our behavioral experiments on a molecular level. These models track the fluctuations of 'clock' proteins throughout the day, and can allow us to make connections between observed activity and theoretical protein levels at that time of day. The results from our model can act as a springboard for further investigation into the molecular circadian oscillators in spiders.

2.2. Methods

Study Species

Adult females of *Anelosimus studiosis* were collected from their webs at night in Washington Co. TN, Summer, 2012 and 2017. Adult females of *Latrodectus mactans* and *Parasteatoda tepidariorum* were collected from their webs at night in Washington Co. TN, July and August 2017. Care of the animals followed Association for the Study of Animal Behavior/Animal Behavior Society (ASAB/ABS) guidelines, and the animals were released near the site of collection following experiments.

Locomotor activity

Following collection, individuals were entrained to 12 hours of light and 12 hours of dark (LD 12:12, Light from 0800-2000) in the laboratory for three days in clear plastic containers (6 cm diameter X 3.6 cm), during which time the spiders were fed once. Monitoring began after the three-day laboratory pre-entrainment. Individuals from *L. mactans* (N=20), *A. studiosis* (N=22), and *P. tepidariorum* (N=17) were placed in clear glass or plastic tubes (15 mm diameter X 100 mm length), and inserted into a temperature-controlled (25 ± 0.5 ^{o}C) locomotor activity monitor (model LAM25, Trikinetics Inc., Waltham, Massachusetts). In the monitors, individuals were first under an LD 12:12 (Lights from 0800-2000) for five days, followed by ten days of total dark (DD). Light during photophase was provided by four vertically mounted, 32 W fluorescent tubes (illuminance ~1400-1600 lux).

Activity for each individual was measured by recording number of interruptions of three infrared beams transmitting across the middle of their tube. Each interruption was registered as an event, and events were counted in 1-minute bins. Activity patterns were analyzed using Clocklab Analysis 6 Software (Actimetrics, Wilmette, IL, U.S.A.). Activity was first plotted as a double-plotted actogram to allow for visual inspection. Significant periods in activity patterns were determined using the chi-squared periodogram (Sokolove and Bushell 1978). Activity patterns showing a significant period under the chi-squared periodogoram were confirmed using the Lomb-Scargle periodogram. Lomb-Scargle periodograms determines periodicity using a least squares fit of sinusoidal waves, and is better suited for activity data with large gaps (Dongen et al. 1999). For *P. tepidariorum* and *A. studiosis*, periods were only reported for an individual if both the chi-squared and Lomb-Scargle periodograms returned significance ($p < 0.01$). For *L. mactans*, their free-runs changed over the course of the experiment. Actograms were divided into thirds (7 day periods) and a period was reported for each third. A total period of an individual is reported as the average of each of the thirds. Furthermore, because their free-run data was generally noisier, we reported any significant periods using only the Lomb-Scargle periodogram since it is better suited for noisy data (Ruf 1999; Van Dongen et al. 1999)

Numerical Simulations

Using Smolen et al. (2002), we investigated the effect of light on PER oscillations (an in-depth discussion of model can be found in Appendix 1). Light was modeled as a step-function increase in the degradation of PER (k_{dp}). Systems were first exposed to 5 days of simulated LD 12:12 followed by DD. We calculated the proportion of the total period spent in the rising phase during the LD phase, and compared those proportions against the proportion in the DD phase (Figure 1). These proportions were calculated for a range of periods, from 23 h down to 17 h.

2.3. Results

Entrainment profiles

We first measured the ratio of daytime to nighttime activity of each species, known as the DiNoc ratio, in order to determine when each species is more active. Across all three species, DiNoc ratios (mean daytime activity – mean nighttime activity / mean total activity) showed predominantly nocturnal locomotor activity (DiNoc ratio < 0; Table 1). Within *A. studiosus*, mean activity, averaged across each 24-hour period of each individual, showed sporadic peaks throughout photophase. Activity had a sharp peak shortly after lights-off (<1 h), which leveled off ~2 hours after lights-off, before dropping ~1 h before lights-on (Figure 1). Both *P. tepidariorum* and *L. mactans* showed low-levels of consistent activity during photo-phase. Compared to *A. studiosis*, activity peaked slower in *P. tepidariorum* and *L. mactans*, at ~1 h after lights-off. *P. tepidariorum*'s activity steadily declined throughout scotophase, whereas *L. mactans*'s activity continued at a low level throughout mid-scotophase, before dropping off ~2 h before lights-on (Figure 1).

