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Age and Seasonal Change in the Chronobiology of a Spider with an Exceptionally Long-Period Circadian Clock

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Age and Seasonal Change in the Chronobiology of a Spider with an Exceptionally Long-Period

Circadian Clock

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A thesis

presented to

the faculty of the Department of Biological Sciences

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In partial fulfillment

of the requirements for the degree

Master of Science in Biology

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by

Shae Elizabeth Crain

December 2019

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Darrell Moore

Karl Joplin

Keywords: circadian rhythm, seasonality, chronoecology, behavioural rhythm, aging, free-
running period

ABSTRACT

Age and Seasonal Change in the Chronobiology of a Spider with an Exceptionally Long-Period

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by

Shae E. Crain

This study examines locomotor activity in samples of *Frontinella pyramitela* collected over its

active season (April-October) to investigate whether seasonality and/or age may help explain the

exceptional variability typically found in spider clock systems. Despite its noteworthy variability

(%CV= 7.7), we have found that *Frontinella* has a mean free-running period of 28.4±2.18 hours

that does not significantly vary over time. There is no correlation between day length and free-

running period, indicating that varying length of FRP is not a function of photoperiod length. In

LD 12:12 h, the window of activity is significantly smaller in April, gradually widening as the

season continues, which may be evident of a shift in foraging strategy. *Frontinella*'s clock

appears to mature before its entrainment mechanisms are fully developed, and towards the end of

its season, there is strong evidence of circadian misalignment which may be a product of

physiological age.

DEDICATION

I dedicate this thesis to all of the spiders who contributed to this study.

ACKNOWLEDGEMENTS

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CHAPTER 1. INTRODUCTION

The Circadian System

An organism's circadian clock exists as a network of self-regulating oscillators that facilitates (entrains) rhythmic coordination with the day/night cycle (Aschoff 1960). This manifests as physiological and behavioral output that oscillates at a period (*tau*) of around 24 hours in the absence of external cues (Pittendrigh 1993). Such rhythmic output is known as a free-running period (FRP). As shown in Table 1.1, free-running periods tend to be highly stable with low inter-individual variability (Czeisler et al. 1999).

Table 1.1 Mean Free-Running Period (τ) and Percent Coefficient of Variation in Various Taxa

Species	Mean τ	%CV	Author
Golden Hamster	23.9	0.30	Czeisler et al, 1999
Lake chub *(Couesius plumbeus)*	25.5	0.98	Kavaliers, 1978
Wasp *(Nasonia giraulti)*	23.2	3.34	Bertossa et al, 2013
Rat	24.4	0.57	Honma et al, 2006
Honeybee*s (Apis mellifera)*	23	2.17	Moore & Rankin, 1985
Flesh fly *(Sarcophaga crassipalpis)*	24.1	1.29	Prohaska et al., 2018
Lesotho mole rat *(Cryptomys hottentotus)*	24.1	0.92	Schöttner et al, 2006
Human	24.2	0.55	Czeisler et al, 1999
Drosophila PER mutants			
per₁, per₁	28.6	1.75	Konopka & Benzer, 1971
per₁, perₒ	30.6	4.25	Konopka & Benzer, 1971

Detailed examination of internal clock mechanisms in model organisms have shown that the circadian clock is a tiered system of multiple (peripheral) clocks regulated by a core oscillator, or 'master clock' (Bell-Pedersen et al. 2005). This 'master clock' mechanism involves

specialized clock genes and proteins which oscillate in a perpetual, negative feedback loop of

specifically-timed translation and transcription processes (Kumar 2017).

Since circadian systems are rarely exactly 24 hours, an organism's clock entrains daily by

remaining in a phase position relative to its zeitgeber (time giver). The most influential zeitgeber

is light availability (photoperiod) (Hirschie Johnson et al. 2003). To entrain to the 24-hr day, a

photic cue is passed on from a photoreceptor to the master clock. That cue continues downstream

from the master clock to its peripheral components which regulate internal and external

processes. The entire clock system then shifts itself to realign its components to the phase angle

of the photo cue. Figure 1.1 illustrates the daily entrainment process undergone by the circadian

clock.
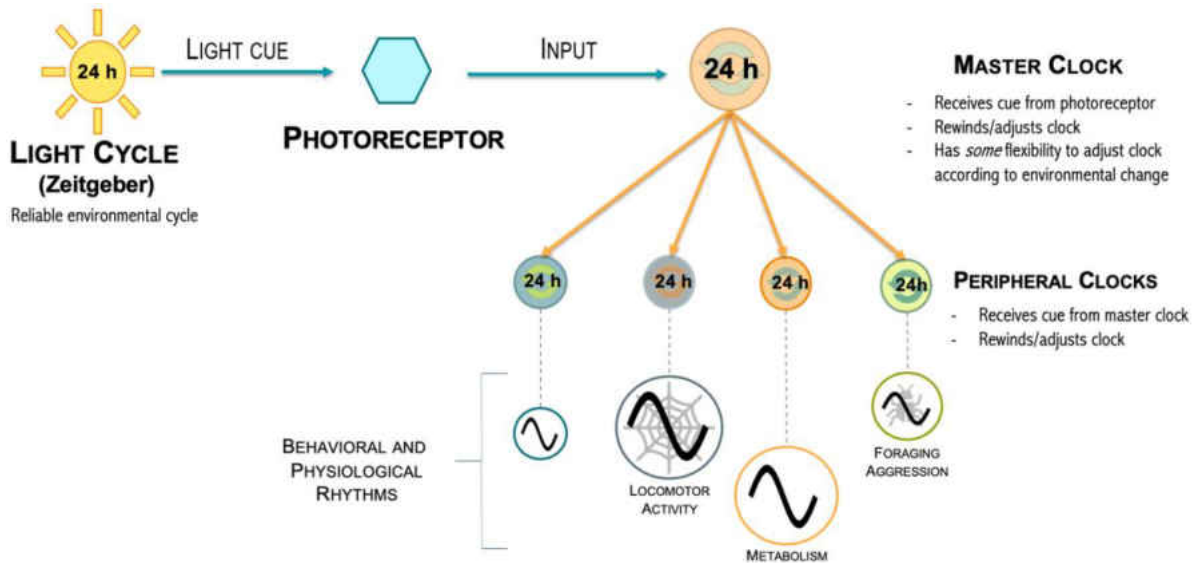
Figure 1.1 Representation of the multi-oscillatory circadian clock system entraining to an
external cue

It is important to note that the circadian system possesses a certain range of plasticity to

entrain to varying photoperiod lengths resulting from seasonality (Aschoff 1960). However,

peripheral clocks do not necessarily entrain at the same rate, therefore a clock too far out phase with its zeitgeber can result in a transient state (West and Bechtold 2015). Abrupt shifts in phase relationships such as jetlag and shift work are a form of *chronodisruption* that result in *circadian misalignment* (Brown et al. 2019). It has been argued that internal desynchrony within the clock system is physiologically damaging and has been associated with a number of metabolic and neurological pathologies (Barnard and Nolan 2008, Brown et. el, 2019).

It is suggested that circadian clocks are adaptive in that they instill internal order while also reinforcing coordination with their external environment. The "circadian resonance hypothesis" maintains that synchrony with the external environment is the main selective pressure indicating an adaptive significance (Pittendrigh and Bruce 1957). A key understanding from studies investigating the resonance hypothesis suggests that coordination with the environment is one of the most essential facets of circadian organization. Synchrony is important to survival; any loss of resonance may result in significantly reduced fitness and lifespan (Ouyang et al. 1998; DeCoursey et al. 2000). Supporting evidence demonstrates how arrhythmic strains of *Synechococcus* do best in an arrhythmic environment while long and short-clock mutants fair best in light: dark cycles best fitting their circadian clocks (Ouyang et al. 1998). In constant conditions, chipmunks with ablated superchiasmatic nuclei (SCN)- the location of the mammalian master clock- had the same survival rates as sham-lesioned controls. However, when released into natural conditions, SCN-ablated individuals suffered higher mortality rates than the control (DeCoursey et al. 2000). Collectively, these studies support the rationale that the most important element regarding the circadian clock's function is synchronicity, both external and internal.

Recently, investigations into the circadian patterns of wild-caught spiders challenged the traditional idea that entrainable rhythms are under selective pressures to resonate with the solar day (Moore et al. 2016; Jones et al. 2018; Garmany et al. (in press); Mah et al. (in press)). Not only does FRP vary among species, there is also significant variation within individuals. While most spider species' free-running periods average within 2 hours of the solar day (Seyfarth 1980; Suter 1993; Ortega-Escobar 2002; Soriano-Morales et al. 2013; Jones et al. 2018), the discovery of *Cyclosa turbinata*'s (Araneidae) free-running period of 18.5 hours is the most remarkable to date (Moore et al. 2016). Although comparable to the 19-h (%CV= 2.05 %) per$_s$ *Drosophila* mutants (Konopka and Benzer 1971), it is not known whether *Cyclosa's* variability (%CV= 12.50%) is a reflection of underlying genetic variation or a reflection of individuals' experience prior to collection. Table 1.2 provides a summary of average free-running period of various spider species.

