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ABSTRACT. Circadian rhythms are endogenous cycles that control physiological and behavioral changes that can be affected by environmental factors which allow most eukaryotic organisms to synchronize their daily activities with the 24-hour day. *Parasteatoda tepidariorum,* the common house spider, demonstrates a short-period circadian clock averaging 21.6 hours when left in constant darkness, yet they are able to entrain to a 24-hour light cycle. We tested whether these spiders were able to use non-photic Zeitgebers to entrain to the 24-hour day. Periodic presentation of food and disturbance were not found to be effective cues for the spiders' entrainment. A few individuals were clearly able to entrain to an 8 ºC amplitude temperature cycle, while most did not.

KEYWORDS: Behavioral rhythms, chronoecology, circadian clock, non-photic entrainment, wild clocks

INTRODUCTION

Circadian rhythms are daily oscillations produced by endogenous clocks that control physiological and behavioral changes including mating, predation, activity, etc. Most eukaryotic organisms have an internal circadian clock that allows them to maintain their physiological and behavioral cycles in phase with the 24-hour day (Panda et al. 2002). Common characteristics associated with circadian clocks include synchronization with (entrainment to) the 24-hour day, the ability to persist in the absence of an external cue including a light-dark-cycle (free-running), and temperature compensation in the period of oscillations (Pittendrigh, 1960). An advantage of the synchronized cycles generated by the internal clock is the ability for organisms to anticipate daily events and changes in the environment (Yerushalmi and Green, 2009). These internal clocks can respond to or be sensitive to environmental factors and can also influence changes in behavior (Jones, Akoury, Hauser, & Moore , 2011).

The ability to entrain to a light cycle indicates sensitivity to light levels, which is already well-known as a strong Zeitgeber, but not much is known about effects of non-photic cues for physiological and behavioral regulation or circadian entrainment in spiders. Some non-photic cues provided for circadian control reported in other organisms include temperature cycles and timed meals. Many organisms have been shown to be capable of temperature entrainment with activity patterns aligning with the temperature cycles, including *Drosophila,* cockroaches, and mammals (Lee and Montell, 2013; Roberts, 1962; Buhr et al., 2010). As for food cycles, anticipation for a meal provided at a consistent time has been observed in organisms (for review see Mistlberger, 1994) including bees (Moore et. al., 1989), fish, rats, and other mammals (Davis and Bardach, 1965). Based on the entrainment ability to non-photic cycles observed in other organisms, here we test spiders' ability to entrain to non-photic cues including daily temperature cycles and periodic presentation of food and general disturbance.

The species *Parasteatoda tepidariorum* Koch, 1841 was utilized for observation of non-photic cues on entrainment ability for spiders in our experiments. This species is a nocturnal spider that consistently demonstrates a short-period circadian clock averaging 21.6 hours when left in constant darkness (Garmany et al. 2019), yet they are able to entrain to (synchronize with) a 24-hour light cycle. Organisms that are out of resonance with the 24-hour day typically demonstrate negative consequences that impact health and fitness, but some spider species, including *P. tepidariorum*, appear to be exempt from the consequences of being out of resonance

(Moore et al, 2016). An organisms' circadian clock that is closer to 24 hours will have an advantage of increased fitness and will not experience the negative consequences of being out-of-phase with the solar day (Ouyang et. al., 1998). Based on the ability of *P. tepidariorum* to have a clock that strays from the typical 24-hour internal clock but still have the ability to remain synchronized with a light-dark cycle, one might wonder why these organisms do not suffer any consequences as a result of the mismatch. Other spider species have been reported to have short circadian clocks as well including *Cyclosa turbinata* Walchenaer, 1842 with an 18.5-hour clock (Moore et al. 2016) and *Metazygia wittfeldae* McCook, 1894 with a 22.7-hour clock (Jones et al., 2018). There is an assumption that the internal circadian clock allows for anticipation of regular events and for quick synchronization to environmental changes.

The ability for spiders, and other organisms, to be synchronized closely with the 24-hour day allows for physiological and behavioral processes to be consistent with and anticipate environmental changes. We tested whether *P. tepidariorum* were able to use 24-hour periodic external cues (Zeitgebers) other than light such as temperature, food, and disturbance to remain in sync with the solar day and to maintain their regular daily cycles. With prey having their own activity patterns, sensitivity to the presence of prey would allow for spider to have the opportunity to optimize food availability. Also, considering that disturbance could potentially indicate the presence of predators or prey, we predicted that *P. tepidariorum* will be capable of entraining to regularly timed food sources and disturbances in the absence of any light cues. Since temperature changes during the day reflect the natural light-dark cycle, we also predicted that this species of spider will be capable of entraining to a temperature cycle. The goal of this study was to observe the sensitivity of *P. tepidariorum* to various Zeitgebers and the effect on their entrainment ability. We expect these spiders to be sensitive enough to these changes to have the ability to synchronize their physiology and behaviors to non-photic cycles given the hypothesized advantages related to predation and prey availability. Sensitivity to non-photic cues would allow spiders to have an advantage of adapting to changes in the environment or manipulating activity based on food resources or predator activity.

METHODS

Study Species. – The common house spider, *P. tepidariorum* (Araneae: Theridiidae), is found near buildings and structures dispersed throughout North America (Bradley 2013). Collection of female *P. tepidariorum* spiders ranged from the end of April through the beginning of May. The spiders were collected in Washington County, Tennessee, USA, and housed in cups when experiments were not being performed. The mating status, exposure to artificial light, and age past adulthood was unknown.

Locomotor Activity Analysis – Individuals were placed in 25 mm diameter x100 mm length glass tubes with loose, plastic caps that were then placed in a locomotor activity monitor (model LAM25, Trikinetics Inc. Waltham, Massachusetts). The locomotor activity monitors were then placed into an environmental chamber which is temperature- and light-controlled. Light exposure was provided by two horizontal 32 W fluorescent tubes with an illuminance of approximately 1400-1600 lux at the activity monitor placement. In the environmental chambers, open containers filled with water maintained high humidity levels throughout the experiment. Activity bouts were recorded in one-minute bins and were measured by disruptions of infrared beams bisecting the long axis of the glass tubes. Resulting data was analyzed through Clocklab Analysis 6 Software (Actimetrics, Wilmette, IL, U.S.A.), aiding in the detection of significant periodicities using Lomb-Scargle periodogram analyses which allows for the analysis of data with frequent or large gaps (Dongen, H.P.A., Olofsen, Hartevelt, & Kruyt, 1999). All data displayed in the periodograms were analyzed with p-value of 0.001 to determine any significant periodicities.

Food and Disturbance Entrainment – Spiders were distributed into four different categories depending on the given external cue and receiving time of that cue: morning feeding, morning disturbance, evening feeding, and evening disturbance. The morning group received the external cue at 10 a.m. while the evening group received the external cue at 10 p.m. The spiders were placed into a temperature-controlled environmental chamber (22 ± 0.5 ºC) under a light-dark (LD) cycle with 12 hours of light and 12 hours of dark. Exposure to this LD cycle lasted for 5 days before feedings and disturbances were introduced. For feedings, spiders received 1 termite for the chosen time while disturbances were puffs of air. Feedings and disturbances occurred for 2-3 days in the LD cycle followed by 7-8 days of constant darkness (DD). After a total of 10 days of receiving an external cue, the spiders continued in DD for 12 days without any food or disturbances.

Temperature Entrainment – The spiders were placed into a LD cycle consisting of 12 hours of light and 12 hours of dark along with a temperature cycle for 3 days. The temperature cycle presented was an 8 ºC difference with 26 ºC beginning at 2 p.m. and 18 ºC occurring at 2 a.m. which was presented in DD for 7 days following the 3 days of LD. Following the first temperature cycle in DD, the temperature cycle was removed and set at 22 ºC, and the spiders were left in DD for 7 days. The same temperature cycle was then re-introduced as the second temperature cycle for 7 days. The experimental design is shown below in Figure 1.

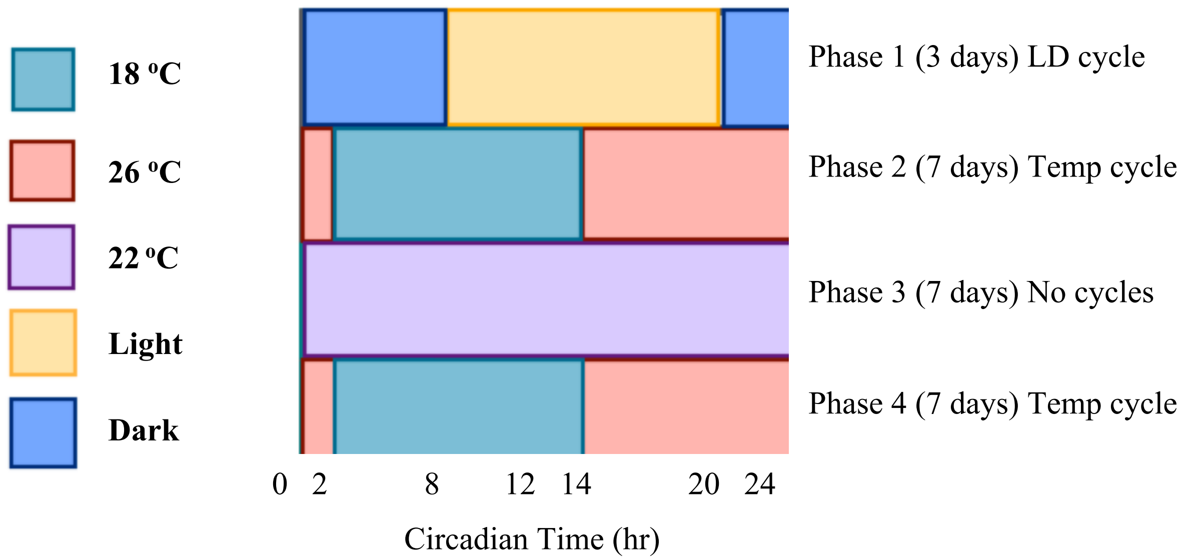


Figure 1 – Temperature entrainment experimental design.

RESULTS

Actogram Interpretation – All actograms (top panels) are double-plotted as a visual method to observe continuous 48 hours of activity. Dark periods are represented by gray background while light periods are represented by the white background. Lomb-Scargle periodograms (bottom panels) indicate significant periodicities ($P < 0.001$) of locomotor activity during the DD conditions as well as stages of the experiment with a presented external cue. Entrainment is shown in the actograms when the activity synchronizes with an environmental cycle or external cue (Foster & Kreitzman, 2004). Activity displayed on actograms as a vertical line that synchronizes with the time of a presented external cue is the criteria for an individual to be considered entrained to the cycle whether it be food, disturbance, or temperature. Free-running activity occurs when individuals are presented to constant conditions which allows for the endogenous circadian rhythms to be displayed and interpreted as an individual's internal circadian clock (Foster & Kreitzman, 2004). For this experiment, free-running activity occurs when there are constant dark conditions.

Morning Feeding Group – The morning feeding group consisted of 16 individuals subjected to a food source provided at 10 a.m. for 2 days under LD, followed by 8 days under DD conditions with a food source, and ending with 12 days without a food source under DD conditions. There were 2 individuals removed from the original 16 since a significant periodicity for 1 of the 4 stages of the experiment was not detected through Lomb-Scargle analysis. There were 11 individuals that exhibited activity around 10 a.m. during the LD feeding stage of the experiment. All 14 individuals appeared to have some degree of consistent activity around 10 a.m. during the presented food source. Only 2 of the 14 individuals demonstrated a difference in periodicities between the constant dark feeding stage and the constant dark without feeding. According to activity and actograms, there were 3 individuals that appeared to anticipate the morning feedings. These conclusions and the periodicities for each individual is shown in Table 1 below. A two-sample t-Test for correlated samples, shown in Table 2, revealed that there was a significant difference between feeding vs constant conditions stages in the experiment for the individuals that appeared to entrain to the morning feedings. There was not a significant difference between the feeding vs constant conditions stages for the individuals that did not appear to entrain.

