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Circadian rhythms of locomotor activity in *Metazygia wittfeldae* (Araneae: Araneidae)

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Abstract. Internal clocks, or circadian rhythms, are nearly ubiquitous across taxa (e.g., animals, plants, fungi, and cyanobacteria), and it is widely believed that a biological clock benefits organisms by enabling them to schedule behavioral and physiological changes in anticipation of predictable changes in environmental conditions. Theory and evidence suggest it is important that the internal clock resonate closely with the 24-h daily cycle. Recently, however, *Cyclosa turbinata* (Walckenaer, 1841) (Araneidae) was revealed to have a circadian clock with a period of about 19 h, which was presumed to be anomalous. Here, we report on the behavioral rhythms of a nocturnal orbweaver, *Metazygia wittfeldae* (McCook, 1894), from the same family. Under laboratory conditions of a 12:12 h light:dark cycle, we found that locomotor activity initiates shortly after dark, reaching a peak early in the dark phase, continuing at a lower level throughout the remaining dark phase, and then diminishing shortly after lights-on. Locomotor activity continued to cycle under constant dark conditions with a mean free-running period of 22.7 h. We also found a second component in the free-running activity (mean 11.5 h) which correlated very tightly with the free-running period. Thus, *M. wittfeldae* has what can be considered a typical circadian clock resonating with the 24-h day. Notably, however, there were two outliers close to the 19-h period observed in *C. turbinata*, suggesting that there may be sufficient variation in clock period among araneid spiders upon which selection could act leading to the short-period clocks in *C. turbinata*.

Keywords: Behavioral rhythm, circadian clock, chronoecology

Endogenous clocks, or circadian rhythms, are nearly ubiquitous among animal taxa (Johnson & Kondo 2001), and it is widely believed that the advantage of having an internal clock allows individuals to anticipate daily environmental changes and schedule behavioral and physiological changes appropriately. Circadian rhythms are detected as oscillations in behavior or physiology, with periods of approximately 24 h that persist in the absence of external cues (i.e., 'free-running'). Another property of circadian rhythms is that they are entrainable by external cues, such as changes in light levels over the course of a day, keeping biological changes synchronized with daily environmental changes. In spiders, circadian rhythms in locomotor activity have been described in several families including Lycosidae (Ortega-Escobar 2002), Ctenidae (Seyfarth 1980), and Linyphiidae and Theridiidae (Suter 1993). Circadian oscillations also are reported in visual sensitivity (Yamashita & Nakamura 1999), and in antipredator behavior (Jones et al. 2011) of spiders in the family Araneidae.

In all of the studies of spiders listed above, the reported circadian periods are 'typical' (i.e., within two hours of the natural 24-h daily cycle). Theory predicts that appropriate anticipation and scheduling of biological changes require that the circadian clock resonate closely with the natural 24-h cycle, and this has been supported empirically (Woelfle et al. 2004; Spoelstra et al. 2016). Recently, however, it was reported that *Cyclosa turbinata* (Walckenaer, 1841) (Araneidae) has an exceptionally short-period clock regulating locomotor activity (Moore et al. 2016). With a mean free-running period (FRP) of activity of 18.5 h, *C. turbinata* has the shortest known naturally-occurring circadian period, comparable to 20-h and 18-h mutants in hamsters (Konopka & Benzer 1971; Monecke et al. 2011), the 19-h *per*^{S} mutant in *Drosophila* (Ralph & Menaker 1988), and 19-h laboratory strains of the adzuki bean beetle (Harano & Miyatake 2010). The short-period clock in *C. turbinata* was presumed to be unusual given general circadian theory and previous studies of spiders (Moore et al. 2016). However, to our knowledge, there are no other reports of circadian periods in araneid spiders, so we do not yet know if *C. turbinata* is, in fact, exceptional. We begin to address this in the present study by examining diel and circadian patterns of locomotor activity in another araneid spider *Metazygia wittfeldae* (McCook, 1894).

METHODS

Study species.—*Metazygia wittfeldae* is an orbweaver common to the eastern US (Bradley 2013). The species is described as nocturnal, building or replacing its orb after dark, foraging through the night, and then hiding in a retreat during the day (Levi 1977). We collected adult females from their webs at night in Washington Co. TN, in late April 2017. Care of the animals followed ASAB/ABS guidelines, and the animals were released near the site of collection following experiments.