Table 1. DiNoc Ratio for Study Species

	A. studiosus	*P. tepidariorum*	*L. mactans*
Mean DiNoc Ratio	-0.819	-0.701	-0.757
Standard Error	0.0417	0.0539	0.0466

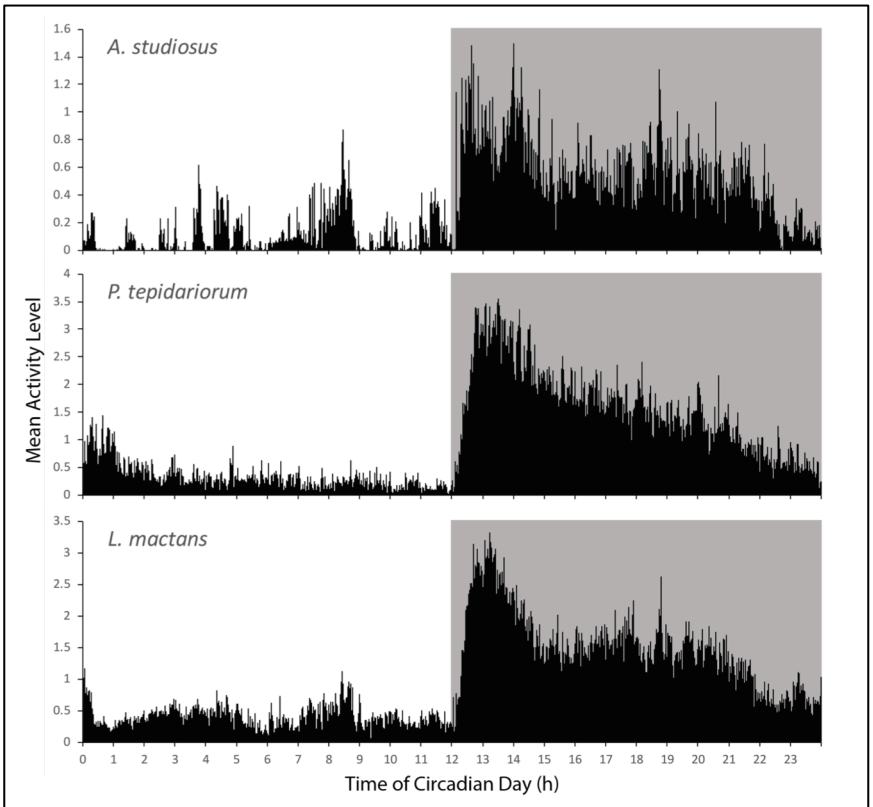

Figure 1. Mean activity levels across species *A. studiosus* (N=17), *P. tepidariorum* (N=22), and *L. mactans* (N=20).
Shown in black are mean activity levels, in 1-min bins, averaged across 5 days of exposure to light-dark cycle.
Shading indicates scotophase.

Circadian free-runs

A. studiosus

Each individual (N = 23) showed significant periods on both chi-squared and Lomb-Scargle periodograms (Figure 2; Mean = 23.1035, SEM = 0.4795). However, there appears to be a wide distribution in free-running periods, ranging from 20.83 to 28.58 h.