ID	DD Feeding Periodicity	DD Free-Run Periodicity		Entrained to Feedings	Activity Around Feedings	Anticipation of Feedings
6.1	17.5	21.92		N	Y	N
6.2	23.33	21.92		Y	Y	N
6.3	26.5	26.33		N	Y	N
6.5	24	22.25		Y	Y	Y
6.6	21.67	21.67		N	Y	N
6.7	23.58	24.5		N	Y	N
6.8	22.83	22.25		N	Y	N
13.1	21.58	22.08		N	Y	N
13.2	22.67	20.58		N	Y	N
13.3	22.25	21.08		N	Y	Y
13.4	22.75	25.42		N	Y	N
13.5	23.25	23		N	Y	N
13.6	23.58	23.58		N	Y	Y
13.7	22.92	23.08		N	Y	N

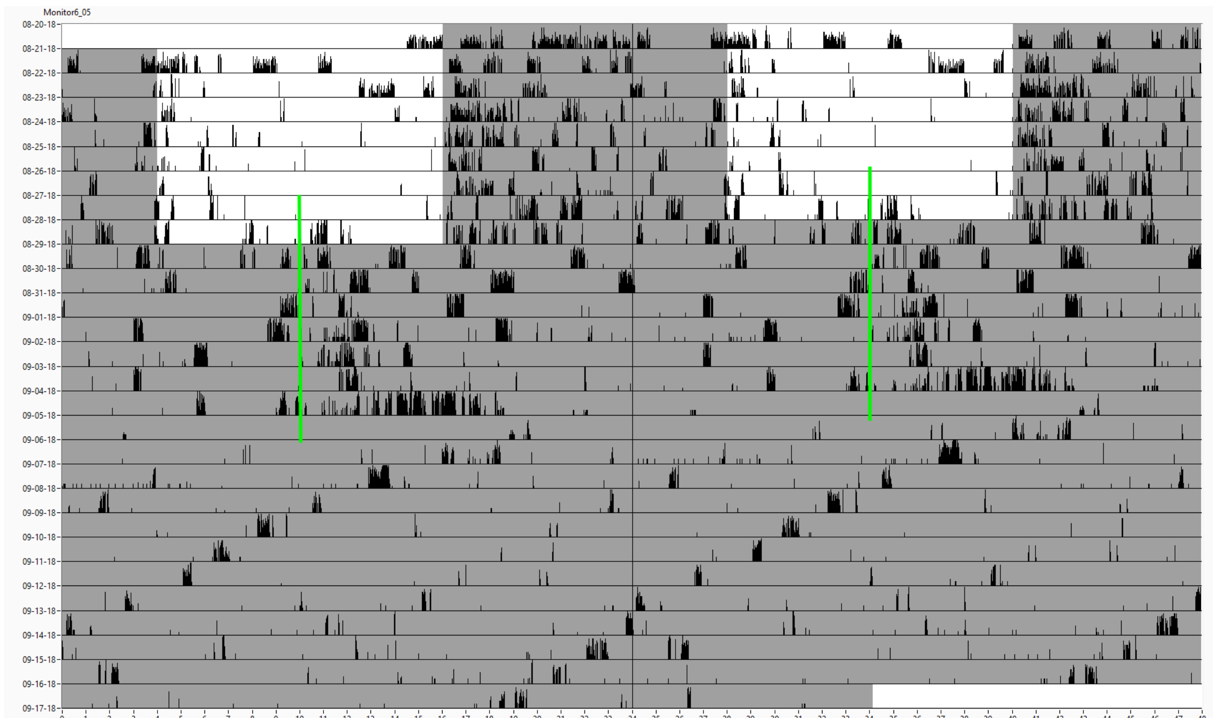
Table 1 – Periodicities of individuals from the morning feeding group for the DD feeding stage compared to the stage of DD without feedings. Individual IDs are in a format that indicates monitor and slot number for recording purposes. Based on the periodicities, either a Y (yes) or N (no) is noted for entrainment activity for each individual. Based on actograms, either a Y or N is noted for displayed activity around 10 a.m. during feedings as well as anticipatory behavior to the feedings.

	Individuals that entrain to morning feedings (2)		**Individuals that do NOT entrain to morning feedings (12)**	
	DD Feeding Periodicity	**DD Free-Run Periodicity**	**DD Feeding Periodicity**	**DD Free-Run Periodicity**
Mean	**23.67**	**22.09**	**22.59**	**22.96**
Standard Error	**0.335**	**0.165**	**0.5889**	**0.5030**
One-tailed t-Test	**P = 0.0341325**		**P = 0.237399**	
Two-tailed t-Test	**P = 0.068265**		**P = 0.474798**	
Table 2 – Two-sample t-Test (correlated sample) for the individuals that appeared to entrain to the morning feedings and those that did not for both stages of the experiment.				

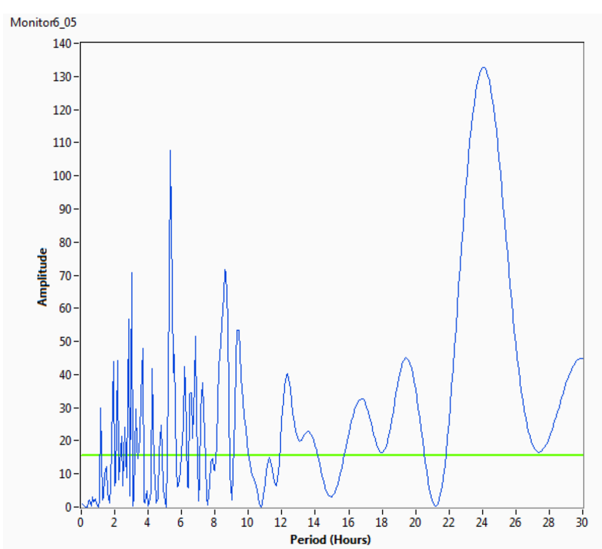
Actogram 2A is an example of an individual that does appear to entrain to the provided food source presented at 10 a.m. According to periodograms B and C, there is a difference between the feeding stage of the experiment and the free-running period. The periodicity during the feeding stage for Actogram 2A was 24 hours while the free-running period during constant conditions was 22.25 hours. However, the locomotor activity in the post-feeding free-run does not extrapolate back to the onset of activity during the feeding. This individual does exhibit anticipation for thermophase during the first temperature cycle.

Actogram 2D is an example of an individual that does not appear to entrain to the provided food source. In the comparison between figures B and C, there is not a difference between the feeding stage of the experiment and the free-running period for this individual. The periodicity during the feeding stage for Actogram 2D was 23.25 hours while the free-funning period during constant conditions was 23 hours. This individual does not exhibit anticipation for thermophase during the first temperature cycle since the individual free-runs through the temperature cycle.

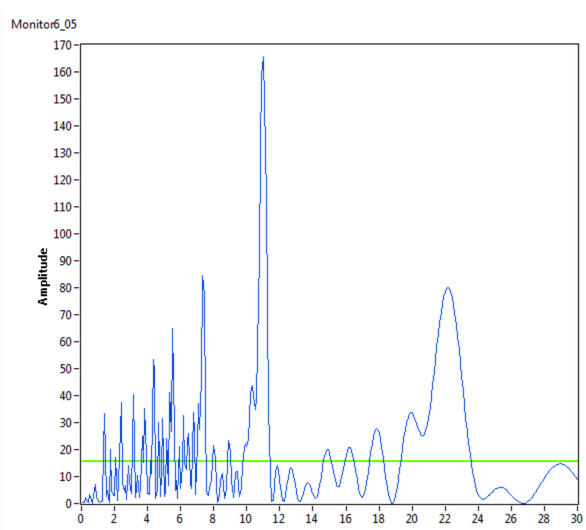
2A



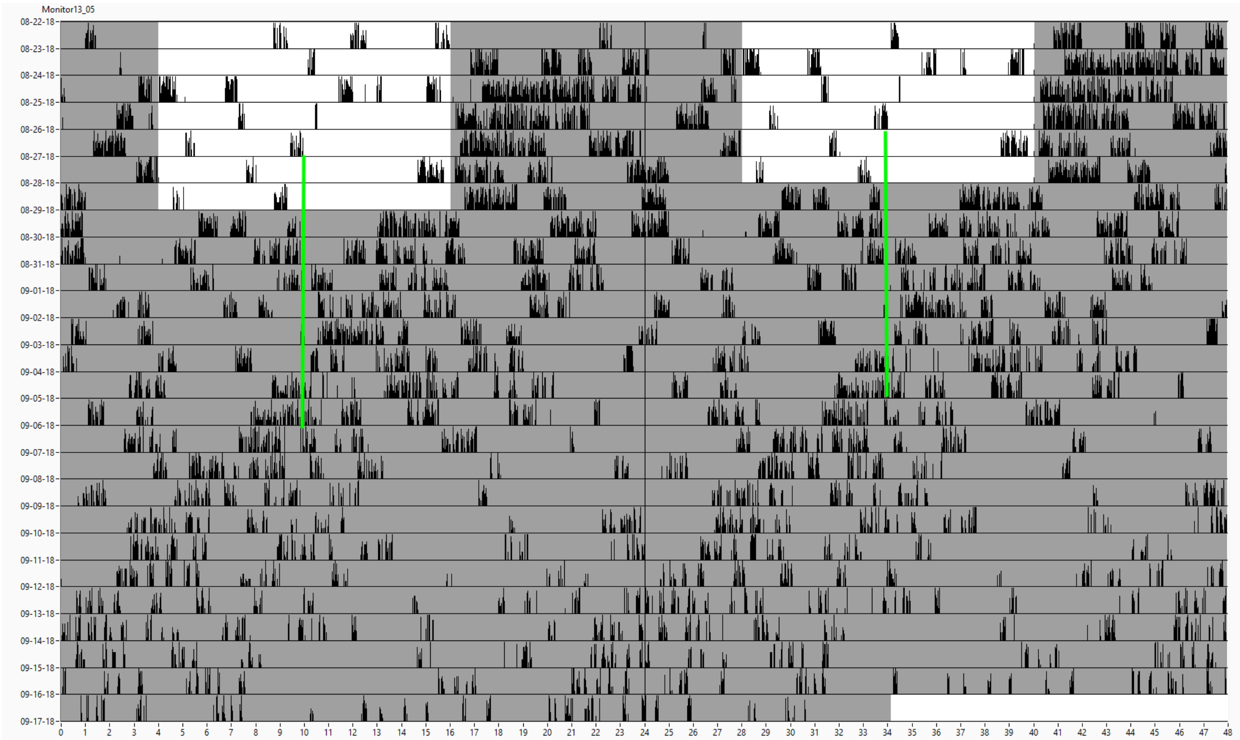
B



C



2D


Monitor13_05

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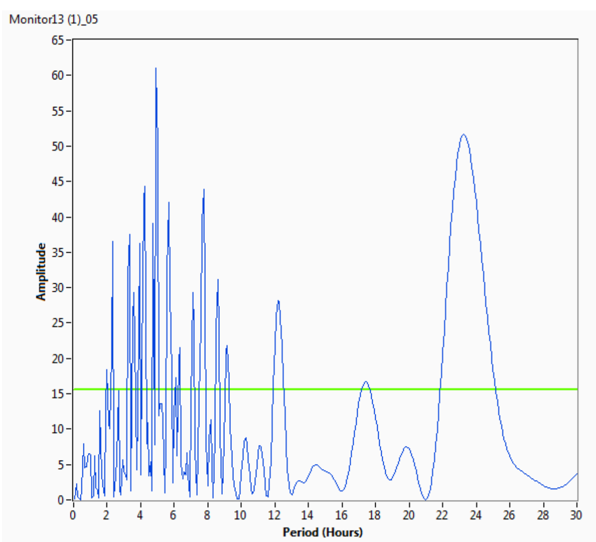
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Amplitude
Period (Hours)

F

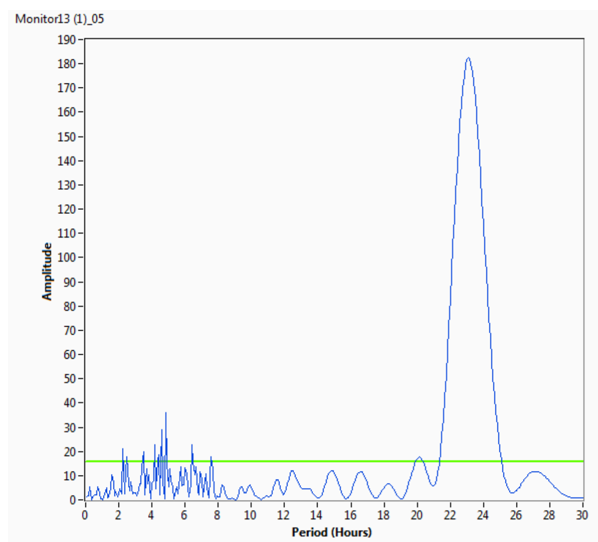

Monitor13 (1)_05
Amplitude
Period (Hours)

Figure 2 - Double-plotted actograms belonging to the morning feeding group illustrating entrainment and free-running locomotor activity in 2 females of *P. tepidariorum*. Actograms (top panels) depict the timing of activity for 5 days under a LD 12:12 h cycle, then 2 days under a LD 12:12 h cycle with a provided food source at 10 a.m., followed by 8 days under constant dark (DD) conditions with a food source at 10 a.m.(indicated by the vertical green line), and ending with 12 days under DD conditions without a food source. The periodograms (bottom panels) depict the significant periodicities for the different stages of the experiment with the left panel representing days with the provided food source and the right panel representing constant dark conditions.