Locomotor activity.—To observe entrainment to the laboratory LD 12:12 environment, activity monitoring began within 24 h of collection. Individuals were placed in 25 mm diameter X 100 mm length, clear glass tubes and inserted into a locomotor activity monitor (model LAM25, Trikinetics Inc., Waltham, Massachusetts). Activity within each tube was measured *via* interruption of three infrared beams transmitting through the midpoint of the tube: each interruption was registered as an event. Events were counted in 1-min bins and analyzed using Clocklab Analysis 6 Software (Actimetrics, Wilmette, IL, U.S.A.). Activity was depicted graphically by double-plotted actograms to facilitate visual recognition of periods. Significant periods were detected using two different periodogram analyses, chi-square and Lomb-Scargle. The chi-square periodogram (Sokolove & Bushell 1978) is broadly applicable for analyzing circadian data. Using a form of Fourier spectral analysis, the Lomb-Scargle periodogram is better suited to analyze records with large or frequent gaps (Van Dongen et al. 1999). The use of these two complementary methods, rather than just one, provides additional support for determinations of period. Because of the sparse nature of activity bouts exhibited by these spiders, we accepted circadian periodicities only if indicated by both methods.

Spiders ($n = 43$) were monitored in a temperature-controlled environmental chamber (24 ± 0.5 °C) under a light-dark cycle of 12 hours of light and 12 hours of dark (LD 12:12) for five days and then under constant darkness (DD) for ten days thereafter. Lights-on occurred at 07:00 h and lights-off at 19:00 h. Light during photophase was provided by four vertically mounted, 32 W fluorescent tubes; the illuminance was approximately 1400–1600 lux at the level of the activity monitor. Six spiders were excluded from the analyses because they did not perform locomotor activity in all five days of LD entrainment.

RESULTS

Entrainment profiles.—Nearly all of the spiders analyzed ($n = 37$) performed locomotor activity throughout the 5 days of LD 12:12 h entrainment. Locomotor activity in these animals was almost exclusively nocturnal with a DiNoc ratio (daytime activity – nighttime activity/ total activity; Suter & Benson, 2014) very close to -1 (mean = -0.96, SEM = 0.006). In each day of entrainment, activity began about 30 min after lights off, peaking 1–2 h into scotophase, then continuing at a lower level throughout scotophase, before tapering off to nothing an hour after lights on (Fig. 1). Overall activity was higher in the first night of entrainment than in subsequent nights. Aside from the initial peak of activity in early scotophase, there were no consistent patterns thereafter. The onset of activity after lights out became remarkably precise after two days of entrainment (mean delay for entrainment days 3–5 = 31.39 min, SEM = 1.27 min; Fig. 2). No correlation was detected between an individual's delay in onset of activity after lights-off in LD and its free-running period.

Circadian free-runs.—Of the 37 spiders analyzed, 28 exhibited free-running periods that were significant at $P < 0.001$, for both chi-square and Lomb-Scargle periodograms (Mean FRP = 22.7 h, SEM = 0.24 h). Actograms revealed that, in most individuals, the main burst of free-running activity under constant dark (DD) conditions extrapolates back to the peak of activity in early scotophase of entrainment (Fig. 3). Also, the onset of free-running activity was very precise for many individuals, resulting in remarkably straight (as visualized on the actograms) free-runs and distinctly sharp primary peaks in the periodograms.

In 18 individuals, a second periodicity was identified in the free-runs which was significant at $P < 0.001$, for both chi-square and Lomb-Scargle periodograms (Fig. 3a–d). The mean FRP of these second components was 11.47 h (SEM = 0.1 h), which is nearly exactly half the FRP of the main component. Moreover, the main FRP and second components were highly correlated within individuals (Spearman rank-order correlation: $r_s = 0.985$, df = 16, $P < 0.000001$). This second component is particularly distinct in some of the actograms (Fig. 3a–c), and, by inspection, it extrapolates back to late scotophase of the LD 12:12 entrainment. However, we do not detect any peaks of activity under LD 12:12 that would correspond to this second component, even when considering only individuals in which periodogram analyses showed a significant second component under DD.