Table 1.2 Summary of average free-running period (τ) and perfect coefficient of variation in various spider taxa (Araneae)

Species	Mean τ	%CV	Author
Cyclosa turbinata	18.5	12.40	Moore et al, 2016
Metazygia wittfeldae	22.7	5.67	Jones et al., 2018
Parasteatoda tepidariorum	21.2	5.14	Garmany et al. (in press)
Anelosimus studiosus	23.1	10.54	Mah et al. (in press)
Latrodectus mactans	24.7	11.87	Mah et al. (in press)

Previous unpublished results have found the average free running period of *Frontinella pyramitela*'s locomotor activity to be 28.83 h (Lomb-Scargle Periodogram, n= 10), comparable to the 29-hr *per*$_L$ mutants in *Drosophila* (Konopka and Benzer 1971). As with other spiders, (Moore et al. 2016; Jones et al. 2018; Garmany et al. (in press); Mah et al. (in press)), *Frontinella pyramitela* displays a remarkable range of inter-individual variability in FRP with a

percent coefficient of variation of 7.44 as compared to the homozygous *per₁* mutant's %CV of 1.75 (Table 1.1).

While many spider species' chronobiology has been examined, time of year has never been a factor taken into consideration. This study monitored locomotor activity in samples of adult *Frontinella pyramitela* collected over its active season (April-October) to investigate whether seasonality or age may help explain its exceptionally long and variable clock. Alternatively, if mean free-running period in *Frontinella pyramitela* does not vary by month of collection, the variability may be a reflection of underlying genetic variation within and among species.

Variability in free-running period may result from a number of factors (Hirschie Johnson 2003). Circadian rhythms and their association with age have been investigated in various taxa, with particular focus on pre- and post-ontogenetic development and old age (Witting et al. 1994, Pittendrigh and Daan 1974, Page and Block 1980, Koh et al. 2006). For example, Page and Block (1980) observed that free-running period in cockroach nymphs fluctuates as they undergo development.

The circadian system has been found to break down from senescence, therefore variation in FRP may result from the physiological effects associated with old age (Pittendrigh 1960, Weinert 2000, Nakamura et al. 2011). There is no consistency in changes to the clock in elderly individuals; effects vary by taxa and experimental design. In rats, FRPs in active wakefulness, body temperature, and drinking behavior were found to be significantly shorter in old rats as compared to young rats (Witting et al. 1994). Changes recorded in elderly mice observed in the laboratory were found to vary by individual- FRPs in some individuals remaining detectable under death where others were lost in late-middle age (Weinert and Weinert 1998.)

It has also been established in the laboratory that an individual's FRP can be modulated by photoperiodic history (Aschoff 1960; Pittendrigh and Daan 1974; Kumar 2017). This phenomenon, known as aftereffects, provides evidence that circadian clocks possess a certain amount of plasticity in order to adjust accordingly to shifts in light cycle [26]. A representation of the clock's plasticity can be seen latitudinally (Pittendrigh and Takamura 1989, Pivarciova et al. 2016), altitudinally (Vanlalhriatpuia et al. 2007), and seasonally (Kavaliers 1978; Bloch et al. 2006).

It has been postulated that spiders' circadian rhythms respond very strongly to light cycles (Jones et al. 2018). Responses to the shifts in daylength tend be greatest at temperate latitudes where seasonality is most apparent (Kumar 2017). It has been established in the laboratory that an individual's FRP can be modulated by length of preceding photoperiod cycle (Aschoff 1960; Pittendrigh 1960; Page and Block 1980). It may be that *Frontinella*'s varying FRP is a result of aftereffects resulting from seasonal change in photophase length. If that is the case, length of average FRP should display a correlation with natural daylength.

Frontinella is stenochronic in its life history- it is active for only one season before it dies in the fall (Manuel 1976). Therefore, shifts in *Frontinella*'s circadian behavior found in later months may be the result of age. A number of studies have provided evidence on how circadian disruption and degradation may be linked with physiological age (Weinert 2000; Barnard et al. 2008). If that is the case, the average "robustness" of circadian rhythm should be lowest as the season ends (August- October)- FRPs will be less stationary and not as detectable by periodogram analysis ($p > 0.05$). If *Frontinella* does show similar patterns of circadian disruption in the late season, this may be evidence that the pathways in aging and sleep/wake cycles may conserved along with flies and mammals (Koh et al. 2006; Nakamura et al. 2011).

While many spider species' chronobiology has been examined, time of year has never been a factor taken into consideration. This study explored locomotor activity in samples of adult *Frontinella pyramitela* collected over its active season (April-October) to investigate whether seasonality or age may help explain its exceptionally long and variable clock. Alternatively, if mean free-running period in *Frontinella pyramitela* does not vary by month of collection, the variability may be a reflection of underlying genetic variation within and among species.

CHAPTER 2. AGE AND SEASONAL CHANGE IN THE CHRONOBIOLOGY OF A SPIDER WITH AN EXCEPTIONALLY LONG-PERIOD CIRCADIAN CLOCK

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Keywords: circadian rhythm, seasonality, chronoecology, behavioural rhythm, aging, freerunning period

ABSTRACT: This study examines locomotor activity in samples of Frontinella pyramitela collected over its active season (April-October) to investigate whether seasonality and/or age may help explain the exceptional variability typically found in spider clock systems. Despite its noteworthy variability (%CV= 7.7), we have found that Frontinella has a mean free-running period of 28.4±2.18 hours that does not significantly vary over time. There is no correlation between daylength and freerunning period, indicating that varying length of FRP is not a function of photoperiod length. In LD 12:12 h, the window of activity is significantly smaller in April, gradually widening as the season continues, which may be evident of a shift in foraging strategy. Frontinella's clock appears to mature before its entrainment mechanisms are fully developed, and towards the end of its season, there is strong evidence of circadian misalignment which may be a product of physiological age.

INTRODUCTION

Nearly all living organisms have a circadian system that enables them to retain internal synchrony and resonate with the day/night cycle. [1,2,3]. The circadian clock is a network of self-regulating oscillators which, even in the absence of synchronizing cues (a Zeitgeber), will free-run at a period (tau) close to 24 hours. Free-running periods (FRP) tend to be highly stable with low inter-individual variability [4]. It is hypothesized that organisms perform best when their circadian clock is in resonance with the surrounding environment [5, 6]. If the clock remains too far out of phase with its Zeitgeber, the circadian system must realign its components to the new phase angle. Circadian misalignment occurs when the master pacemaker and its peripheral components fail to achieve synchronicity [6,7]. It has been found that continuous

circadian disturbance may result in reduced fitness and lifespan [5, 8, 9] as well as contributes to a number of metabolic and neurological pathologies [7, 10, 11].

Recently, investigations into the circadian patterns of wild-caught spiders challenged the traditional idea that circadian rhythms are close to 24 hours [12, 13, 14, 15]. Not only does FRP vary among species, there is also significant variation within individuals (Table 2.1).

Variability in free-running period may result from a number of factors [17]. Circadian rhythms and their association with age have been investigated in various taxa, with particular focus on pre- and post-ontogenetic development and old age [18, 19, 20, 21]. The circadian system has been found to break down from senescence, therefore variation in FRP may result from the physiological effects of aging [19, 22, 23, 24]. It has also been established in the laboratory that an individual's FRP can be modulated by photoperiodic history [1, 20, 25]. This phenomenon, known as aftereffects, provides evidence that circadian clocks possess a certain amount of plasticity in order to adjust accordingly to shifts in light cycle [26]. A representation of the clock's plasticity can be seen latitudinally [27, 28], altitudinally [29], and seasonally [30, 31].

Table 2.2 Mean Free-Running Period (τ) and Percent Coefficient of Variation in Various Taxa

Species	Mean τ	%CV	Author
Golden Hamster	23.9	0.30	Czeisler et al, 1999
Lake chub *(Couesius plumbeus)*	25.5	0.98	Kavaliers, 1978
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Lesotho mole rat *(Cryptomys hottentotus)*	24.1	0.92	Schöttner et al, 2006
Human	24.2	0.55	Czeisler et al, 1999

Table 2.1 continued.

Drosophila PER mutants

per_l, per_l	28.6	1.75	Konopka & Benzer, 1971
per_l, per_o	30.6	4.25	Konopka & Benzer, 1971

Table 2.2 Summary of average free-running period (τ) and perfect coefficient of variation in various spider taxa (Araneae)

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Latrodectus mactans	24.7	11.87	Mah et al. (in press)

While many spider species' chronobiology has been examined, time of year has never been taken into consideration. This study monitored locomotor activity in adult *Frontinella pyramitela* throughout its active season (March-October) to investigate whether seasonality or senescence may help explain its exceptionally variable clock.

METHODS

Study Species

Frontinella pyramitela (=communis) (Araneae: Linyphiidae), or, the bowl and doily spider is named for its distinct, non-sticky sheet web system composed of a horizontal bowl laying above a flat sheet of silk. Both the bowl and "doily" are surrounded by an entanglement of silk [31]. The spider rests on the underside of the bowl structure where it preys upon flying insects that fall into it having been knocked down by the silk above [32]. Individuals are 3-4 mm in body length and are dark brown, almost black, in color, with stripes of white on the abdomen [33]. They are common in temperate environments in North America, found in areas of low vegetation, fields, and low branches. The species is stenochronic, juveniles overwintering in the

soils and subsequently emerging in late spring [33] Due to *Frontinella's* life history, this study is carried out under the assumption that an individual's age is representative (± 1 month) of the time in which they were collected (i.e. individuals collected and tested in April are younger than individuals collected and tested in October).