Morning Disturbance Group – The morning disturbance group consisted of 16 individuals subjected to a disturbance provided at 10 a.m. for 2 days under LD, followed by 8 days under DD with a disturbance, and ending with 12 days under DD. There were 8 individuals removed from the original 16 since a significant periodicity for 1 of the 4 stages of the experiment was not detected through Lomb-Scargle analysis. Only 1 of the individuals demonstrated activity around 10 a.m. during the LD disturbance stage. There were 6 individuals of the 8 that appeared to have some degree of consistent activity around 10 a.m. during the presented disturbance. None of the 8 individuals exhibited a difference in periodicities between the DD disturbance stage and DD without a disturbance. According to the actogram activity, none of the individuals appeared to anticipate the morning disturbance. These conclusions and the periodicities for each individual is shown in Table 3 below. While there is a significant difference between the DD disturbance and DD without a disturbance, these individuals exhibit shorter periodicities during the provided external cue compared to the free-run periodicities which does not indicate entrainment activity.

ID	DD Disturbance Periodicity	DD Free-Run Periodicity		Entrained to Disturbance	Activity Around Disturbance	Anticipation of Disturbance
6.9	20.5	22.75		N	Y	N
6.13	22.92	22.42		N	Y	N
6.14	22.42	22.5		N	Y	N
6.16	20.42	21.58		N	Y	N
13.9	21.42	21.92		N	Y	N
13.13	20.08	21.58		N	N	N
13.14	22.5	22.92		N	Y	N
13.15	22.33	23.75		N	N	N
Individuals that did NOT entrain to morning disturbance:						
Mean	21.57	22.43		One-tailed t-Test		P = 0.014883
Standard Error	0.3941	0.2603		Two-tailed t-Test		P = 0.029766

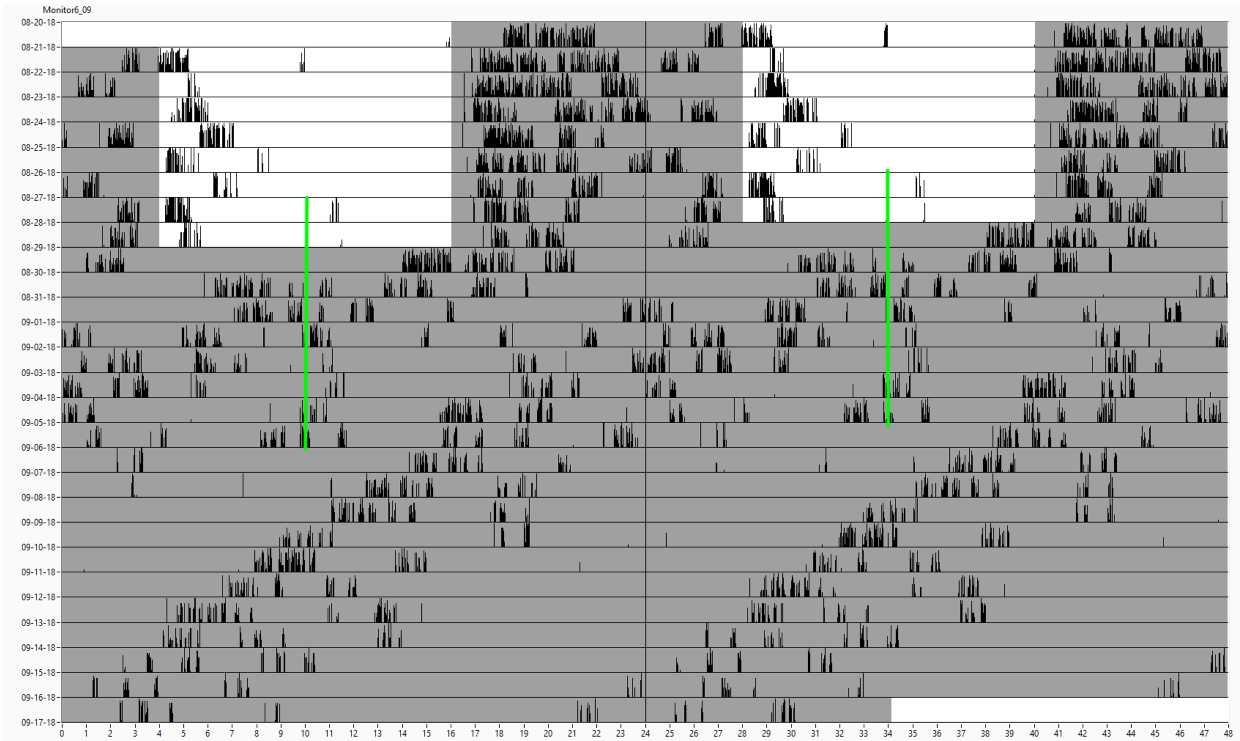
Table 3 – Periodicities of individuals from the morning disturbance group for the DD disturbance stage compared to the stage of DD without a disturbance. Individual IDs are in a format that indicates monitor and slot number for recording purposes. Based on the periodicities, either a Y (yes) or N (no) is noted for entrainment activity for each

individual. Based on actograms, either a Y or N is noted for displayed activity around 10 a.m. during disturbance as well as anticipatory behavior to the disturbance.

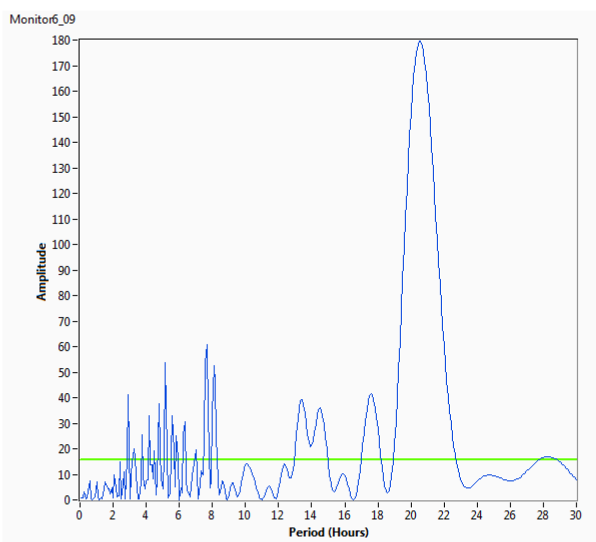
Actogram 3A is an example of an individual that does appear to entrain to the provided disturbance presented at 10 a.m. but also shows potential free-running activity during the stage of the experiment with the provided external cue. According to periodograms B and C, the periodicity during the feeding stage for Actogram 3A was 20.5 hours while the free-running period during constant conditions was 22.75 hours.

Actogram 3D is an example of an individual that does not appear to entrain to the provided disturbance throughout the experiment. In the comparison between figures B and C, there is not a difference between the feeding stage of the experiment and the free-running period for this individual. The periodicity during the feeding stage for Actogram 3D was 21.42 hours while the free-funning period during constant conditions was 21.92 hours.

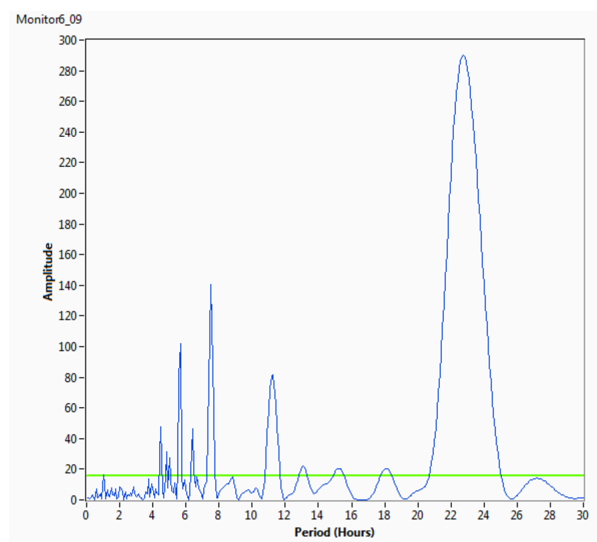
3A


Monitor6_09

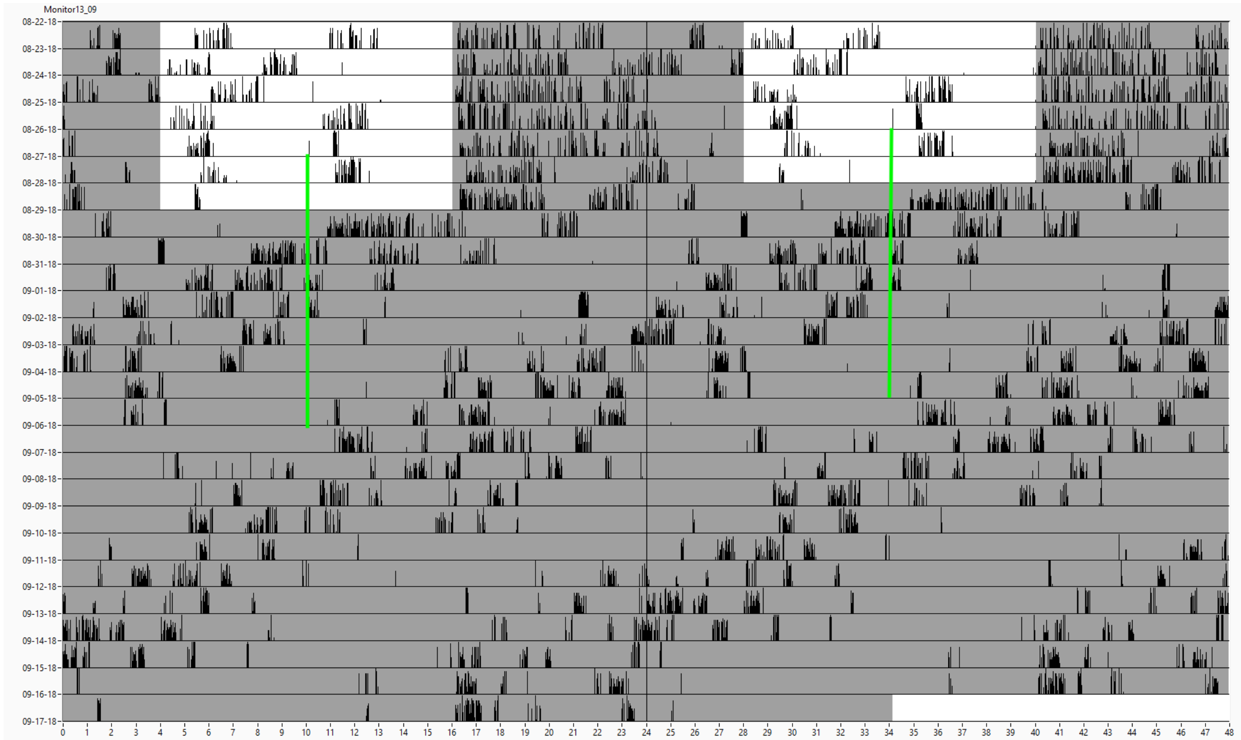
B


Monitor6_09
Amplitude
Period (Hours)

C


Monitor6_09
Amplitude
Period (Hours)

3D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09-08-18
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09-10-18
09-11-18
09-12-18
09-13-18
09-14-18
09-15-18
09-16-18
09-17-18
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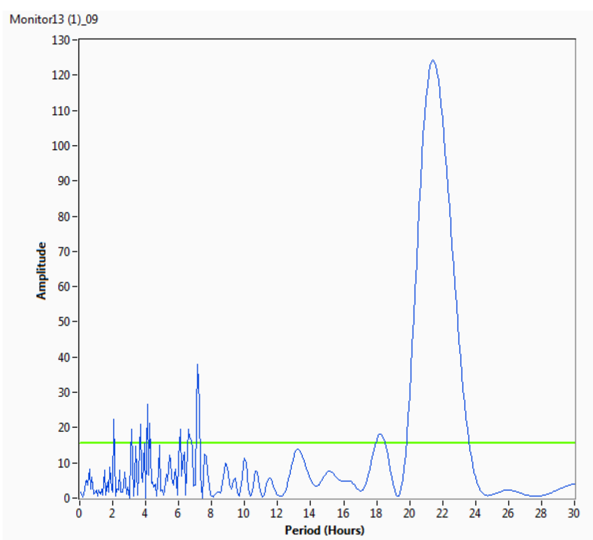
E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Amplitude
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Period (Hours)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