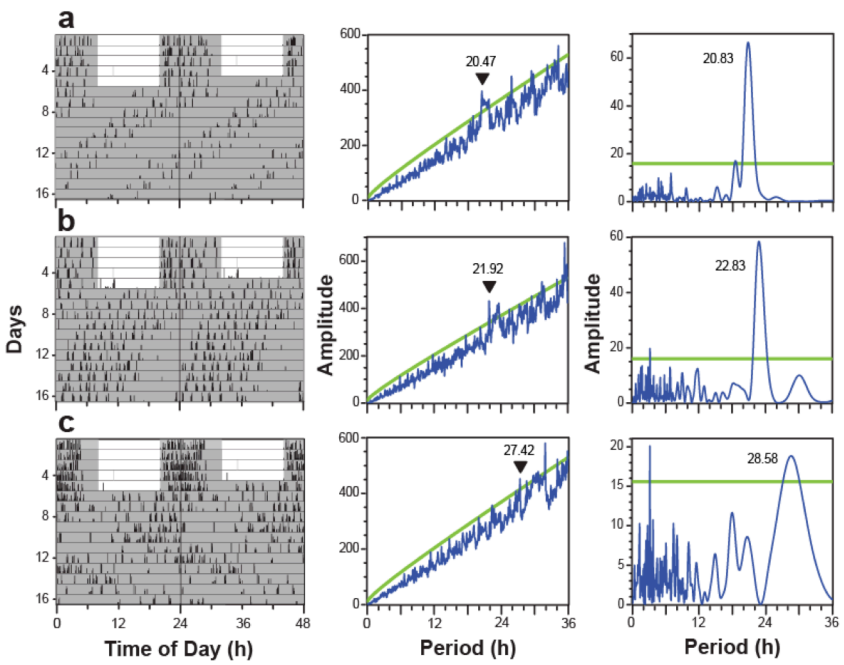

Figure 2. Double-plotted actograms and two periodograms for three different individuals of *A. studiosus.*
Actograms (left-column) depict timing of activity for five consecutive days under LD 12:12 cycle, followed by constant dark (DD) conditions thereafter. Dark periods are represented by gray background.
Chi-square periodograms (middle-column) and Lomb-Scargle periodograms (left-colmun) indicate significant periodicities ($p < 0.001$) of locomotor activity under DD.

P. tepidariorum

Each individual (N=17) exhibited significant free-running periods ($p < 0.001$), for both chi-square and Lomb-Scargle periodograms (Mean FRP = 21.6, SEM = 0.26 h). Furthermore, 16 out of 17 individuals showed a significant secondary period in Lomb-Scargle periodogram (Figure 3, indicated by black triangle).

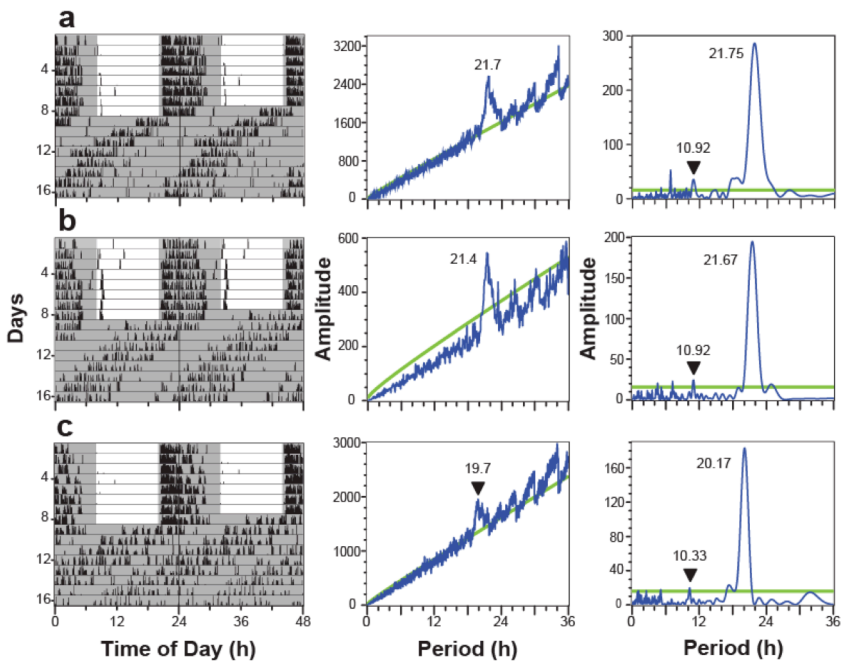

Figure 3. Double-plotted actograms and two periodograms for three different individuals of *P. tepidariorum.*
Actograms (left-column) depict timing of activity for eight consecutive days under LD 12:12 cycle, followed by constant dark (DD) conditions thereafter. Dark periods are represented by gray background.
Chi-square periodograms (middle-column) and Lomb-Scargle periodograms (left-colmun) indicate significant periodicities ($p < 0.001$) of locomotor activity under DD.
All three individuals show significant second period of activity under DD in Lomb-Scargle periodogram (indicated by black triangle).