Collection and Housing

Adult females were collected from Washington Co., Tennessee between May and September 2018. All individuals were captured from their webs and kept in 60 ml plastic cups for transport. In the laboratory individuals were assigned a number and housed in plastic, 473 ml cups with lids, the straw holes plugged with a cotton ball to ensure ventilation and easy feeding access. Prior to any assessment, spiders were kept for five days in a temperature-controlled room (25₀ C) under a light: dark cycle consisting of 12 hours of day and 12 hours of night (LD 12:12) with a one-hour transition ramp. Upon building a web in their container, individuals were fed either a termite worker or *Drosophila sp.* and watered three times weekly.

Assessing Locomotor Activity

To examine locomotor activity, a total of 98 individuals distributed over the course of the season were placed in clear tubes (24 x 100 mm) and placed in a locomotor activity monitor (Trikinetics LAM 25) for 12:12 h light: dark (LD) conditions for 4-5 days followed by 10 days of constant darkness (DD). Conditions were controlled by placing the monitor in an environmental chamber at 24 degrees (± 0.5 ₀C). To prevent escape and ensure ventilation, a small section of sheer voile fabric was placed onto one end of the tube and then covered with a plastic top. The other end of the tube was covered by a vinyl cap. Locomotor activity was measured continuously by the crossing of three infrared light beams bisecting each tube (in

cross-section). The crossing of one beam registers as a count. Activity counts and periodicity were analyzed using Clocklab 6 (Actimetrics, Wilmette, IS, U.S.A.). Analyses were conducted with one-minute bins unless otherwise stated. Total counts are visually depicted with double-plotted actograms which graphically display an individual's activity over time. Any data containing an individual that died before five days of constant darkness was discarded.

LD Assays

LD counts for each individual were averaged onto a 24-hour entrainment profile for each month (Figure 3A). Phase angles of entrainment (PAE) for onset, acrophase, and offset, were extracted from Clocklab and converted into zeitgeber time (ZT) to provide a metric for examining the relationship between individual PAE and its zeitgeber. Zeitgeber time 0 (ZT0) represents the beginning of the dark phase, while ZT12 equates to lights on. Average onset and offset were required to calculate each individual's length of active phase, hereon referred to as the "activity window".

When quantifying circadian disruption, one must use more than one metric to avoid false "positives" [7]. To search for evidence of circadian misalignment, the variability in activity onset was determined by calculating the standard deviation between five days of an individual's onset phase position in LD. To estimate the degree of nocturnality under LD conditions, alpha (light activity) and rho (dark activity) counts were used to calculate the DiNoc ratio ($Activity_{alpha} - Activity_{rho} / Activity_{total}$) [34] of each individual. Interdaily stability is represented as a ratio of the variance of activity in the period *tau* and the overall activity variance. In LD, it is used to measure both consistency in activity from day to day and the strength of synchronization between an individual's LD rhythm and its zeitgeber [7, 35]. The ratio ranges from 0 to 1- a measurement closer to 1 indicating a more constant variance.

DD Assays

Significant free-running periods (FRPs) were detected using Lomb-Scargle periodogram analysis which performs best with nonstationary data [36, 37], based on an interval of at least five days. Due to *F. pyramitela*'s tendency to produce uneven and/or sparse activity distributions, any periodicity detected at an alpha level of > 0.05 was not ruled significant. In order to compare interdaily stability of free-running period among individuals, rhythms in DD were transformed to circadian time (CT) by using the "Set Tau" option on Clocklab. Only the first 5 days were selected as *Frontinella*'s FRP appears highly non-stationary- prone to fluctuation in period and phase. In order to conduct the nonparametric analysis in CT, rhythms should be stationary [38]. When set to CT, and rounded to a whole number, interdaily stability in DD was able to be extracted from Clocklab for statistical analysis.

Statistical analyses

All statistical analyses were performed in R, version 3.5.1. Datasets used in statistical analyses were examined for normality (Shapiro-Wilke) and constant variance (Bartlett's test); data is reported as mean ± standard deviation unless otherwise stated. To assess the importance of time of year on free-running period, interdaily stability, and phase angles of entrainment, we performed separate ANOVAs with month of collection as a factor. Between-group differences were reported significant if $p < 0.05$ If required, a Tukey multiple comparison of means test was used in post-hoc analysis (Tukey HSD). Linear regression analysis was used to test whether the day of year significantly predicted individuals' phase angles of entrainment and whether activity window increased over time. The relationship between interdaily stability in LD vs DD in the overall sample as well as by month was determined by linear regression.

RESULTS

Constant Conditions

Frontinella pyramitela exhibits an average free-running period of 28.4 hours (Lomb-Scargle Periodogram, n= 76). Despite high variability (Table 2.3), mean FRP at the population level does not significantly vary across months (Figure 2.1A, ANOVA, p = .107). Representative actograms and periodograms are shown in Appendix B. Significance of Lomb-Scargle periodograms (Figure 2.1B) does decline (AugSep= 46% individuals with FRP > 0.001; October=61% individuals with FRP > 0.001).

Early in the season, locomotor activity appears to be fairly consolidated and "neat": actograms and periodograms in many individuals depicting two, separate components that appear in sync with one another (Figure 1A, Appendix B). In comparison, most actograms in August-October appear to show both components running out of sync, activity becoming most dispersed and losing overall pattern (Figure 2C, E, Appendix B). In October there were individuals with a severe decrease in activity, yet there was considerable inter-individual variability. Many periodograms displayed more than one significant peak (Figure 1B, D, Figure 2B, Appendix B). Many of these peaks were close to half the period (Figure 1D, Appendix B) where others showed distinct, ultradian components (Figure 2D, F, Appendix B).

Entrainment Profiles

In LD 12:12 conditions, 89 of 91 individuals displayed locomotor activity, which was mostly confined to the dark cycle (Figure 2.1). The degree of nocturnality in LD 12:12 was quantified by DiNoc ratios of locomotor activity in each individual (Figure 2.1, Appendix A)), mean DiNoc ratio being – 0.91 ± 0.19. In April (n = 15), every individual was exclusively nocturnal (DiNoc = -1) with the exception of one (DiNoc = -.77).

Linear regression analysis was used to test whether the day of year significantly predicted

individuals' phase angles of entrainment. It was found that average activity window increased

with earlier onsets and later offsets as the season continued (Table 2.6). Despite the overall
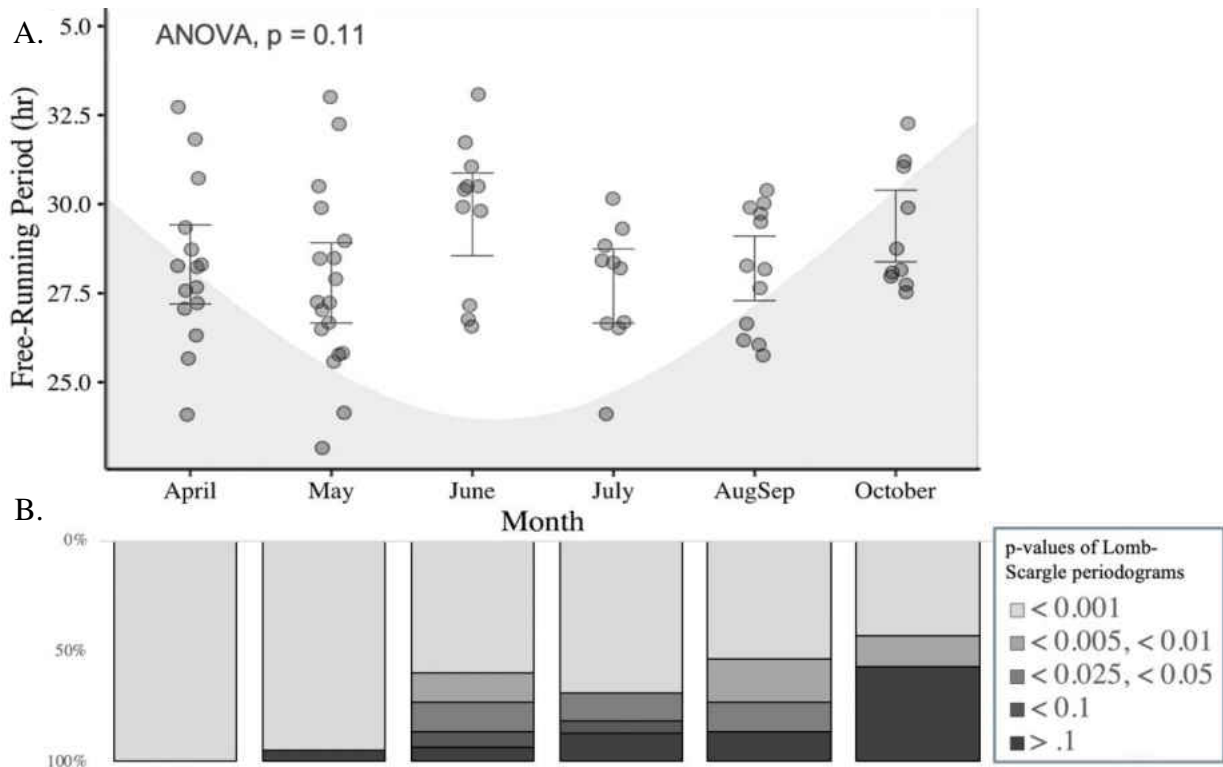
Figure 2.1 Locomotor activity of *Frontinella pyramitela* recorded in constant dark conditions (DD). AugSep represents the months of August and September. (A.) Free-running period (τ) of individuals collected by month with 95% confidence intervals (n=76). The FRP of each individual is represented by one dot. All individuals depicted showed an FRP with a significance of at least < 0.05 (Lomb-Scargle). (B.) The percentage of significance by Lomb-Scargle periodogram by month (n=98).

average entrainment profiles depicting what appears to be a phase advance at the population

level, this is representative of only a few individuals (Figure 2.2A). April stands apart from the

rest of the season, having the shortest window of activity (5.14 ±1.19, Table 2.4). Activity

onsets in April begin significantly later than the following months (ANOVA, F= 12.29, *p* <

0.05). The "window" expands as the season continues, activity of some individuals spanning into

early photophase.