F

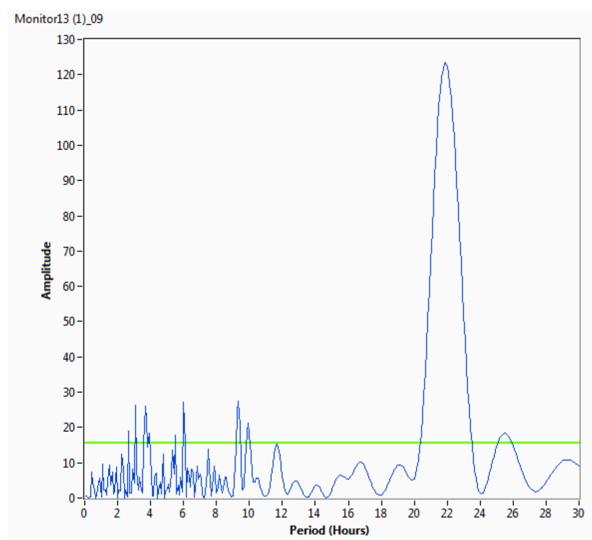

Monitor13 (1)_09
Amplitude
0 10 20 30 40 50 60 70 80 90 100 110 120 130
Period (Hours)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Figure 3 - Double-plotted actograms belonging to the morning disturbance group illustrating entrainment and free-running locomotor activity in 2 different females of *P. tepidariorum*. Actograms (top panels) depict the timing of activity for 5 days under a LD 12:12 h cycle, then 2 days under a LD 12:12 h cycle with a provided disturbance at 10 a.m., followed by 8 days under DD with a provided disturbance at 10 a.m. (indicated by the vertical green line), and ending with 12 days under DD conditions without a disturbance. The periodograms (bottom panels) depict the significant periodicities for the different phases of the experiment with the left panel representing days with the provided disturbance and the right panel representing constant dark conditions.

Evening Feeding Group – The evening feeding group consisted of 16 individuals subjected to a food source provided at 10 p.m. every day for 3 days under LD, followed by 7 days under DD conditions with a disturbance, and ending with 12 days under DD conditions. There were 6 individuals removed from the original 16 since a significant periodicity for 1 of the 4 stages of the experiment was not detected through Lomb-Scargle analysis. There were 4 individuals that demonstrated activity around 10 p.m. during the LD disturbance stage of the experiment. There were 9 individuals of the 10 that appeared to have some degree of consistent activity around 10 p.m. during the presented food source. None of the individuals demonstrated a difference in periodicities between the DD feeding stage and the DD without feeding. While individual 13.18 does have a periodicity of 24.08 in the feeding stage, there was hardly any activity as shown in Actogram 4A. According to activity and actograms, only 1 individual appeared to anticipate the evening feedings. These conclusions and the periodicities for each individual is shown in Table 4 below. There was not a significant difference between the feedings and the constant conditions stages for the individuals that did not appear to entrain to the evening feedings.

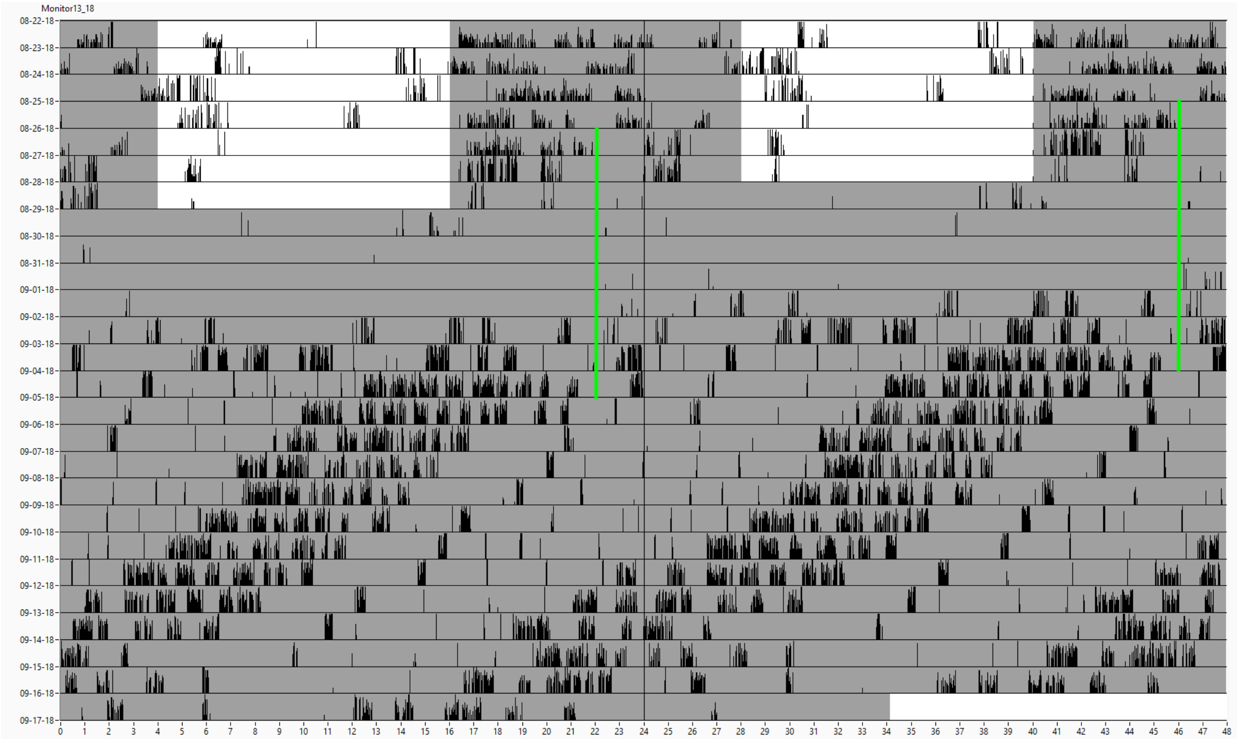
ID	DD Feeding Periodicity	DD Free-Run Periodicity		Entrained to Feedings	Activity Around Feedings	Anticipation of Feedings
6.17	21.58	20.92		N	Y	N
6.20	25.25	21.25		N	N	N
6.21	22.08	21.67		N	Y	N
6.22	21.67	24.17		N	Y	N
6.24	23	22.08		N	Y	N
13.17	23.08	22.08		N	Y	Y
13.18	24.08	22.25		N	Y	N
13.22	23.17	24.08		N	Y	N
13.23	22.25	23.67		N	Y	N
13.24	20.83	21.75		N	Y	N
Individuals that did NOT entrain to evening feedings:						
Mean	22.70	22.39		One-tailed t-Test		P = 0.3078055
Standard Error	0.4118	0.3691		Two-tailed t-Test		P = 0.615611

Table 4 – Periodicities of individuals from the evening feeding group for the DD feeding stage compared to the stage of DD without feedings. Individual IDs are in a format that indicates monitor and slot number for recording purposes. Based on the periodicities, either a Y (yes) or N (no) is noted for entrainment activity for each individual. Based on actograms, either a Y or N is noted for activity around 10 p.m. during feedings as well as anticipatory behavior to the feedings.

Actogram 4A is an example of an individual that does not appear to entrain to the provided food source presented at 10 p.m. but does show inconsistent and disturbed activity. According to periodograms B and C, there is a difference between the feeding stage of the experiment and the free-running period. The periodicity during the feeding stage for Actogram 4A was 24.08 hours while the free-running period during constant conditions was 22.25 hours. This individual does not exhibit anticipation for thermophase during the first temperature cycle since there was not any activity during the temperature cycle.

Actogram 4D is an example of an individual that does not appear to entrain to the provided food source. In the comparison between figures B and C, the periodicity during the feeding stage for Actogram 4D was 22.08 hours while the free-funning period during constant conditions was 21.67 hours. This individual does not exhibit anticipation for thermophase during the first temperature cycle since the individual free-runs through the temperature cycle.

4A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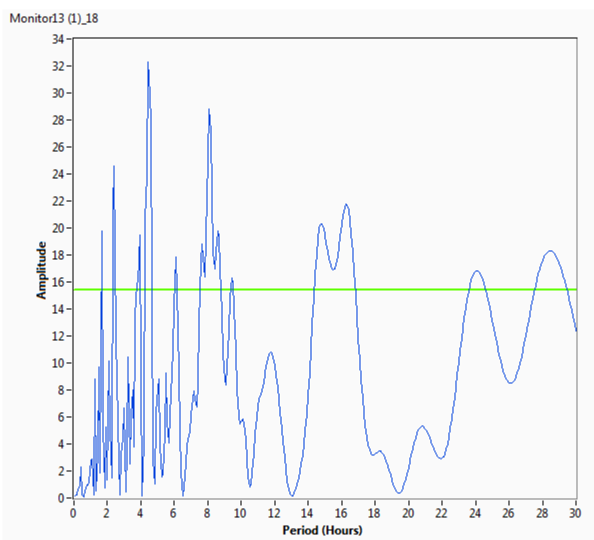
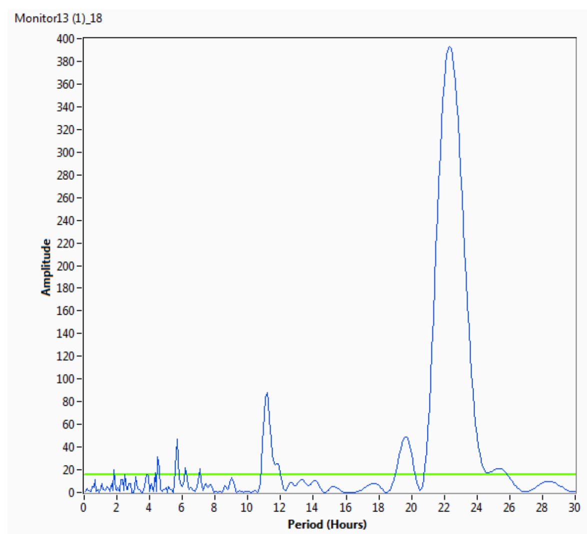
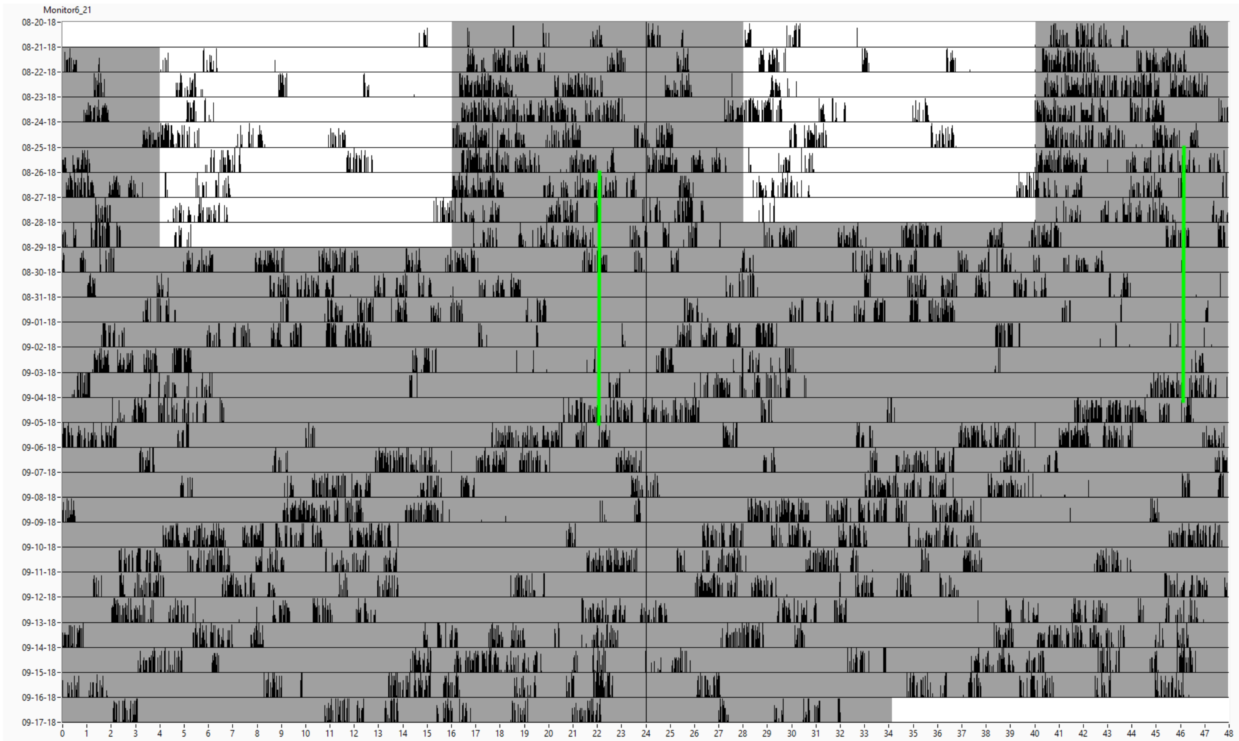
B