L. mactans

At first glance, free-running data for *L. mactans* (N=22) appears to be highly noisy, without any apparent rhythmicity (Figure 4a). Chi-squared periodograms do not report any significant periods. However, Lomb-Scargle periodograms, which are better suited for noisy data (Ruf 1999; Van Dongen et al. 1999), reported weakly significant periods (Mean FRP = 24.7 h, SEM = 0.63 h). Furthermore, in 10 out of 22 individuals, the free-running period changes throughout DD (Figure 4). Lomb-Scargle Periodograms calculated separately for each third of the experiment (6-7 days) report different periods, differing by as much as 8 h. Furthermore, periodograms of 20 out of 22 individuals suggest ultradian peridodicity (period ~2-5 h; ultra- = 'shorter', -dian = 'day'), although they are still weakly rhythmic (Figure 5).

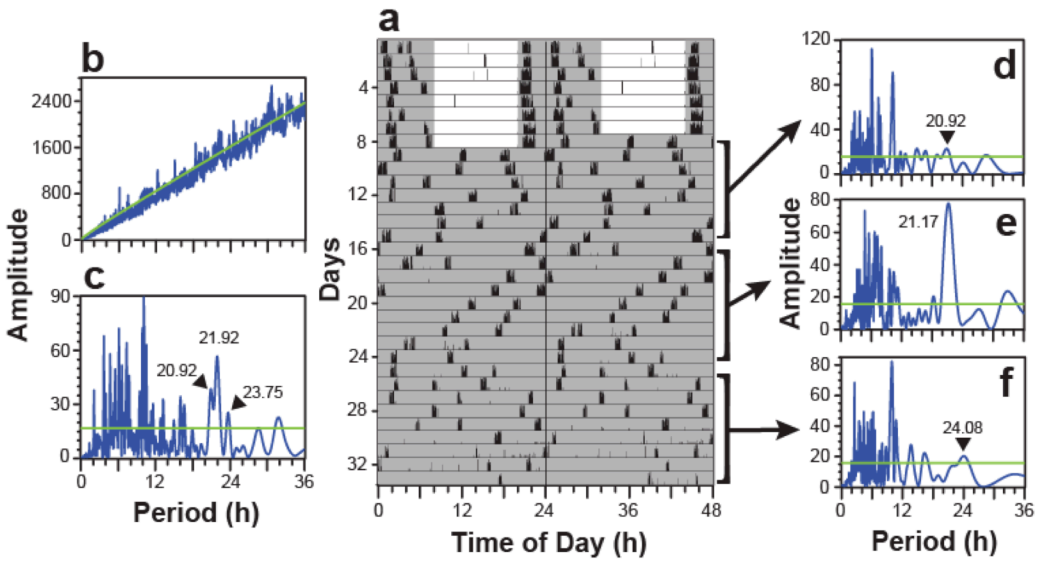

Figure 4. Actogram of *L. mactans* individual shows changing free-running period throughout DD conditions.
a. Actogram for one *L. mactans* individual.
b. Chi-squared periodogram of entire DD is inconclusive.
c. Lomb-Scargle periodogram of entire DD shows multiple significant periods.
d., e., f. Lomb-Scargle periodogram of first, second, and third of DD (respectively) show changing free-running period