Table 2.3. Mean free-running period (τ) of *Frontinella pyramitela* as measured by Lomb-Scargle periodogram. AugSep represents the months of August and September. Only individuals with a significance of at least $p < 0.05$ were used

	April (n=15)	May (n=18)	June (n=11)	July (n=10)	AugSep (n=12)	October (n=10)	Overall (n=76)
Mean τ							
Mean	28.3	27.7	29.8	27.7	28.2	29.3	28.4
Min, Max	24.1,32.8	23.2,33.1	26.6,33.1	24.1,30.4	25.8,30.4	27.5,32.3	23.2,33.1
%CV	8.0	9.3	7.1	6.3	6.1	5.8	7.7

Table 2.4. Mean activity window and phase angles of entrainment for *Frontinella pyramitela*

	April (n=15)	May (n=19)	June (n=14)	July (n=13)	AugSep (n=13)	October (n=15)	Overall (n=89)
Activity Window							
Mean (SD)	5.14 (1.2)	7.23 (2.1)	10.6 (3.1)	10.7 (3.6)	9.6 (3.2)	11.8 (2.33)	9.03 (3.5)
Median	5.7	7.5	9.9	10.9	8.3	12.1	9.1
[Min, Max]	[2.6, 6.4]	[3.6,10.6]	[6.2,17.2]	[0.9, 17.3]	[5.5, 15.3]	[5.7, 15]	[0.9, 17.3]
Onset							
Mean (SD)	4.5 (1.2)	1.9 (0.84)	1.6 (0.9)	2.2 (1.3)	2.3 (1.1)	1.9 (1.6)	2.4 (1.5)
[Min, Max]	[3.1, 7.3]	[1.2, 3.7]	[0.2, 3.4]	[0.2, 5]	[0.7, 4.7]	[0.2, 5.8]	[0.2, 7.3]
Acrophase							
Mean (SD)	7 (1)	4.7 (1.9)	5.2 (1.2)	5.8 (1.7)	5.8 (0.7)	4.7 (2.3)	5.5 (1.8)
[Min, Max]	[5, 8.8]	[1.2, 7]	[3.6, 8.2]	[2.1, 8.2]	[4.8, 7.4]	[-0.04, 7.8]	[-0.04, 8.8]
Offset							
Mean (SD)	9.6 (0.7)	9.2 (2.3)	12.2 (2.6)	12.9 (3)	11.9 (2.8)	13.7 (2)	11.4 (2.8)
[Mix, Max]	[8.3, 10.9]	[5.3, 12.1]	[8.6, 18]	[6, 19]	[8.7, 18]	[10.6, 18.1]	[5.3,19]

Table 2.5. Mean interdaily stability of *Frontinella pyramitela* in individuals collected over its active season.

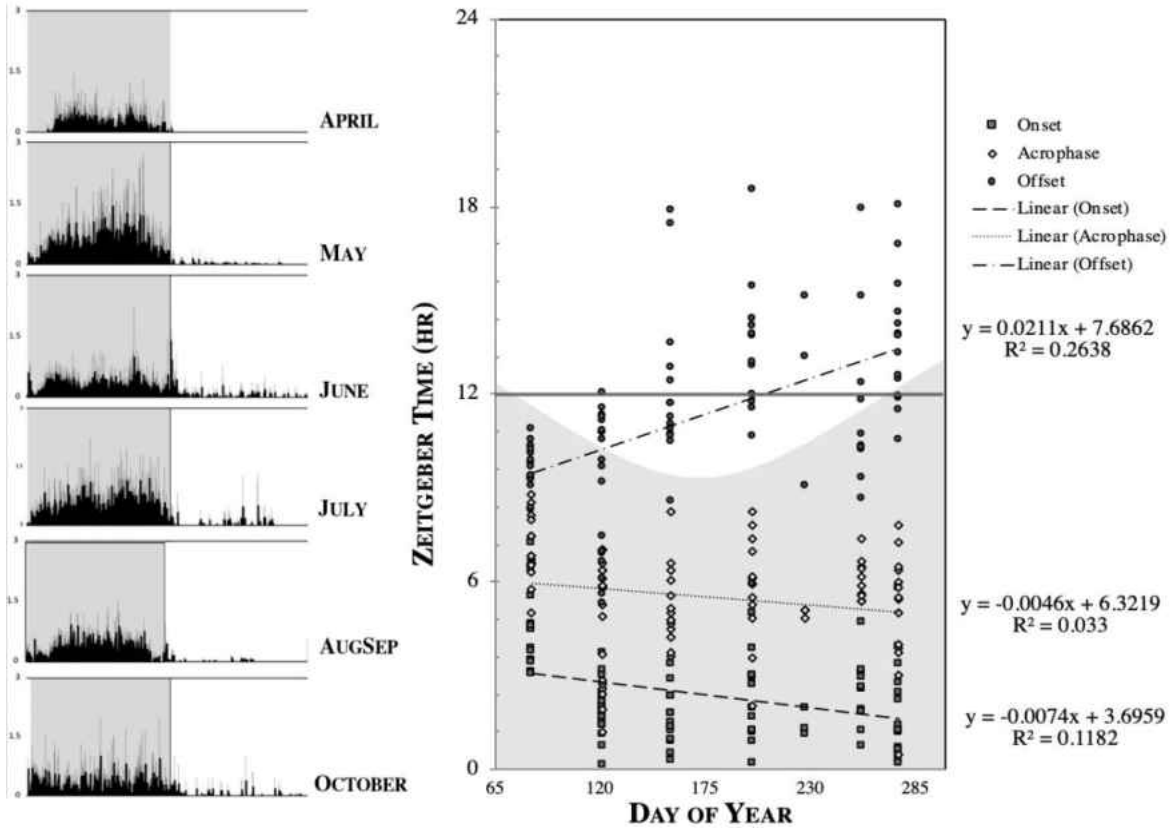|	April (n=15)	May (n=18)	June (n=14)	July (n=12)	AugSep (n=15)	October (n=13)
Interdaily Stability (LD)						
Mean (SD)	0.38 (0.1)	0.56 (0.1)	0.53 (0.2)	0.58 (0.2)	0.54 (0.1)	0.31 (0.1)
Interdaily Stability (DD)						
Mean (SD)	0.28 (0.04)	0.39 (0.1)	0.41 (0.1)	0.43 (0.1)	0.31 (0.1)	0.36 (0.1)

Figure 2.2 Locomotor activity of *Frontinella pyramitela* in light: dark conditions (LD). (Left) Entrainment profiles for pooled locomotor activity, showing average counts in one-minute intervals. Dark background indicates the dark portion of the LD 12:12 h cycle. (Right) Phase angles of entrainment for each individual plotted by day of year in which they were collected. The shaded background indicates the daylengths of the natural environment. The solid grey line at ZT 12 represents the transition from dark to light in laboratory conditions.

Table 2.6. Linear Regression Analysis of Phase Angles of Entrainment and Activity Window

	Window	Onset	Acrophase	Offset
Day	0.029***	- 0.008***	- 0.005*	0.021***
	(0.004)	(0.002)	(0.003)	(0.004)
Constant	4.004***	3.691***	6.322***	7.695***
	(0.799)	(0.405)	0.512	(0.695)
Number of Observations	88	88	88	88
R_2	0.355	0.127	0.034	0.284
Adjusted R_2	0.347	0.117	0.022	0.276
Residual Std Error (df = 86)	2.722	1.380	1.744	2.366
F Statistic	47.28***	12.54***	2.99*	34.13***
Note:				*p < 0.1; **p < 0.05; ***p < 0.01