Monitor13 (1)_18
Amplitude
Period (Hours)

C


Monitor13 (1)_18
Amplitude
Period (Hours)

4D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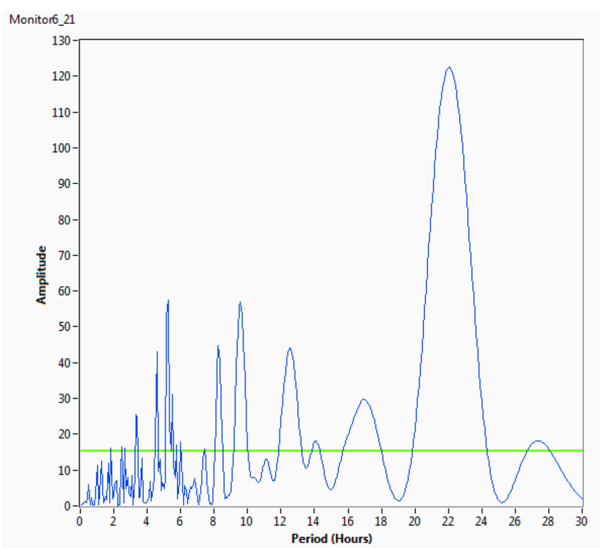
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48

E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Amplitude
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Period (Hours)
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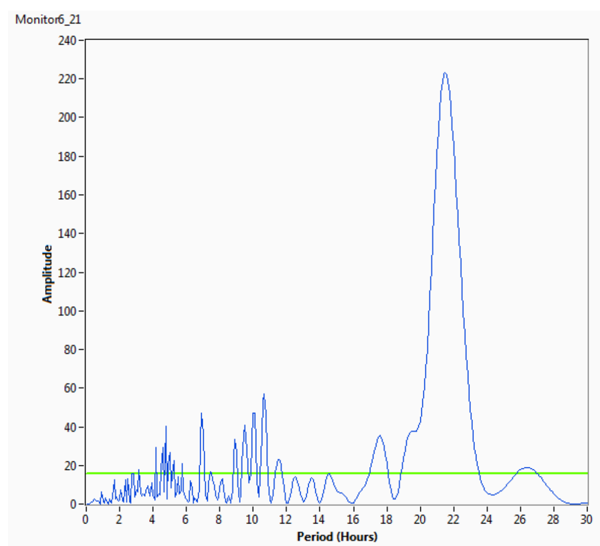
F


Monitor6_21
Amplitude
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Period (Hours)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Figure 4 - Double-plotted actograms belonging to the evening feeding group illustrating entrainment and free-running locomotor activity in 2 females of *P. tepidariorum*. Actograms (top panels) depict the timing of activity for 4 days under a LD 12:12 h cycle, then 3 days under a LD 12:12 h cycle with a provided food source at 10 p.m., followed by 7 days under DD conditions with a provided food source at 10 p.m. (indicated by the vertical green line), and ending with 12 days under DD conditions without a food source. The periodograms (bottom panels) depict the significant periodicities for the different phases of the experiment with the left panel representing days with the provided food source and the right panel representing constant dark conditions.

Evening Disturbance Group – The evening disturbance group consisted of 16 individuals subjected to a food source provided at 10 p.m. for 3 days under LD, followed by 7 days under DD with a disturbance, and ending with 12 days under DD. There were 6 individuals removed from the original 16 since a significant periodicity for 1 of the 4 stages of the experiment was not detected through Lomb-Scargle analysis. There were 3 of the individuals that demonstrated activity around 10 p.m. during the LD disturbance stage of the experiment. There were 8 individuals that appeared to have some degree of consistent activity around 10 p.m. during the presented disturbance cue. Only 1 of the 10 individuals exhibited a difference in periodicities between the DD disturbance stage and the DD without a disturbance. According to actogram activity, the same individual appeared to anticipate the evening disturbance. These conclusions and the periodicities for each individual is shown in Table 5 below. An analysis was not performed on the individuals that did appear to entrain to the evening disturbance cue since there was only 1 individual for the group. There was not a significant difference between the disturbance and the constant conditions stages for the individuals that did not appear to entrain to the evening disturbance cue.

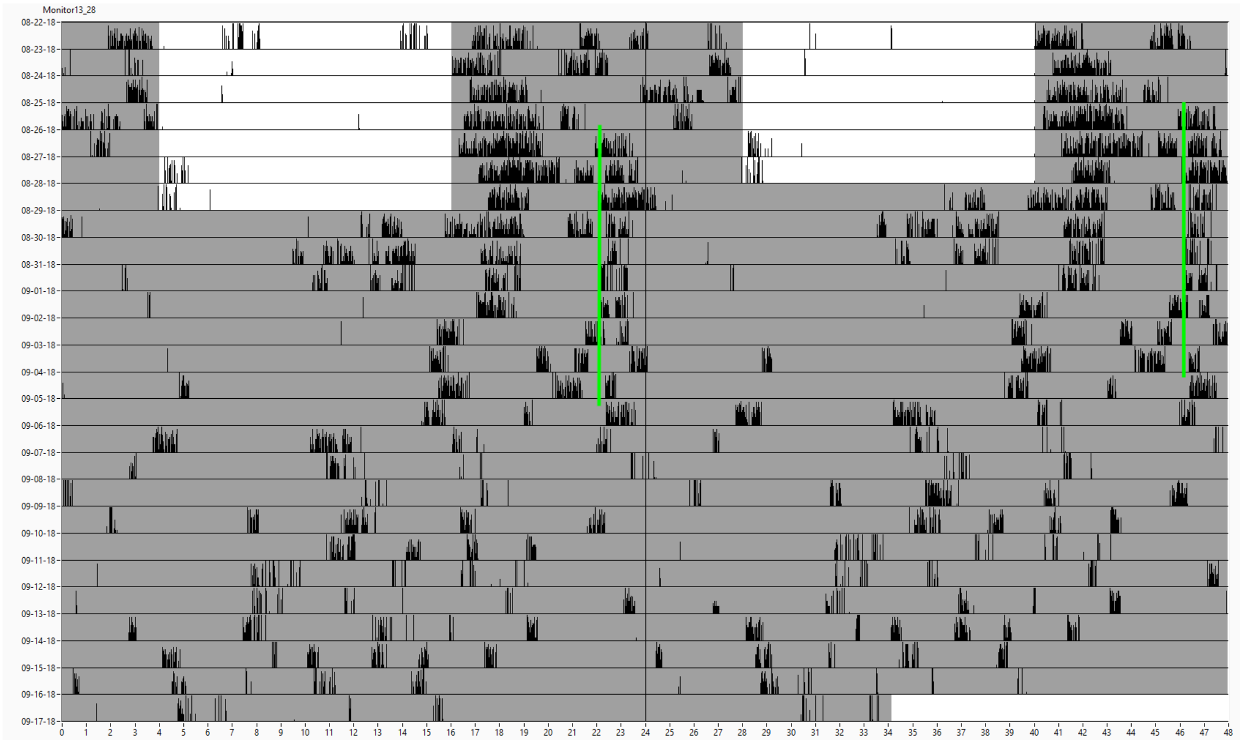
ID	DD Disturbance Periodicity	DD Free-Run Periodicity		Entrained to Disturbance	Activity Around Disturbance	Anticipation of Disturbance
6.27	22.83	22.25		N	N	N
6.30	21.33	21.17		N	N	N
6.31	21.08	23.42		N	Y	N
6.32	21.67	22.58		N	Y	N
13.25	23.33	22.25		N	Y	N
13.26	21.75	22.75		N	Y	N
13.28	24.58	23.17		Y	Y	Y
13.29	23.83	24.25		N	Y	N
13.31	21.17	21.17		N	Y	N
13.32	21.58	23.5		N	Y	N
Individuals that did NOT entrain to evening disturbance:						
Mean	22.06	22.59		One-tailed t-Test		P = 0.0981065
Standard Error	0.3355	0.3448		Two-tailed t-Test		P = 0.196213

Table 5 – Periodicities of individuals from the evening disturbance group for the DD disturbance stage compared to the stage of DD without a disturbance. Individual IDs are in a format that indicates monitor and slot number for recording purposes. Based on the periodicities, either a Y (yes) or N (no) is noted for entrainment activity for each individual. Based on actograms, either a Y or N is noted for displayed activity around 10 p.m. during the disturbance as well as anticipatory behavior to the disturbance.

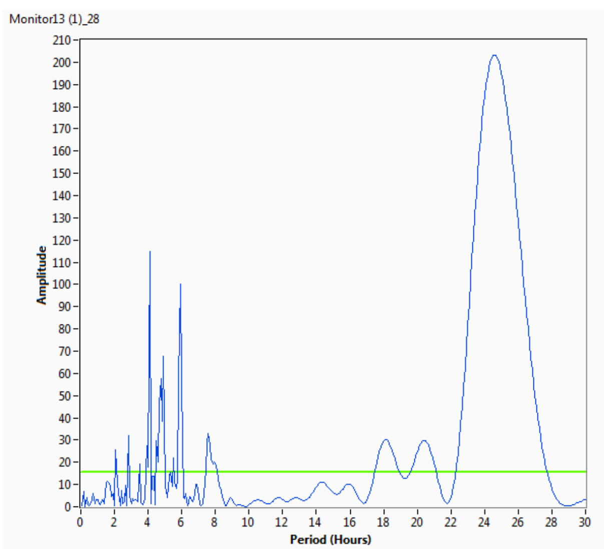
Actogram 5A is an example of an individual that does appear to entrain to the provided disturbance presented at 10 a.m. but also shows potential free-running activity during the stage of the experiment with the provided external cue. According to periodograms B and C, there is a slight difference between the feeding stage of the experiment and the free-running period. The periodicity during the feeding stage for Actogram 5A was 24.58 hours while the free-running period during constant conditions was 23.17 hours. This individual does appear to display anticipation for thermophase during the first temperature cycle.

Actogram 5D is an example of an individual that does not appear to entrain to the provided disturbance throughout the experiment. In the comparison between figures B and C, there is not a difference between the feeding stage of the experiment and the free-running period for this individual. The periodicity during the feeding stage for Actogram 5D was 21.17 hours while the free-funning period during constant conditions was 21.17 hours. This individual does not demonstrate anticipation for thermophase during the first temperature cycle since there was free-run activity throughout the temperature cycle.