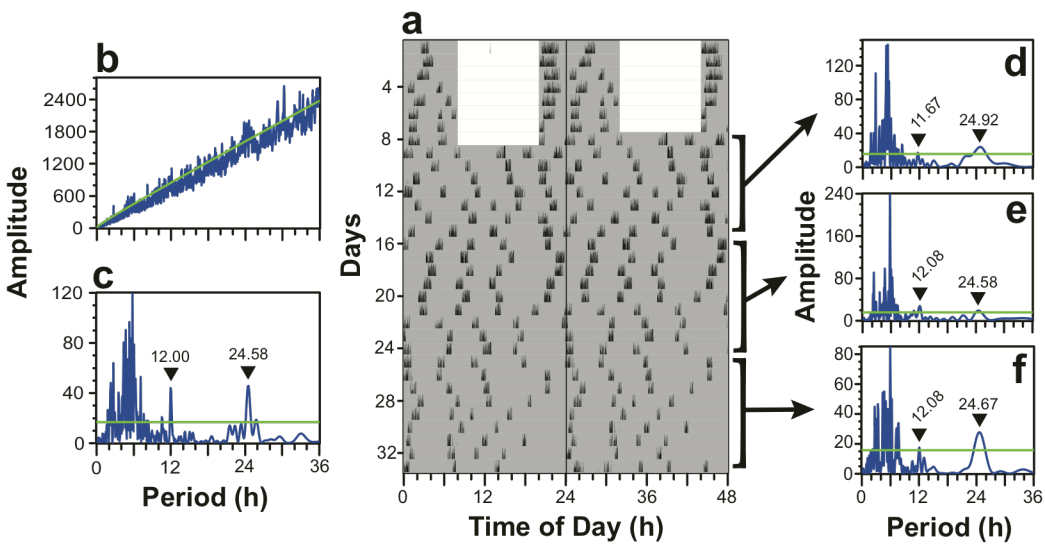

Figure 5. Actogram of *L. mactans* individual shows consistent free-running period and evidence of ultradian rhythms.
a. Actogram for one *L. mactans* individual shows multiple bands of activity in a single day, indicative of an ultradian rhythm.
b. Chi-squared periodogram of entire DD is inconclusive.
c. Lomb-Scargle periodogram of entire DD shows single significant periods.
d., e., f. Lomb-Scargle periodogram of first, second, and third of DD (respectively) show consistent free-running period

2.4. Discussion

Among our three species surveyed, there is a great deal of similarities in their entrainment profiles (Figure 1). However, the free-running behavior of our species varies extensively, both within and among species. Within species, both *A. studiosus* and *L. mactans* show a wide distribution in free-running periods, both ranging between ~20 to 30 h (Figure 6). In context, distributions of free-running periods for humans range from ~23.9 to 24.5 h (Czeisler et al. 1999), and from ~23.8 to 24.1 h for golden hamsters (Ralph and Menaker 1988). So the distributions of *A. studiosus* and *L. mactans* are much wider than we would expect, given the distributions of more canonical circadian systems.

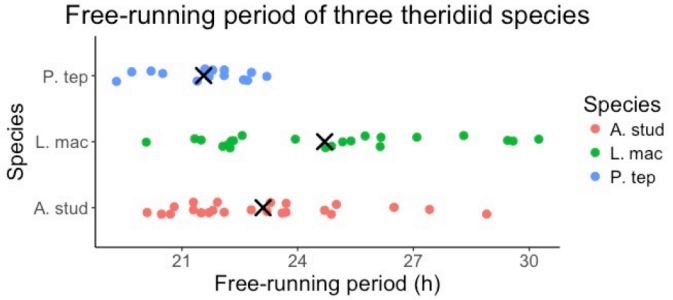

Figure 6. Three theridiid species show wide distribution of free-running periods within species, and wide variation in distribution between species.
X indicates mean free-running period for each species

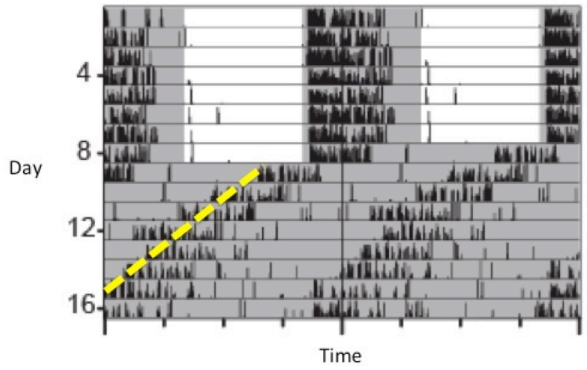

Figure 7. Activity onsets of *P. tepidariorum* do not trace back to last peak of LD