Interdaily Stability

To examine consistency in activity patterns from day to day, interdaily stability for each individual was measured in both LD 12:12 and in constant conditions (DD). To test whether interdaily stability (IS) varied between month of collection, two separate one-way ANOVAs were calculated on both IS in LD and DD. There was a significant effect of month of collection on interdaily stability in LD (Figure 3A, F= 10.93, $p < 0.001$). Results of post-hoc tests (Tukey HSD) reveal that average IS was significantly lower in the early (April) and late season (October) groups (See Table 5 for mean values). Further, individuals collected in the midseason (May- July) did not differ between in IS across May-July. Interdaily stability in DD (Figure 4B) shows the same trend as LD of the midseason (May-July) having the highest mean interdaily stability values (Figure 4B; Table 6). For the total sample, there was a significant difference with month of collection on IS in DD (Figure 3B, ANOVA, F = 4.28, p = 0.002). Post hoc comparisons using the Tukey HSD test indicate that the mean IS in April (M= 0.38, SD= 0.928, Table 4) was the only significant difference between months.
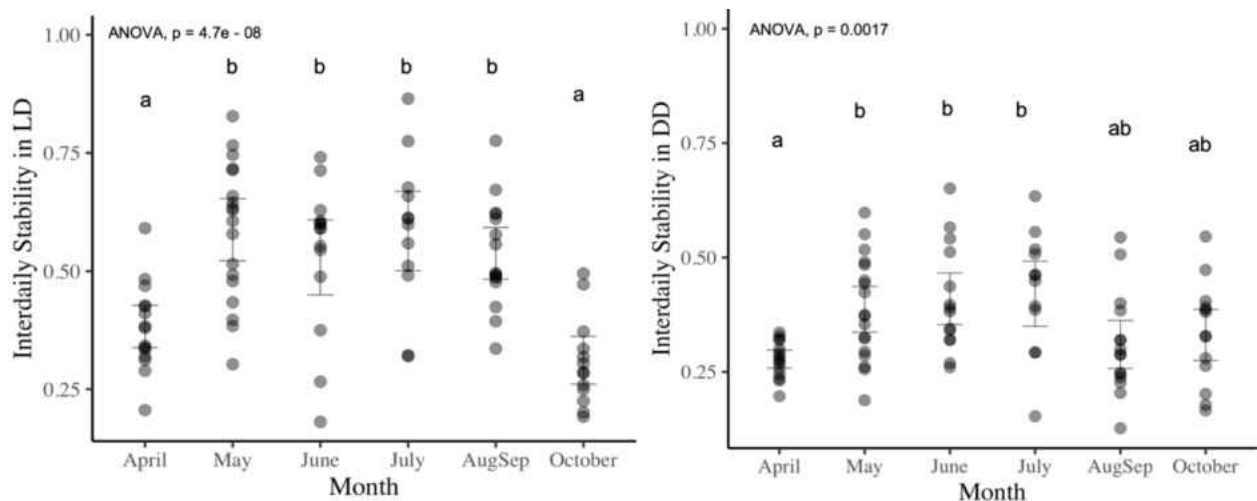

Figure 2.3 Interdaily stability in *Frontinella pyramitela* in LD and DD. p-values of one-way ANOVAs with month as a factor are in the top left of each figure. (Left) Interdaily stability in LD (Right) Interdaily stability in DD.

Linear regression analysis was used to examine the relationship between interdaily stability values in LD and DD within individuals. A significant correlation (Figure 3, Appendix A, $p = 0.003$; Pearson correlation, R= 0.32) was found for the overall sample. When viewed by level of month (Figure 4, Appendix A), April is the only group which has a significant correlation (p= 0.005, $R_{2adj}= 0.43$).

DISCUSSION

Despite considerable variability in free-running period in *Frontinella pyramitela,* the mean remained consistent throughout its adult life. Thus, this eliminates the hypotheses that FRP is affected by seasonal changes in photoperiod or age, and, by process of elimination, lends support to the hypothesis that the variability seen in spider free-running periods reflects underlying genetic variation. While FRP in locomotor activity did not vary over time, there were noticeable changes in certain parameters of its clock system. Entrainment mechanisms appeared to mature after the circadian clock. Activity in both LD 12:12 and constant dark appeared the most consistent in the midseason (May-July).

Age

An organism's behaviour in light/dark conditions may also be indicative of the state of its circadian clock [7, 36]. An indicator of healthy circadian function is when the phase position of activity onset in LD remains consistent from day to day and a departure from such regularity is expected to occur from chronodisruption [6, 7, 22]. In *Frontinella,* the strongest evidence of misalignment in LD 12:12 can be found in the April and October samples- interdaily stability in LD (Figure 3A) is significantly lower than the mid-season (May-July) populations.

While length of FRP did not differ by month of collection, it was found that the detectability of FRP decreased as the season continued (Figure 1B). Research into caloric

restriction has found that age-related decline in physiology results from oxidative damage in taxa ranging from *Drosophila* to human beings [42, 43]. Austad [31] demonstrated that *Frontinella* may also follow this same pattern in aging. Loss of circadian rhythmicity as a function of oxidative damage from aging has been documented in several taxa [21, 22, 41]. *Frontinella* is stenochronic in its life history- it is active for only one season before the adults die in the fall [40], therefore, shifts in *Frontinella*'s circadian behaviour may be the result of physiological age. Further tests are needed to verify if the loss of rhythm detectability in *Frontinella* is a result of physiological age. Koh et al. [21] treated *Drosophila* with the oxidative stress-producing reagent paraquat to show how rhythm fragmentation and breakdown in rhythm detectability were a product of physiological aging. If further tests on *Frontinella* treated with paraquat show similar results as Koh et al., this may establish spiders as a model organism for studying age-associated changes in the circadian system.

Four general profiles can be established when taking all metrices into consideration, each profile most likely occurring at a specific time of year (Figure 4, Table 7). The first profile consists of exclusively the April sample, where all individuals had a highly significant free-running period while also having low values of interdaily stability in LD (0.36 ± 0.093) and DD (0.28 ± 0.04) as compared to the midseason (Figure 3; Table 1, Appendix A). Onset deviation was also significantly higher than May-September (Figure 2, Appendix A). Although Figures 3A and 3B appear to depict the same trend, April is the only sample where average interdaily stability in DD (Figure 3B) is significantly different from the midseason. Interestingly, April is the only month where interdaily stability in LD and DD have a significant correlation (Figure 4, Appendix A). The results in April suggest that *Frontinella* follows the development pattern

found in mice where circadian rhythmicity is achieved before the maturation of its entraining mechanisms [44].

The midseason consists of individuals primarily found from May-July. Individuals with this profile appear to have the most consistent activity patterns in both LD and DD from day to day. Individuals have both a significant free-running period along with high interdaily stability values in LD and DD along with low onset variability.

The mid-late season profile comprises of individuals collected from July-September. Spiders will still show entraining behavior: low onset variability and high interdaily stability in LD. In constant conditions, activity patterns appear episodic. The most significant peaks in Lomb-Scargle periodograms are ultradian (< 20 hrs).

The late season profile is almost entirely exclusive to October. These individuals have the highest variability in activity onset (Figure 2, Appendix A) and interdaily stability values comparable to those seen in the April sample (Figure 3; Table 5). Given that individuals in October were adults nearing the end of their life cycle, the extreme deviation in onsets along with the low ratios of interdaily stability in LD may be the result of senescence. When taking these results into consideration, *Frontinella's* circadian system appears to break down in the opposite order as other taxa [21, 41]. Circadian rhythms become undetectable far earlier than what has been found in age studies of rat, mice, and flies [22].
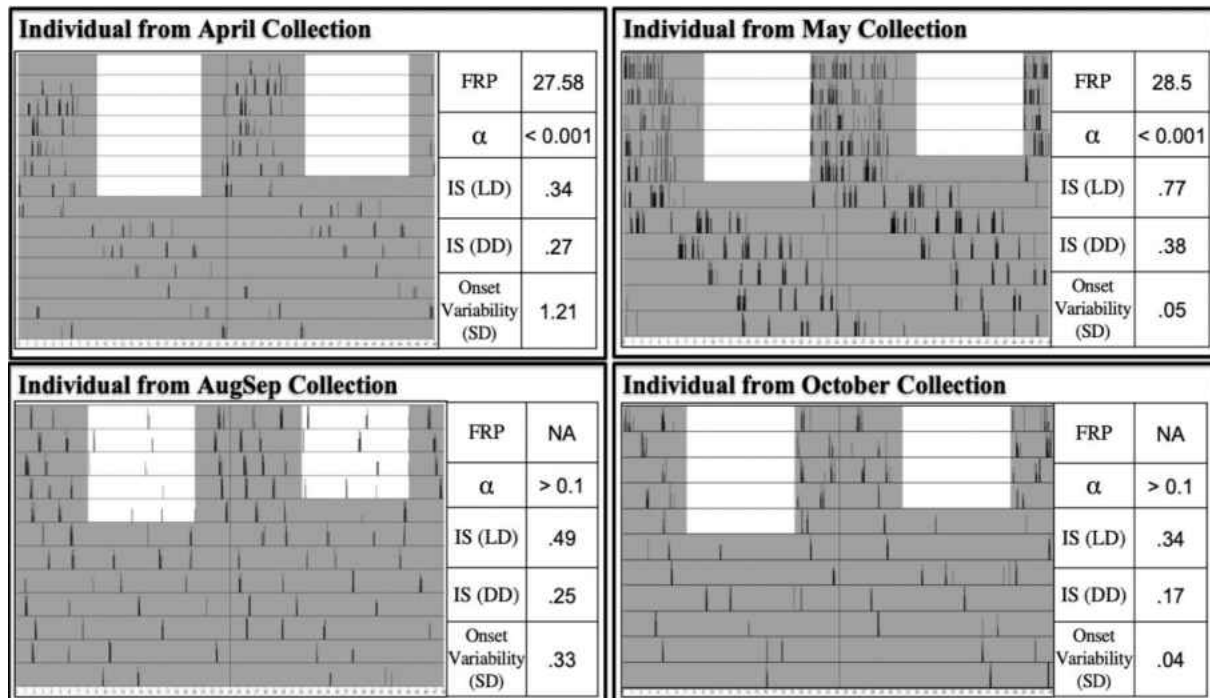
Figure 2.4 Individuals representative of the four general profiles of entrainment and circadian behavior seen in adult *Frontinella pyramitela*