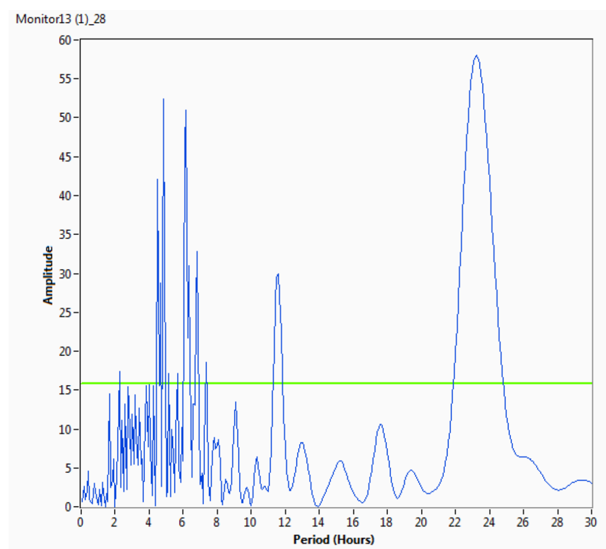
5A


Monitor13_28

B


Monitor13 (1)_28
Amplitude
Period (Hours)

C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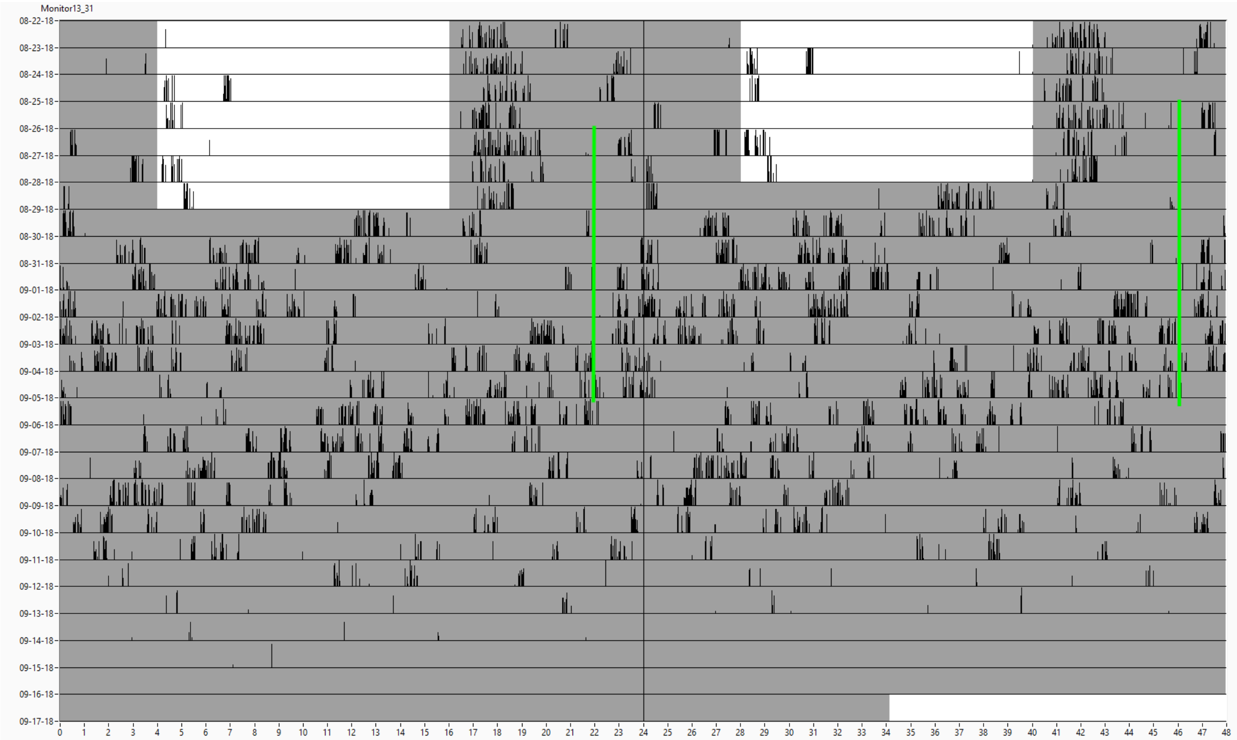
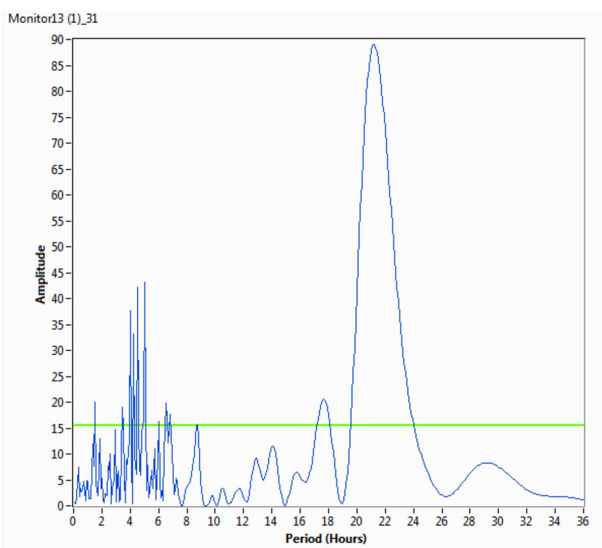
Amplitude
Period (Hours)

5D


Monitor13_31

E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Amplitude
Period (Hours)

F

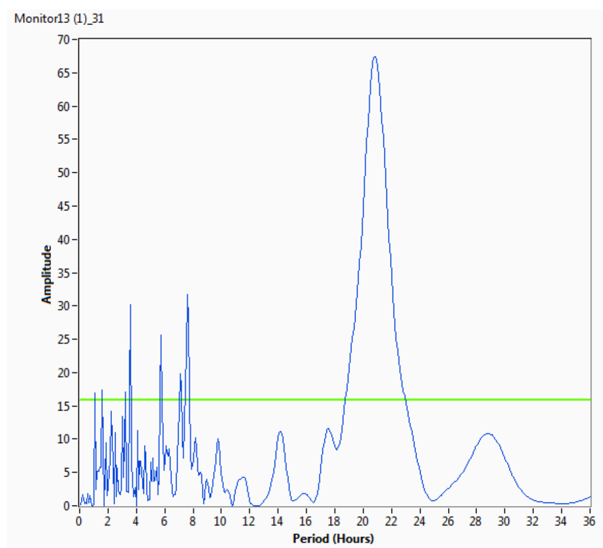

Monitor13 (1)_31
Amplitude
Period (Hours)

Figure 5 - Double-plotted actograms belonging to the evening disturbance group illustrating entrainment and free-running locomotor activity in 2 different females of *P. tepidariorum*. Actograms (top panels) depict the timing of activity for 4 days under a LD 12:12 h cycle, then 3 days under a LD 12:12 h cycle with a provided disturbance at 10 p.m., followed by 7 days under DD conditions with a provided disturbance at 10 p.m. (indicated by the vertical green line), and ending with 12 days under DD conditions without a disturbance. The periodograms (bottom panels) depict the significant periodicities for the different phases of the experiment with the left panel representing days with the provided disturbance and the right panel representing constant dark conditions.

Temperature Experiment – In the temperature experiment, there were 16 individuals that were exposed to 2 temperature cycles separated by a constant conditions stage. The first temperature cycle was presented for 7 days with thermophase occurring at 2 p.m. and chryophase occurring at 2 a.m. Then, constant dark conditions were presented for 7 days and followed by a second temperature cycle with the same conditions as the first temperature cycle. There was 1 individual removed from the original 16 since a significant periodicity for 1 of the 4 stages of the experiment was not detected through Lomb-Scargle analysis. There were 13 individuals that appeared to entrain to the first temperature cycle with activity around 2 p.m. during the warm temperatures while only 3 individuals appeared to entrain to both the first and second temperature cycles with activity around 2 p.m. during the warm temperatures. According to activity and actograms, there were 8 individuals that appeared to anticipate the warm temperatures of the first temperature cycle. Since many of the individuals did not entrain to the second temperature cycle, anticipatory behavior was not identified for that cycle. The average onset for all individuals that demonstrated anticipatory behavior was 10.93 and the thermophase occurred at 14.00 hours with a standard error of 0.6383. These conclusions and the periodicities for each individual is shown in Table 6 below. A one-way ANOVA for correlated samples, shown in Table 7, revealed that there was a significant difference between the first temperature cycle vs the constant conditions stages in the experiment for the individuals that appeared to entrain to the temperature cycle ($P = 0.011819$**)**. There was not a significant difference between the second temperature cycle vs the other two stages of the experiment. For the individuals that did not appear to entrain to the temperature cycle, there was not a significant difference between the feeding vs constant conditions stages ($P = 0.274725$**)**.

ID	Temp. Cycle 1 (M1)	Constant Conditions (M2)	Temp. Cycle 2 (M3)		Temp Cycle 1 Entrainment	Temp Cycle 2 Entrainment	Anticipation of Temp. Cycle 1
1.1	23.58	22.58	22.58		Y	N	N
1.2	23.67	23.17	23.42		N	N	N
1.4	23.58	21.58	23.17		Y	Y	Y
1.5	24.5	22.92	21.67		Y	N	N
1.6	23.58	20	21.92		Y	N	Y
1.7	23.75	23.67	26.42		Y	N	Y
1.8	23.33	21.5	21.42		Y	N	N
1.9	23.33	22.42	21.83		Y	N	Y
1.10	23.42	23.25	25.17		Y	N	Y
1.11	24.92	22.67	22.83		Y	N	N
1.12	23.17	21.33	20.42		Y	N	Y
1.13	22.75	21.42	22.92		N	N	N
1.14	23.42	22.33	21.5		Y	N	Y
1.15	23.58	22.5	25.42		Y	Y	Y
1.16	24.92	25.08	24.67		Y	Y	N

Table 6 – Periodicities of individuals from the temperature experiment for the temperature cycle 1, constant conditions, and temperature cycle 2 stages. IDs are in a format that indicates monitor and slot number for recording purposes. Based on the periodicities and actograms, either a Y (yes) or N (no) is noted for entrainment activity

for each temperature cycle at 2 p.m. for each individual as well as anticipatory behavior to the first temperature cycle.

	Individuals that entrain to one of the temperature cycles (13)			**Individuals that do NOT entrain to one of the temperature cycles (2)**		
	Temp. Cycle 1 (M1)	**Constant Conditions Periodicity (M2)**	**Temp. Cycle 2 (M3)**	**Temp. Cycle 1 (M1)**	**Constant Conditions Periodicity (M2)**	**Temp. Cycle 2 (M3)**
Mean	**23.78**	**22.45**	**23.00**	**23.21**	**22.30**	**23.17**
Standard Error	**0.1663**	**0.3422**	**0.5126**	**0.46**	**0.875**	**0.25**
One-Way ANOVA	**P = 0.011819**			**P = 0.274725**		
M1 vs M2 P<.01 **M1 vs M3 nonsignificant** **M2 vs M3 nonsignificant**				**--------**		

Table 7 - Table 2 –One-way ANOVA (correlated sample) for both the individuals that appeared to entrain to the temperature cycle and those that did not for all three stages of the experiment.

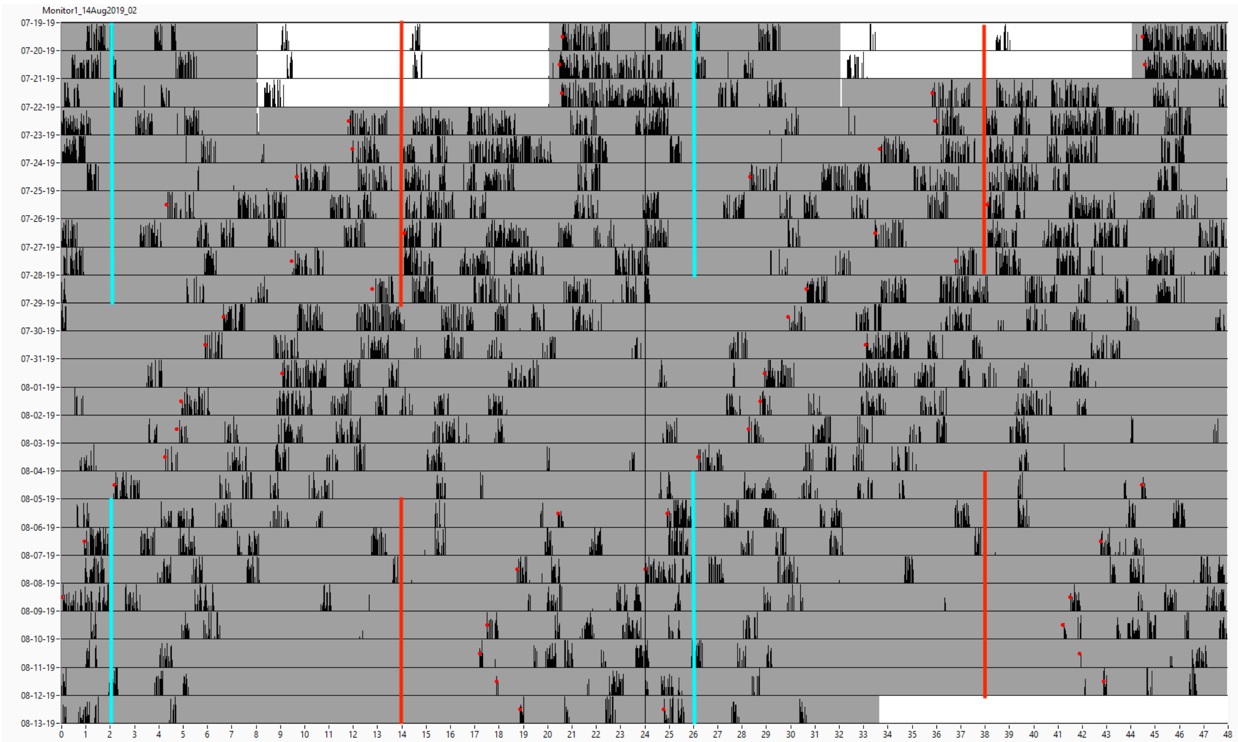
Actogram 6A is an example of an individual that appears to entrain to the first temperature cycle, in constant darkness conditions, but also demonstrates free-running activity during the temperature cycle. This individual displays free-running activity through the constant dark without a temperature cycle along with the second temperature cycle presented. While there is activity concentrated around the time of thermophase, the free-run extrapolates back to activity that is around 21 hours. According to periodograms B, C, and D, there is not a difference between the periodicities in the first temperature cycle stage, constant darkness stage, or the second temperature cycle stage of the experiment. The periodicity during the first temperature cycle for Actogram 6A was 23.67 hours while the free-running period during constant conditions was 23.17 hours and the second temperature cycle was 23.42 hours. There is also not any anticipation displayed for thermophase during the first temperature cycle since the individuals exhibits free-running activity during the temperature cycle.