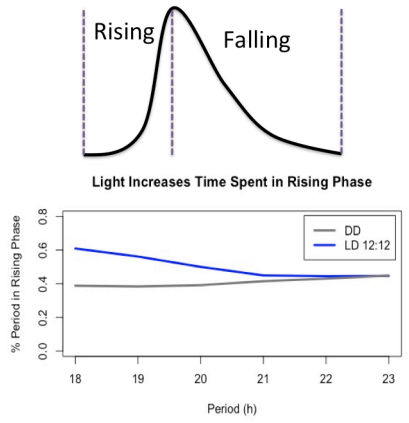

Figure 8. Smolen et al. (2002) model predicts inhibition of rising phase in shorter period clocks

Furthermore, free-running behavior markedly differs among species While, *A. studiosus* have generally consistent free-runs, both *P. tepidariorum* and *L. mactans* display abnormal behaviors. First, in typical circadian free-running data, if you draw a line though the onset of activity each day during DD, it should connect back to onset of activity on the last day of entrainment. However, in 16 of the 17 *P. tepidariorum* individuals, a line through their activity onsets would connect back to mid- to late-photophase, during which there was no activity, rather than the activity onset of the last day of entrainment (Figure 7). Because the free-runs extrapolate back to photophase, we hypothesize that light is strongly inhibiting activity that, in essence, *wants* to begin during late photophase but cannot because light is inhibiting it. Mechanistically, using numerical simulations of a model developed by Smolen et al. (2002; See Appendix 1 for detailed discussion of model), we found that light can inhibit the onset of activity in systems with free-running periods similar to *P. tepidariorum* (~21 h) by increasing time spent in rising phase of the PER oscillation (Figure 8). By inhibiting the rising phase, it takes longer for the system to reach the critical concentration of protein to begin initiating activity, thus delaying activity onset. However, our experiment does not confirm whether light is truly inhibiting activity or if we observed a

phase-shift as a result of the transition from LD to DD. Future experiments using different levels of light are necessary to make the distinction between these possible explanations. If it is inhibition of activity, then the initial burst of activity should spread as the light phase gets progressively dimmer (Figure 9A.). However if it is a phase shift, then the entire burst of initial activity should shift earlier as the lights dim (Figure 9B).

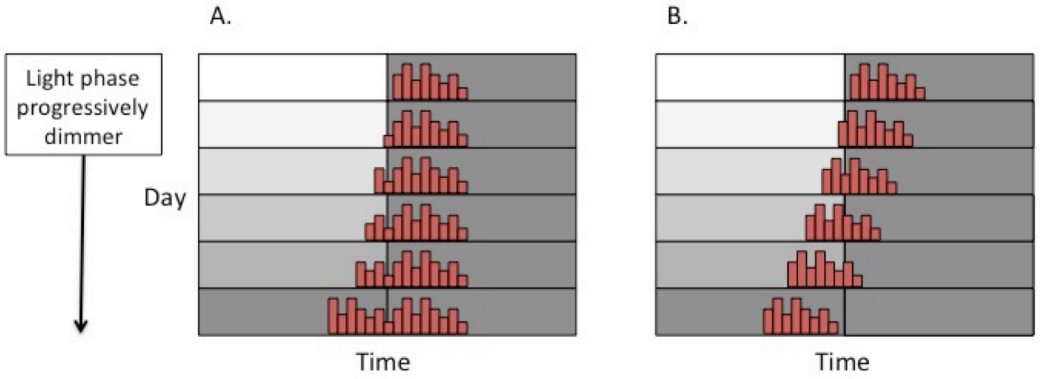

Figure 9. Two possible scenarios of light-dimming experiment in *P. tepidariorum.*
A. Initial activity burst spreads as lights gets dimmer, which suggests light inhibition.
B. Initial activity burst shifts earlier as light gets dimmer, which suggests a phase shift.