Table 2.7 Metrices Associated with the Profiles of Entrainment and Circadian Behavior in *Frontinella pyramitela*

	Likelihood of Occurrence	FRP detectability	IS (LD)	IS (DD)	Onset Deviations
Early Season	April	High (p < 0.001)	Low	Low	High
Midseason	May-July	High (p < 0.001)	Very High	High	Low
Mid-Late Season	July-September	Low (p > 0.05)	High	Low-High	Low
Late Season	October	Low (p > 0.05)	Low	Low	Very High

Chronoecology

  Frontinella overwinters as a juvenile and emerges the following spring as a penultimate adult [40]. Until its web is large enough to provide adequate protection, an individual remains more vulnerable to diurnal predators (wasps, birds) [45]. *Frontinella*'s antipredator strategy relies on the fortification of its three-dimensional web [32, 45]. Web size indicates foraging

30

success in several "Araneoid sheet weavers" [45]. Provided that there is sufficient prey availability and low perturbation of web site, *Frontinella* will opt to remain in the same web as the season continues [46, 47].
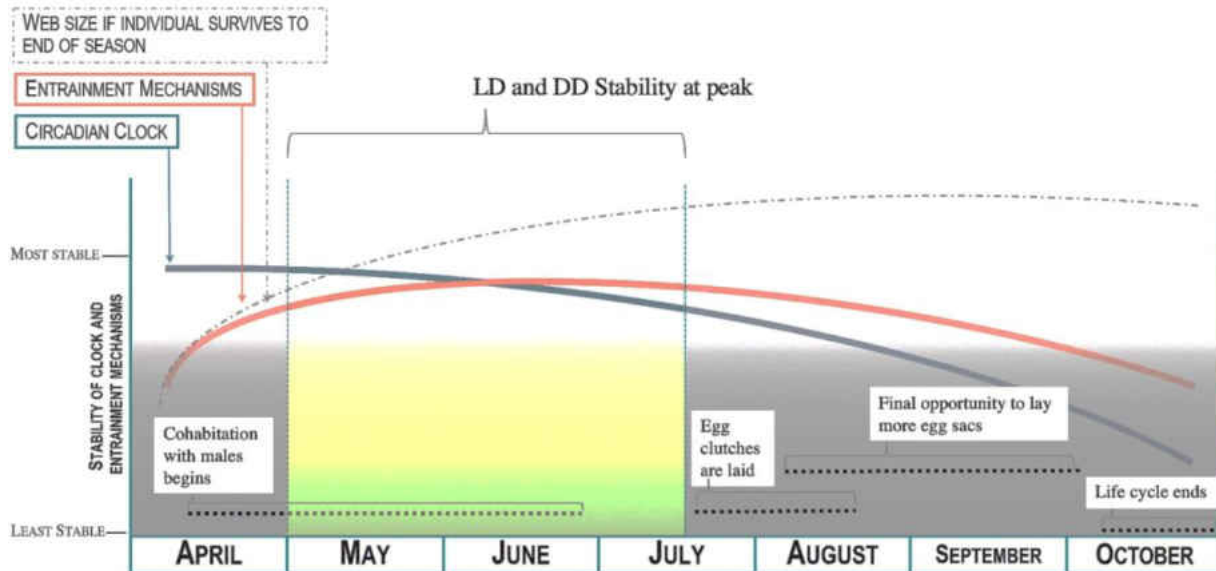

Figure 2.5 An overview of *Frontinella pyramitela*'s clock system with respect to its life history. Growth of web size is only applicable if individual is in an optimal habitat with steady prey availability and low risk of web perturbation.

Presuming sufficient prey availability and no web perturbation, an individual is more likely to have an adequately-sized web by mid-late May. While some studies have found that web-building effort decreases in well-fed spiders [48,], the benefit from continued web investment is likely different for a three-dimensional web builder [45]. As time continues, web size grows, thus reducing predation risk and increasing prey capture efficiency. Therefore, increasing their foraging hours may involve less risk. Consequently, the gradual widening of the "activity window" in LD (Figure 6) may be evidence of temporal plasticity in foraging aggression.

Another significant event occurs around mid-May; male *Frontinella* begin cohabitation in webs with females. During cohabitation , the male ends up ingesting 32% of the female's prey [32], therefore she may have to alter her foraging strategy in order to sustain them both while gathering enough energy for yolk accumulation. Also, if one takes the theory of the "selfish herd" into consideration [48], there is the possibility that having an extra spider (practically of the same size, 33) in the web may lessen the pressures of predation and thus enable her to act more boldly.

By late July/early August, the female will have laid at least one egg sac. Since *Frontinella* can store sperm, there is the possibility of laying more egg sacs. [31, 49]. Reproductive success (lifetime deposition of eggs) in *Frontinella* is proportional to food intake [31, 49]. What is seen in both LD and DD activity patterns beginning in July may be a shift in circadian strategy; their once diel rhythms giving way to a more arrhythmic pattern in a "last ditch" foraging effort to build up enough reserves to increase fecundity.

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CHAPTER 3 CONCLUSION

Despite its variability, mean free-running period in *Frontinella pyramitela* remained consistent throughout its adult life. This leads to the likelihood that the variability seen in spider free-running periods are a reflection of underlying genetic variation. While FRP in locomotor activity did not vary over time, there were noticeable changes in certain parameters of its clock system. Entrainment mechanisms appeared to mature after the circadian clock. Activity in both LD 12:12 and constant dark appeared the most consistent in the midseason (May-July).

The free-running period in *Frontinella pyramitela* did not correlate with previous photoperiods. When put into an ecological context, the dynamics of shifting photoperiod length may only have a significant influence upon spiders depending on their foraging strategy. One orb weaving spider, *Larinioides cornutus* does not rely on crypsis or web fortification to avoid predation (Foelix 2011). Its circadian expression in octopamine levels (Jones et al. 2011) correlate to the diel cycle of predator and prey availability (Watts et al. 2015) enabling *Larinioides* to anticipate the ideal time to both forage and seek refuge. This rhythmic expression in antipredator behaviour minimizes risk and optimizes prey capture. Unlike *Larinioides'*s more restricted and "specialist" approach, *Frontinella*'s antipredator strategy relies on the fortification of its three-dimensional web (Suter 1985). The additional defense may afford it a more "generalist" strategy, thus likely giving a more relaxed relationship with its photoperiod. Testing whether *Larinioides's* free-running period would change as a result of previous photoperiod length would be helpful to help clarify whether spiders' sensitivity to light results from foraging strategy.

Phase angles of entrainment were quantified under a light: dark cycle (LD 12:12) for all samples. However, the activity window is not a result of preceding daylength. This gives rise to the likelihood that *Frontinella's* expanding activity window may be an endogenous phenomenon. Since most spiders lack the cognitive ability to exhibit learned behavior, it is likely that such behavioral oscillations are an innate, repeatable trait. An individual will have an ingrained level of aggression that is part of its overall behavioral phenotype. In the context of antipredator behavior: some individuals may be bolder where others may be more wary, regardless of environment. Such a suite of correlated behaviors found in a population is called a behavioral syndrome (Sih et al. 2004).

We hypothesize that *Frontinella pyramitela* would benefit from anticipating events in its life history, and therefore the expanding activity window of *Frontinella pyramitela may* correspond to the broadening of a foraging window. It would be of interest if tests were carried on individuals collected over time to clarify if antipredator strategy is affecting its phase positioning in the early months.