Actogram 6E is an example of an individual that appears to weakly entrain to the first temperature cycle, then displays free-running activity through the constant dark without a temperature cycle, and then showing re-entrainment to the second temperature cycle presented. According to periodograms F, G, and H, there is a difference between the periodicities in the first and second temperature cycle stages compared to the constant darkness stage of the experiment. The periodicity during the first temperature cycle for Actogram 6E was 22.75 hours, the free-running period during constant conditions was 21.42 hours, and the second temperature cycle periodicity was 22.92 hours. There is also not any anticipation displayed for thermophase during the first temperature cycle since the individuals exhibits free-running activity during the temperature cycle.

Actogram 6I is an example of an individual that appears to entrain to the first temperature cycle in DD, but then appears to free-run through the stage with constant conditions as well as the second temperature cycle. While there is activity concentrated around the time of thermophase, the free-run extrapolates back to activity that is around 20 hours. According to periodograms J, K, and L, there is a difference between the periodicities in the first temperature cycle stage compared to both the constant darkness stage and the second temperature cycle stage of the experiment. The periodicity during the first temperature cycle for Actogram 6I was 24.5 hours while the free-running period during constant conditions was 22.92 hours and the second temperature cycle was 21.67 hours. There is also not any anticipation displayed for thermophase during the first temperature cycle since the individuals exhibits free-running activity during the temperature cycle.

Actogram 6M is an example of an individual that appears to entrain to the first temperature cycle, then displays free-running activity through the constant dark without a temperature cycle, and then showing re-entrainment to the second temperature cycle presented. The free-run activity extrapolates back to the activity that appears to be entrainment to thermophase of the first temperature cycle. According to periodograms N, O, and P, there is a difference between the periodicities in the first temperature cycle stage, constant darkness stage, and the second temperature cycle stage of the experiment. The periodicity during the first temperature cycle for Actogram 6M was 23.58 hours, the free-running period during constant conditions was 22.5 hours, and the second temperature cycle periodicity was 25.42 hours. This individual does exhibit anticipation in small bouts of activity for thermophase during the first temperature cycle.

6A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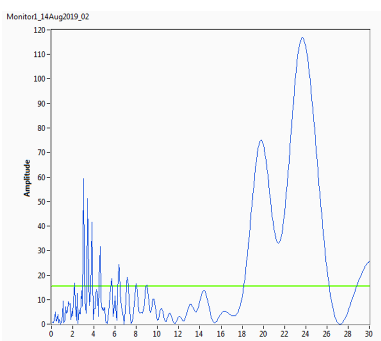
B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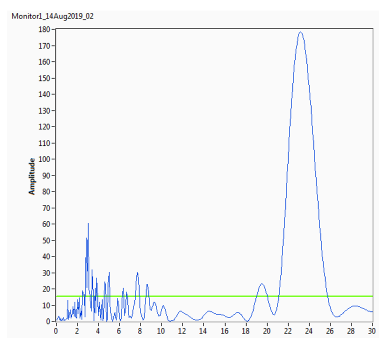
Amplitude
Period (Hours)

C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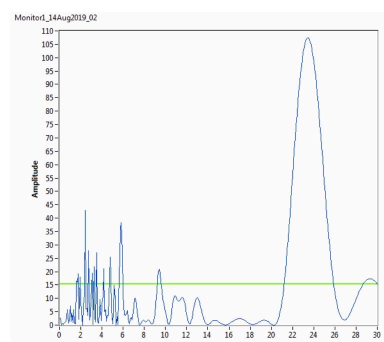
Amplitude
Period (Hours)

D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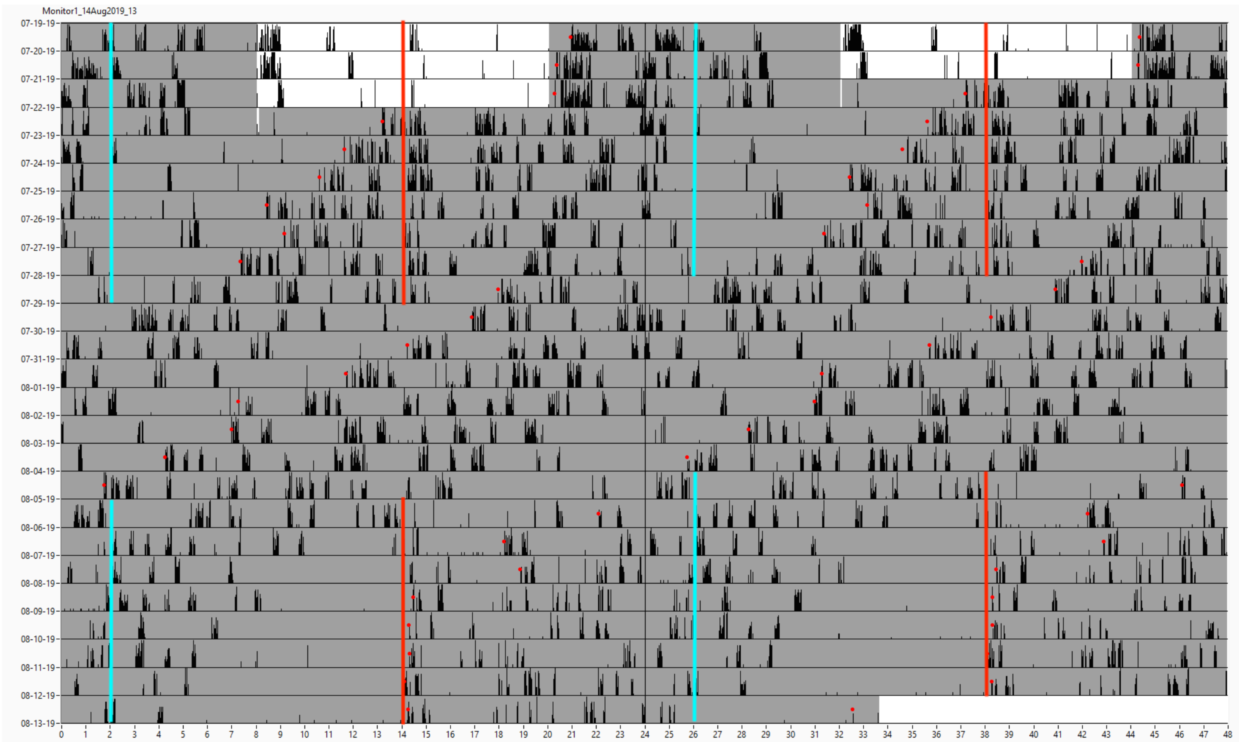
Amplitude
Period (Hours)

6E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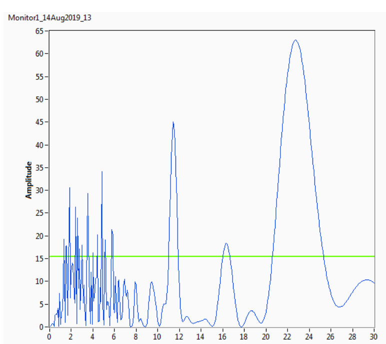
F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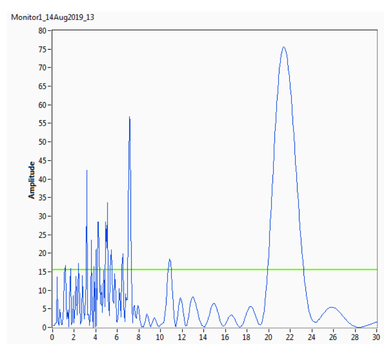
Amplitude
Period (Hours)

G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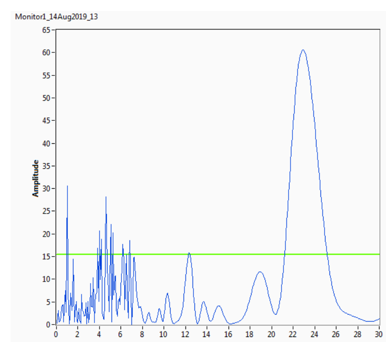
Amplitude
Period (Hours)

H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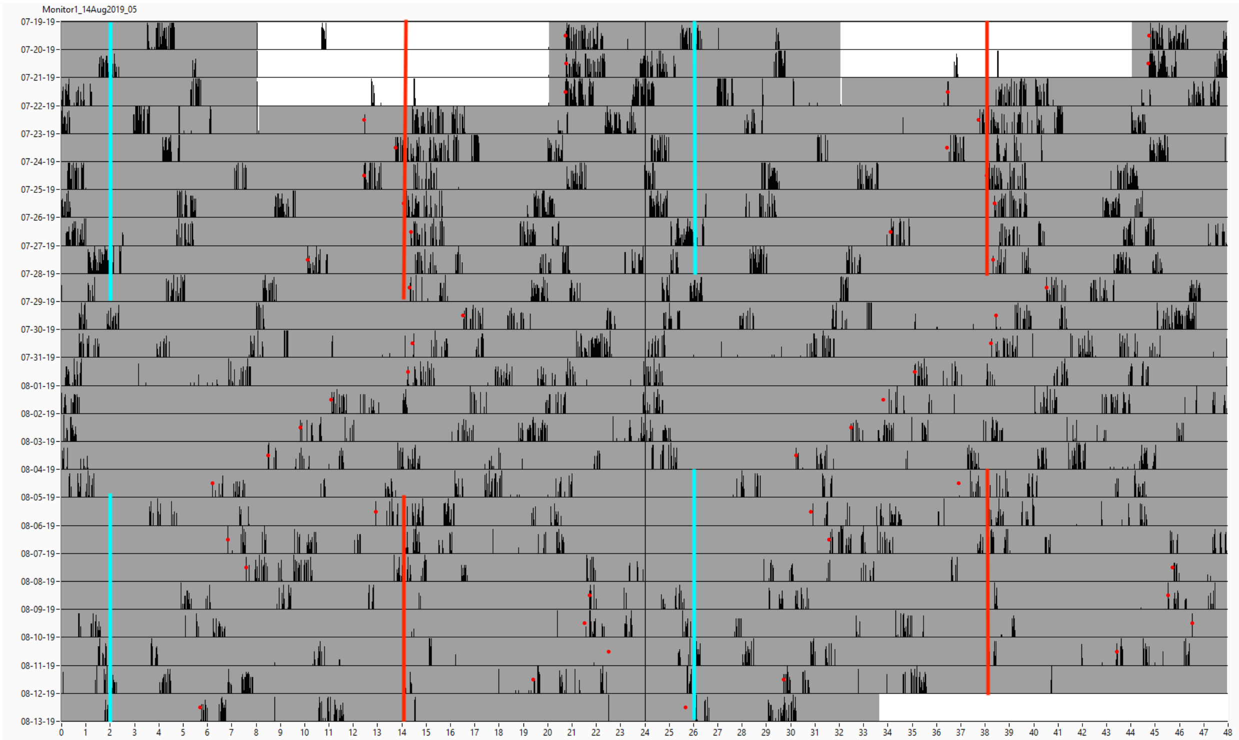
Amplitude
Period (Hours)

6I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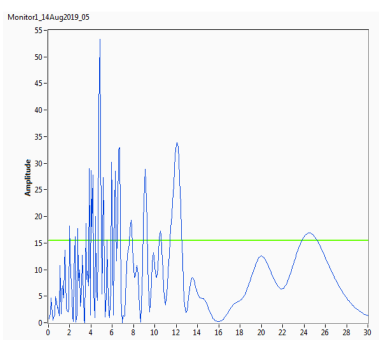
J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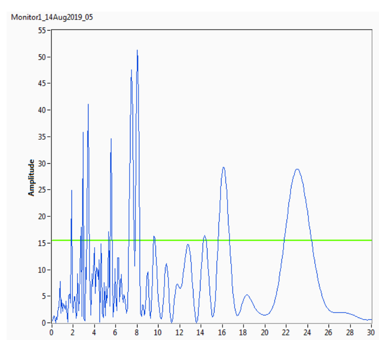
Amplitude
Period (Hours)