Second, in *A. studiosus* and in *P. tepidariorum,* despite the potential masking effect, their free-runs still have periods ~24 h and appear to be typically circadian. *L. mactans*' free-runs, however, show evidence of ultradian peridodicity (period ~2-5 h; ultra- = 'shorter', -dian = 'day'), although they are still weakly rhythmic. First, we can see multiple vertical bands in the actograms for *L. mactans* (Figure 5) that are characteristic of ultradian rhythms (e.g., in the common vole, *Microtus arvalis*; Gerkema and van der Leest 1991). Because ultradian rhythms have periods much shorter than 24 hours, they would peak multiple times over the course of a single day, as opposed to circadian rhythms, which should only peak once in a single day. So multiple, distinct bands of peaks suggest ultradian rhythms in *L. mactans*. Similar bands, however, are not present for either *P. tepidariorum* (Figure 4) or *A. studiosus* (Figure 3). Furthermore, Lomb-Scargle periodograms for *L. mactans* show multiple significant periods that are integer multiples of each other. This suggests a short, ultradian period that is being detected twice in the periodograms.

Given the experimental support for significant fitness costs associated with free-running behavior that is out of resonance with the external environment (Ouyang et al. 1998; DeCoursey et al. 2000; Beaver et al. 2002, 2003; Emerson et al. 2008), we would expect less variation in free-running behavior within and among our three species. In order to explain our high levels of observed variation, we hypothesize spiders have evolved a robust response to light that led to the consistent entrainment profiles among our three theridiid species. Evolving such a robust response to light could have then relaxed selection on free-running behavior, because a robust response to light can iron-out intra- and interspecies variation, allowing for the emergence of the variety of observed behavior (e.g., wide variation in FRP, weak free-running behavior). Relaxed selection of free-running behavior also allows for adaptive evolution, which could allow spiders to evolve unique adaptive free-running mechanisms. This idea of relaxed selection could be extended to the exceptionally short-clock of the trashline-orb weaving spider, *Cyclosa turbinata* (Moore et al. 2016), which belongs to a related family, Araneidae. *C. turbinata* similarly shows a

consistent entrainment profile, but under DD conditions, it has the shortest known naturally-occurring free-running period of 18.74±0.13 h. So our hypothesized relaxed selection could apply to more arachnid families than just Theridiidae. However, our work does not necessarily explain any actual adaptive benefits of the clocks, just that they may be under conditions that would allow for adaptive benefits to arise. Further behavioral (e.g., measuring fitness) and genetic (e.g., identifying arachnid clock genes and examining selection patterns) tests would be necessary to help elucidate adaptive benefits.

Appendix 1. Explanation of Model Equations

The Smolen (2002) model uses a system of two differential equations to describe changes in concentration of PERIOD and CLOCK with parameter fitted from Drosophila data (values found in Table A1).

$$\frac{d[\mathrm{PER}]}{dt} = v_{sP} R_{sP} - k_{dP}[\mathrm{PER}]$$

$$\frac{d[\mathrm{CLOCK}]}{dt} = v_{sC} R_{sC} - k_{dC}[\mathrm{CLOCK}]$$

It is able to recreate stable oscillations in the concentration of both PERIOD and CLOCK (Chapter 1, Figure 1) with a periods ranging from 17 to 23 hours. Furthermore, Smolen (2002) demonstrated that the oscillatory behavior is fairly insensitive to changes in parameters. Changes in parameters of up to 50% still allowed for robust oscillations.

Figure A1. pictorally represents the model.