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Figure 1. Percentages of nocturnality expressed as DiNoc Ratios from April to October. Individuals with a calculation of -1 are represented as exclusively nocturnal. Individuals with a DiNoc ratio over 0 are considered slightly diurnal.
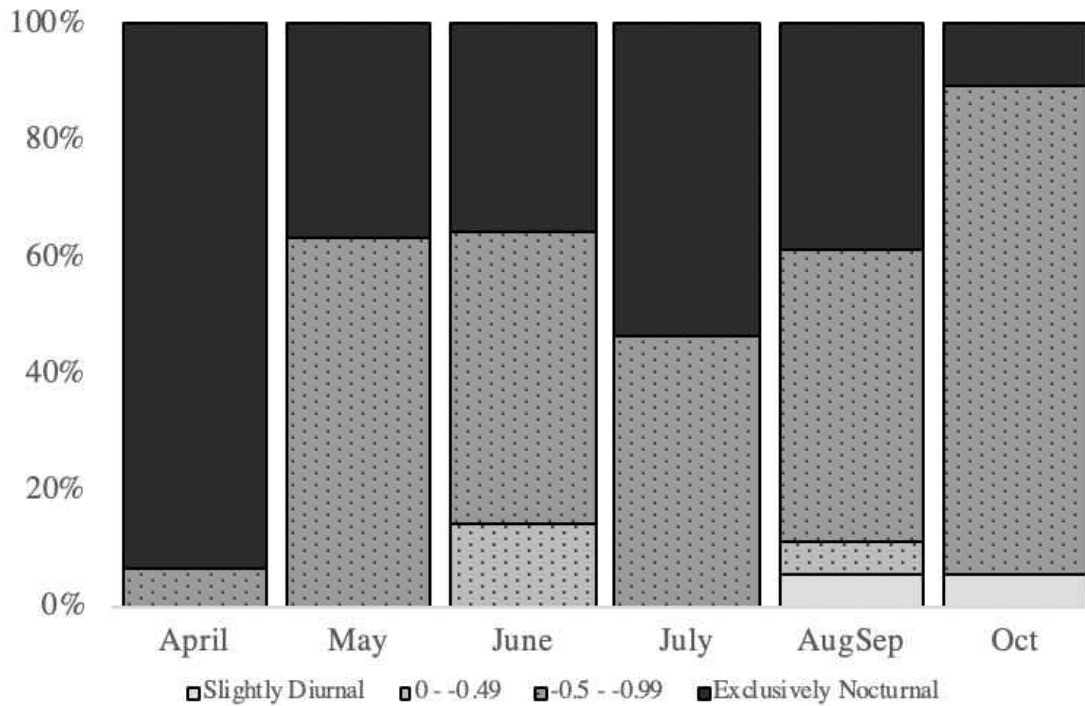
Figure 2. Variation in phase positions in onsets of activity in LD. Each dot represents the standard deviation in one individual's activity onsets over five days in light: dark conditions. Bars represent mean confidence intervals at 95%.
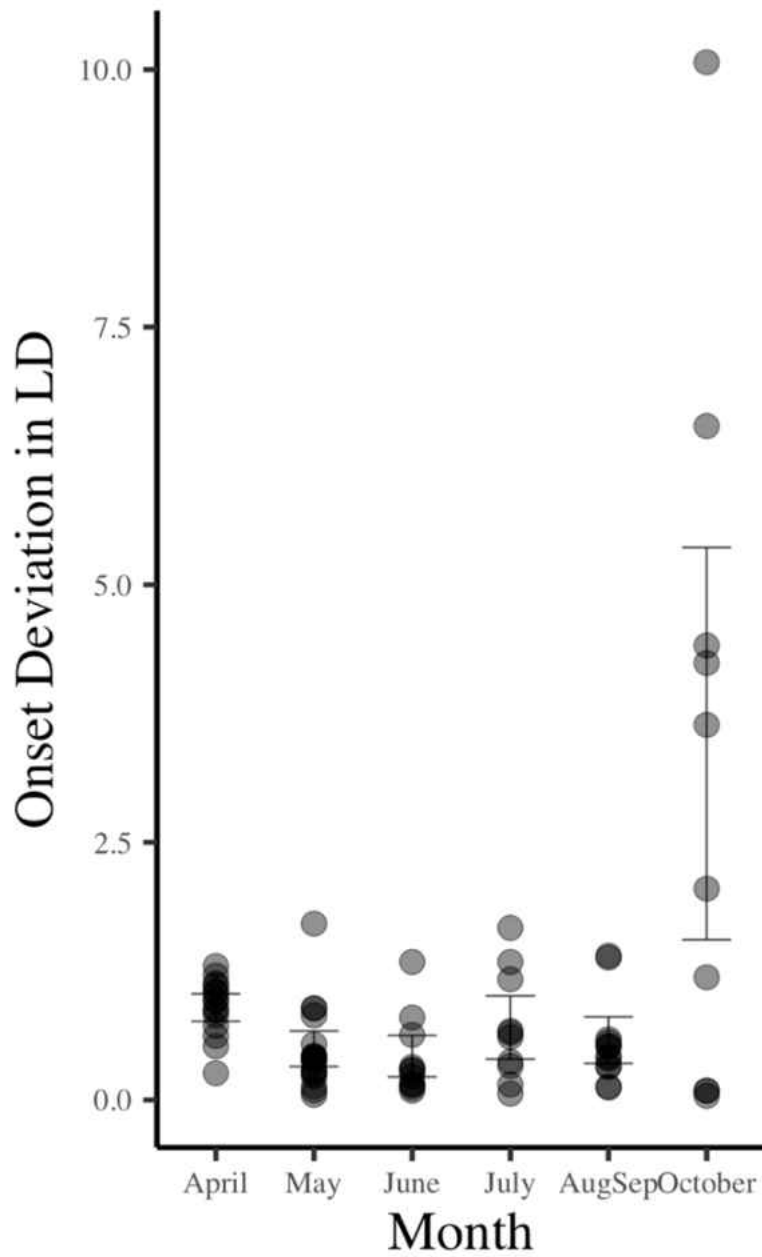
Figure 3. Scatterplot depicting relationship of interdaily stability in LD and DD of *Frontinella pyramitela* in overall population.
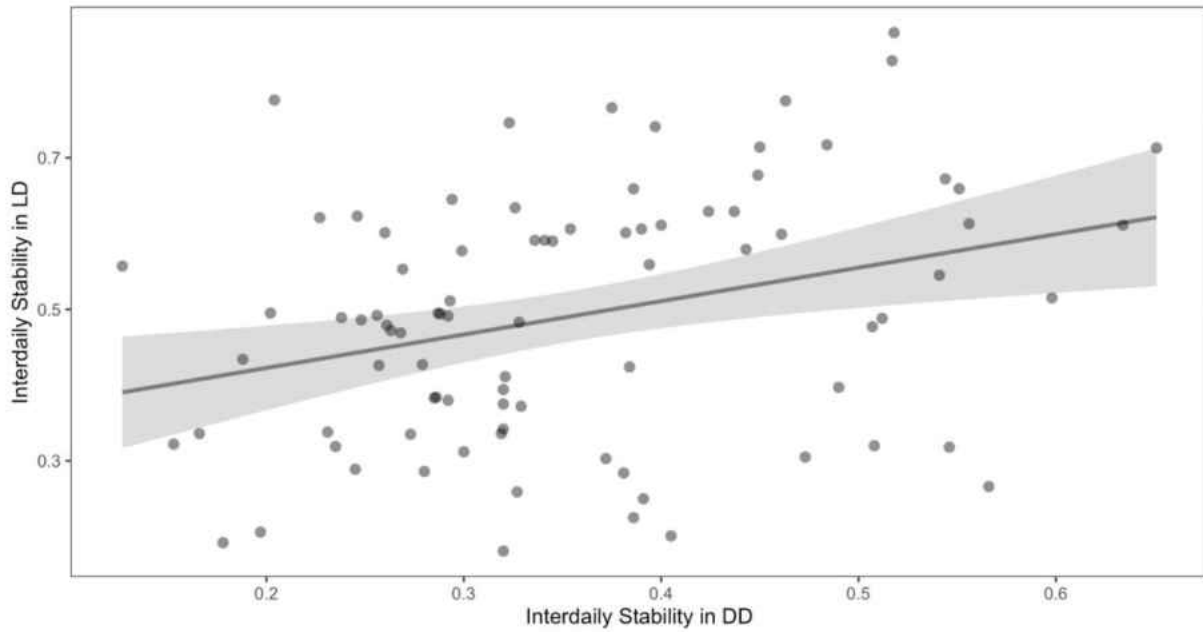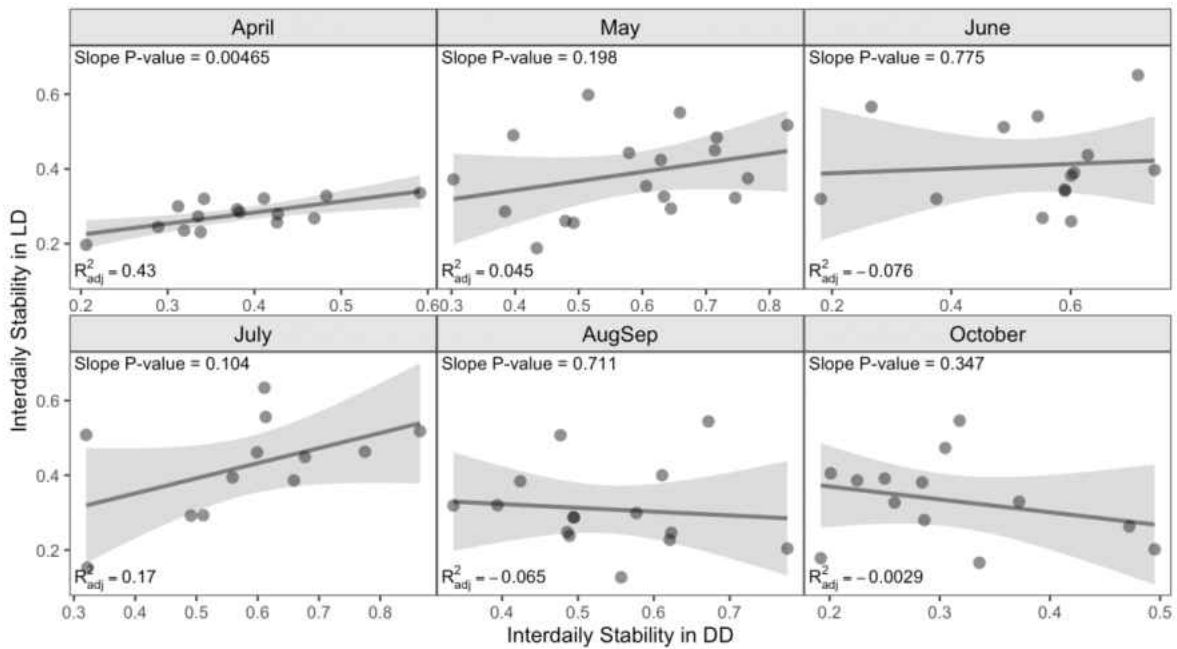

Figure 4. Scatterplots depicting relationship of interdaily stability in LD and DD of *Frontinella pyramitela* by month of collection.