K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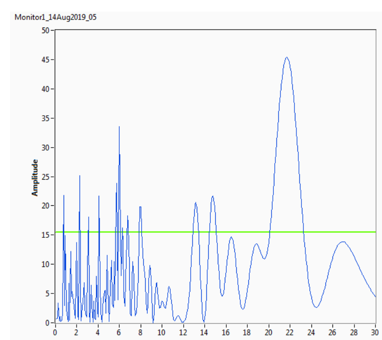
Amplitude
Period (Hours)

L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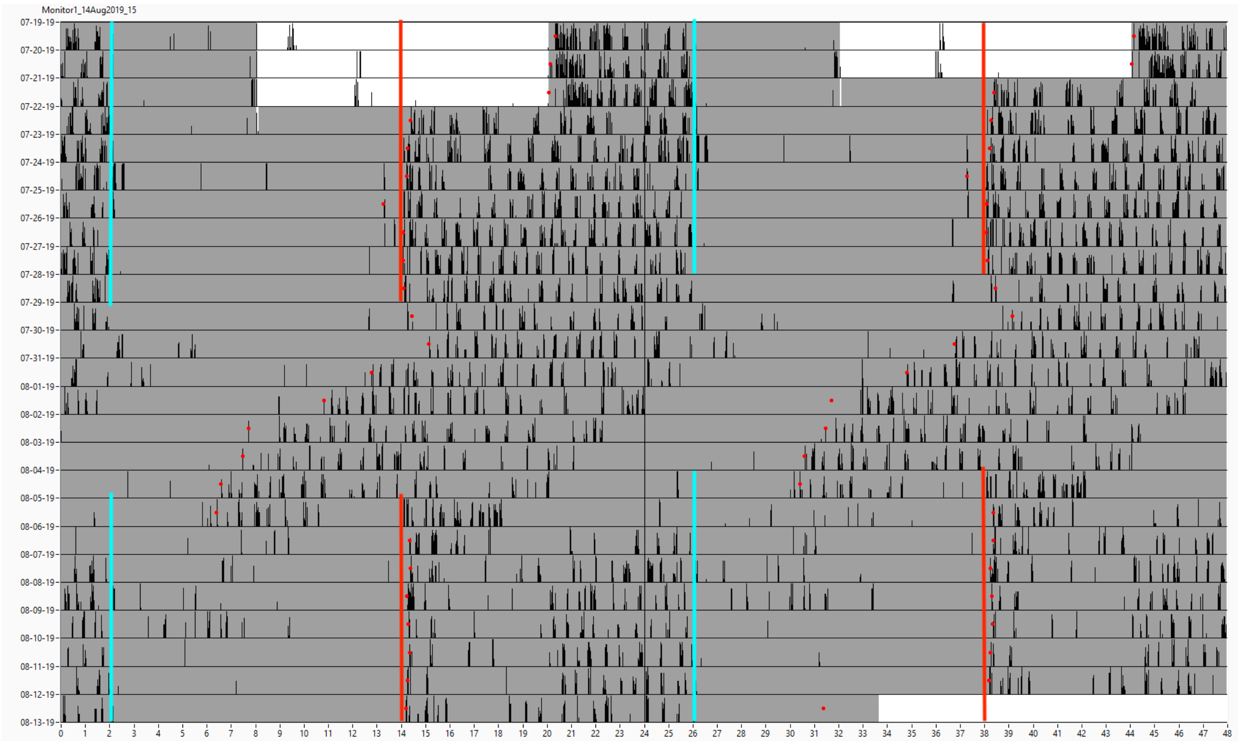
Amplitude
Period (Hours)

6M


Monitor1_14Aug2019_15
07-19-19
07-20-19
07-21-19
07-22-19
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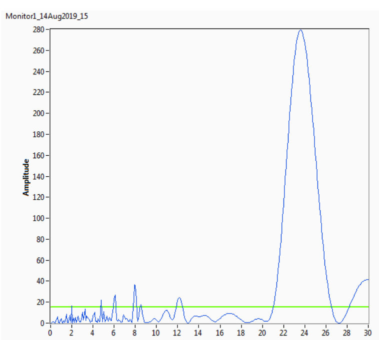
08-13-19

N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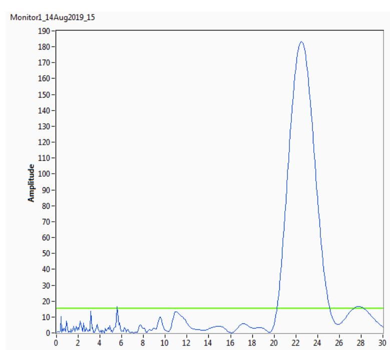
Amplitude
Period (Hours)

O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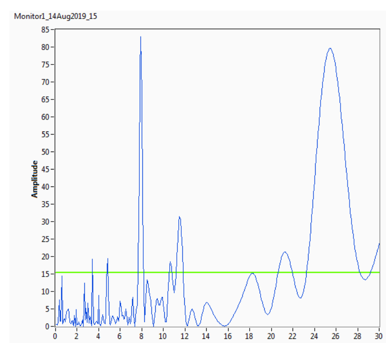
Period (Hours)

P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Amplitude
Period (Hours)

Figure 6 - Double-plotted actograms belonging to the temperature cycle experiment illustrating entrainment and free-running locomotor activity in 4 different females of *P. tepidariorum*. The light portion of the LD cycle occurred from 8 a.m. to 8 p.m. When the temperature cycle was presented to the spiders, the temperature during photophase was 26°C occurring at 2 p.m. (indicated by the vertical red line) while the temperature during scotophase was 18°C occurring at 2 a.m. (indicated by the vertical blue line). Actograms (top panels) depict the timing of activity for 3 days under a LD 12:12 h cycle combined with a temperature cycle, then 7 days under a temperature cycle in DD, followed by 7 days under DD without a temperature cycle, and ending with 7 days under the same temperature cycle in DD. The periodograms (bottom panels) depict the significant periodicities for the different phases of the experiment. The left panel represents days in the first provided temperature cycle, the middle panel represents the days during DD, and the right panel represents the same, second temperature cycle.

DISCUSSION

These results, concerning spiders' limited ability to use food sources or disturbances as non-photic external cues, were unexpected. Considering the spiders' preferred habitat of secluded webs in darker areas, typically with less light, we hypothesized that *P. tepidariorum* would benefit from having the ability to use non-photic external cues for the synchronization of their physiological and behavioral activity with a 24-hour day (Riechert & Cady, 1983).

For the majority of spiders, food cycles were not found to be effective external, non-photic cues for entrainment. Nonetheless, a few individuals appeared to have the ability to entrain to regularly timed food sources, shown in Table 1 and Actogram A, which leads to the conclusion that some spiders are capable of using food as a weak Zeitgeber. Displayed in Table 3 and 5, the majority of spiders were also not observed to be capable of using disturbances as effective non-photic Zeitgebers. For both feedings and disturbances, there were mixed results for anticipation of either external cue, shown in Tables 1,3,4 and 5. More than half of the individuals did not anticipate either external cue, and the group demonstrating the weakest anticipatory behavior being the evening feeding spiders with only 1 individual appearing to anticipate the time leading up to the food source. The majority of individuals from all feeding and disturbance groups demonstrated activity around the time of the provided external cue. This activity that is shown around the time of the given external cues is best interpreted as exogenous activity with the spiders responding to the cue as it is provided. This interpretation explains how almost all individuals show some level of activity around the external cues, but the majority do not show entrainment to that cue, based on Lomb-Scargle analysis. An explanation for why most individuals did not demonstrate entrainment ability to the feeding and disturbance cues would be that the spiders are simply not capable of entraining to these external cues, and the individuals that did appear to entrain were either coincidental or not a normal representation for the species. Other conclusions could be that the spiders were not given enough time (days) to entrain to the provided Zeitgeber or that food is not a day-to-day, life-or-death choice. Spiders can go weeks to months in the absence of any prey or food sources without any fatal consequences.

For the temperature cycles, 13 of the 15 individuals, shown in Table 6, appeared to have entrained to the first temperature cycle presented while only 3 individuals appeared to have entrained to the second temperature cycle. The 3 individuals that entrained to the second temperature cycle were also 3 of the 13 individuals that entrained to the first temperature cycle indicating that these individuals might have been more sensitive to temperature changes relative to the other spiders. Many of the spiders also demonstrated anticipatory activity before thermophase. Temperature changes during the day that reflect the natural light-dark cycle, with the light stage corresponding to warmer temperatures, have been shown to influence entrainment activity in organisms (Lee and Montell, 2013; López-Olmeda et al., 2006). Since *P. tepidariorum* are a nocturnal spider species, temperature changes from warmer to cooler would correspond to dusk and could be utilized as an external cue for these spiders to begin to get active (López-Olmeda et al., 2006).

While many of the spiders subjected to a temperature cycle demonstrated anticipatory behaviors and activity, many of the spiders from the food and disturbance groups did not demonstrate these behaviors. Theoretically, organisms should not be able to anticipate daily events if they are not first entrained to that external cue. Rats have been shown to not exhibit anticipatory activity to daily feedings if the food source is not provided in 24-hour intervals (Mistlberger, 1994). In other words, if the food source was provided every 19 or 29 hours, the rats were not capable of anticipating the meal, but when the meals were provided every 24-hours, the rats did exhibit anticipatory activity (Mistlberger, 1994). For the feeding and disturbance groups, the group that exhibited the most anticipatory behaviors was the group that also had the highest percentage of individuals that demonstrated entrainment to the provided external cue, the morning feeding group. The group that exhibited the least anticipatory behaviors was the group that also had the lowest percentage of individuals that demonstrated entrainment to the provided external cue, the evening feeding group.

There was a lot of variation in ability to entrain among the groups in response to various potential non-photic Zeitgebers tested: temperature, food, and disturbance. From the variation within the feeding and disturbance groups, we can conclude that most activity around the time of the provided external cue was exogenously driven and were simply responses from the spiders as they experienced that cue. Nevertheless, some spiders appeared to be sensitive to regularly provided food sources, and some appear to be sensitive to regularly timed disturbances. In other words, some individuals appear to be capable of using non-photic cues as weak Zeitgebers for the synchronization of their daily physiological and behavioral activities. The variation observed within the temperature group is best interpreted as relative coordination. In other words, the temperature cycle had an effect on the activity exhibited by the spiders, but stable entrainment to the temperature as a Zeitgeber never occurred for some individuals. Most individuals were capable of entrainment to the first temperature cycle, with some of those being able to entrain to the second temperature cycle while others struggling to find the second cycle. Some individuals show clean and clear entrainment to the temperature cycles, some can either entrain to the first or second temperature cycle, and others cannot entrain to any of the presented cycles. One conclusion would be that the timing of the temperature cycle impacts each individual differently. Since a constant conditions stage following the first temperature cycle was presented, this allowed for individuals to free-run from the first temperature cycle for those that entrained to it. Individuals that were capable of entraining to the second temperature cycle were potentially exposed to the cycle during their free-run during a time that allowed them to recognize the temperature cycle for re-entrainment. Others may have not been exposed to the temperature cycle during a time in their natural endogenous cycle that allowed them to re-entrain to the re-introduced temperature cycle. In other words, the ability for entrainment to a temperature cycle could depend on when an individual is exposed to that cycle relative to their own natural circadian clock. An alternative conclusion would be that the difference in temperature was not drastic enough to elicit a response from some of the spiders. This is unlikely though since the majority of spiders did entrain to the first temperature cycle despite not entraining to the re-introduced temperature cycle.

The abilities and limitations of spiders' entrainment to some non-photic cues were observed through this study, but more research should be conducted to attempt to understand how changes in the environment affect activity and the limitations of entrainment for a variety of temperature ranges. There should also be more studies involving the provision of these non-photic cues at different times relative to the individual's endogenous clock to determine if different times throughout an individual's own clock impacts sensitivity to cues, responses, and abilities for entrainment. This study has helped expand our knowledge of spiders' circadian rhythms as well as some of their abilities and limitations. Considering all the variation in activity and responses to external stimulus within this spider species, spiders are an interesting organism to study to help improve our understanding and knowledge of circadian clocks, circadian rhythms, and activity patterns.

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