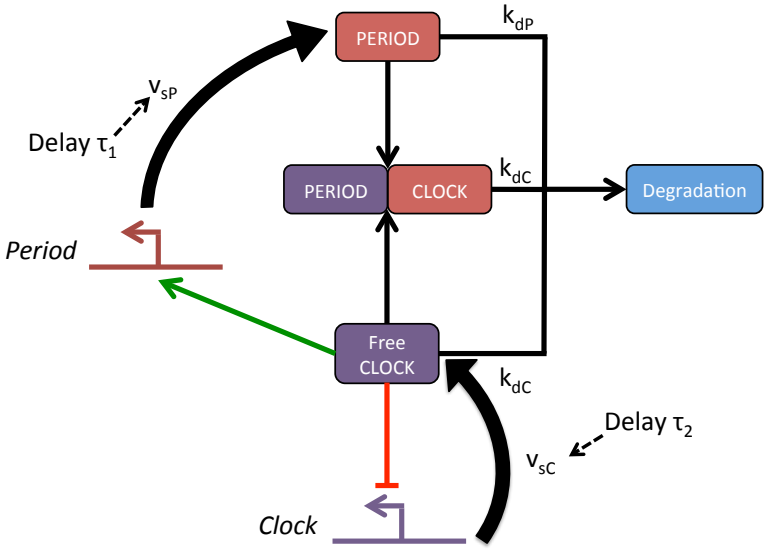

Figure A1. General Schematic of Smolen (2002) model.
Based on Figure 1A of Smolen (2002)

Mechanistically, the *Period* gene is transcribed at a rate v_{sP} that is directly proportional to the concentration of active CLOCK, related by a Hill Function described below, a commonly used function in the description of biochemical interactions (Weiss 1997), including the time delay, τ_1.

$$R_{sP} = \left\langle \frac{[\mathrm{dCLOCK}_{\mathrm{free}}]}{K_1 + [\mathrm{dCLOCK}_{\mathrm{free}}]} \right\rangle \tau_1$$

Physiologically, this time delay represents the post-translational modifications of PERIOD, including multiple phosphorylations (Kloss et al. 2001). Finally, PERIOD either complexes with CLOCK (which occurs instantaneously in this model) or is degraded at a rate of k_{dP}, which is directly proportional to the concentration of PERIOD.

In this model, CLOCK can take one of two forms, free CLOCK and PERIOD-bound CLOCK. Free CLOCK is the active form that promotes the transcription of *Period* (demonstrated by Rutila et al. 1998). Below is the definition of $[\mathrm{dCLOCK_{free}}]$ for R_{sP} equation.

$$[\mathrm{dCLOCK_{free}}] = \begin{cases} [\mathrm{dCLOCK}] - [\mathrm{PER}] \text{ if } [\mathrm{dCLOCK}] > [\mathrm{PER}] \\ 0 \text{ if } [\mathrm{dCLOCK}] \leq [\mathrm{PER}] \end{cases}$$

For CLOCK, the *Clock* gene is transcribed at a rate of v_{sC}, which is described by an inverse Hill Function, making it inversely proportional to the concentration of CLOCK, described below. Similar to PERIOD, CLOCK also includes a time delay, τ_2, between transcription of *Clock* and the production of functional CLOCK. Again, this time delay incorporates the post-translational modifications of CLOCK (Lee et al. 2001).

$$R_{sC} = \left\langle \frac{K_2}{K_2 + [\mathrm{dCLOCK_{free}}]} \right\rangle \tau_2$$

After functional CLOCK has been produced, it will promote the transcription of PERIOD, increasing the transcription rate of PERIOD, v_{sP}. However, when PERIOD is produced, it complexes with CLOCK, rendering it inactive, lowering, v_{sP}. Both CLOCK and the CLOCK-PERIOD complexes can degrade at a constant rate of k_{dC}, which is again directly proportional to the concentration of CLOCK.

Table A1. Parameters in Smolen et al. (2002) Model

Name	Interpretation	Value
τ_1	Time delay in PERIOD production	10
τ_2	Time delay in CLOCK production	10
v_{sp}	Production rate of PERIOD	0.5
v_{sc}	Production rate of CLOCK	0.25
k_{dp}	Degradation rate of PERIOD	0.5
k_{dc}	Degradation rate of CLOCK	0.5
K_1	Hill Coefficient for Production of PERIOD	0.3
K_2	Hill Coefficient for Production of CLOCK	0.1

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