Figure 1. Locomotor activity of *Frontinella pyramitela* from April to June. (Left; A, C, E) Double-plotted actograms of one individual in light: dark (LD) 12:12 h cycle for five days, followed by constant conditions (DD). DD is indicated by a grey background. (Right; B, D, F) Lomb-Scargle periodograms indicating significant (p < 0.001) periodicities in DD. (A, B) An individual representative of the April sample. Note the delayed and slightly variable onsets of activity. (C, D) An individual representative of the May sample. In May, most individuals displayed evidence of increased activity as well as a significant phase advance. (E, F) An individual representative of the June sample.
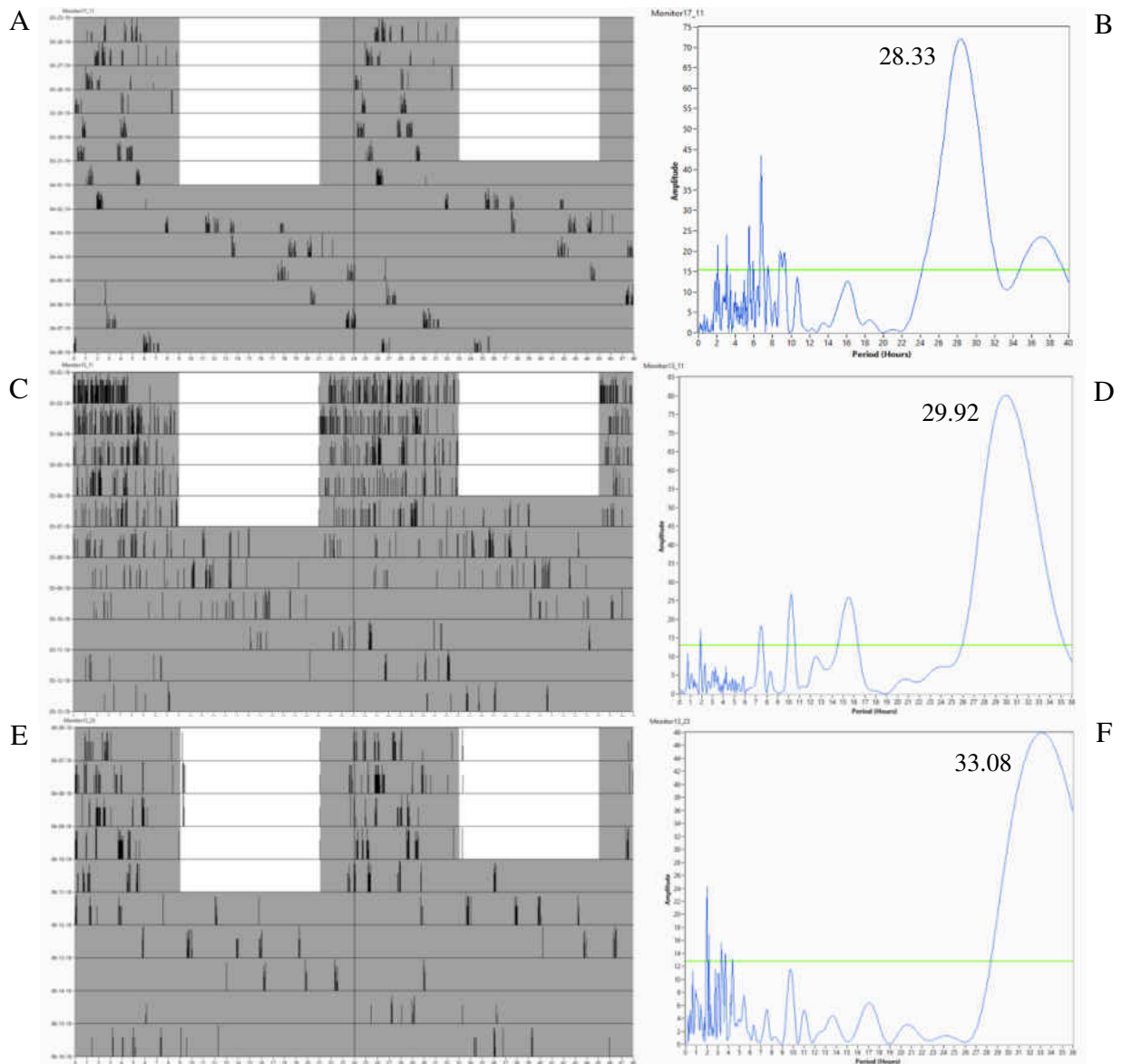
Figure 2. Locomotor activity of *Frontinella pyramitela* from July to October. (Left; A, C, E) Double-plotted actograms of one individual in light: dark (LD) 12:12 h cycle for five days, followed by constant conditions (DD). DD is indicated by a grey background. (Right; B, D) Lomb-Scargle periodograms indicated significant (p < 0.001) periodicities in DD. (A, B) An individual representative of the July sample. (C, D) An individual representative of the AugSep sample. The individual appears to maintain entrainment in LD. Both components appear entirely out of sync with one another in DD. (E) An individual representative of the October sample.
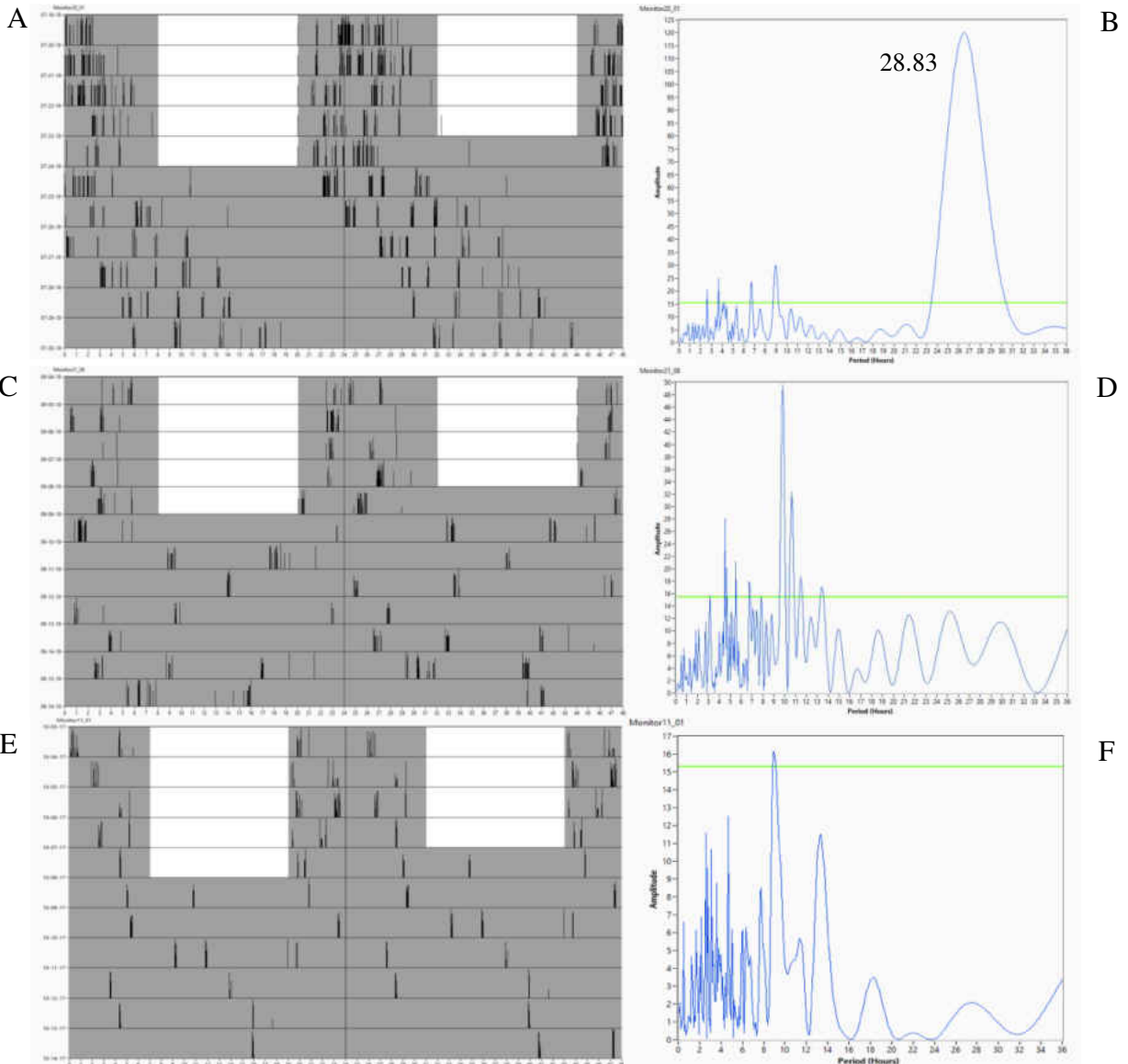
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