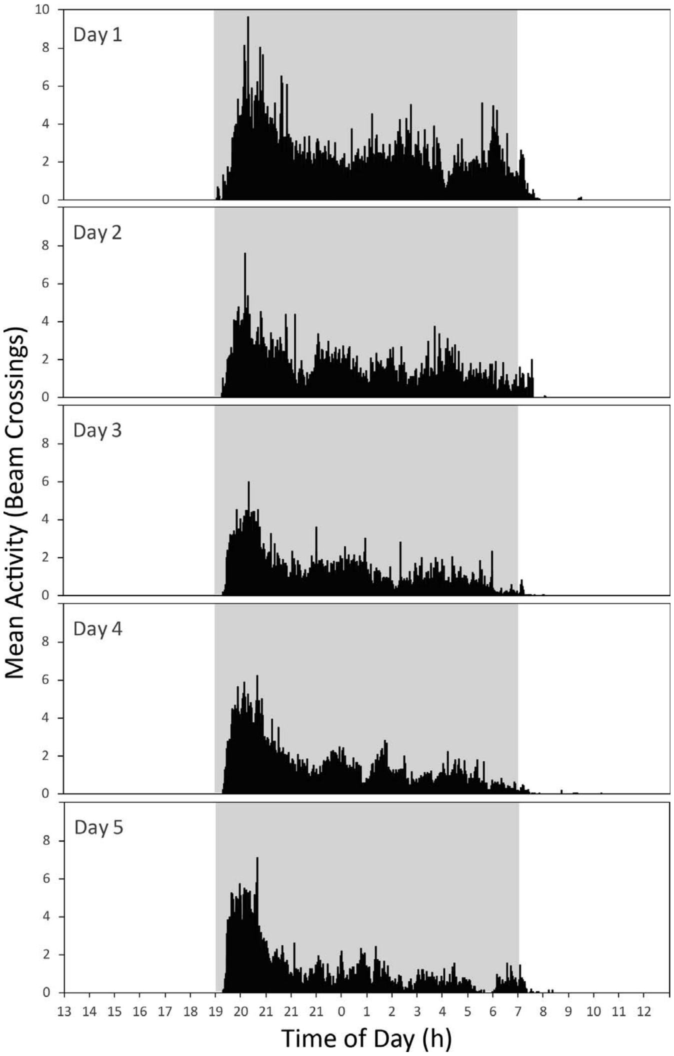

Figure 1.—Pattern of entrainment to LD 12:12 h cycle, showing mean activity levels of 37 individuals, in 1-min bins, for five consecutive days of exposure to the light-dark cycle. Shading indicates scotophase.

The majority of FRPs calculated for *M. wittfeldae* fell within a left-skewed distribution between 21.5 and 23.8 h (Fig. 4). However, there were two outliers with distinctly shorter FRPs of 18.75 and 19.53 h. The actogram and periodogram for one of these outliers are illustrated in Fig. 3e. Interestingly, the FRPs of these two outliers fall within the distribution of 'exceptionally short' free-running periods (Fig. 4) described for *C. turbinata* (Moore et al. 2016).

DISCUSSION

Locomotor activity in the nocturnally foraging *M. wittfeldae* was almost exclusively nocturnal under LD 12:12 conditions, the only exception being that some activity persisted up to an hour after lights on. This contrasts with what was found for eight non-web building species of

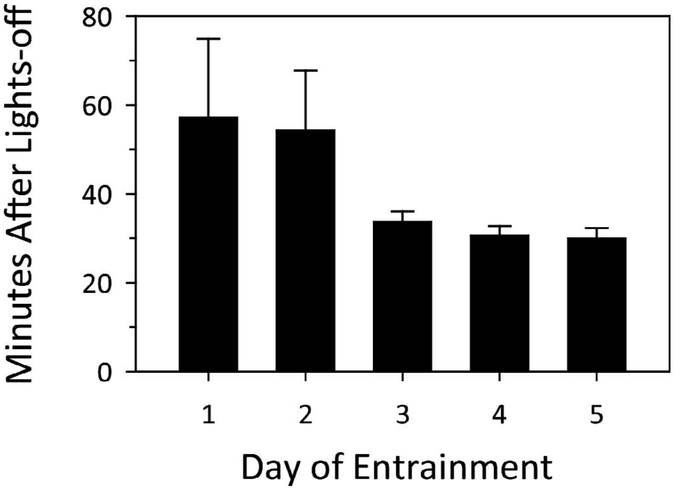

Figure 2.—Delay (in minutes) between lights-off and the beginning of locomotor activity each day for five consecutive days of exposure to the LD 12:12 h cycle. Bars show means (+ SE). Only spiders that exhibited activity during scotophase for all five days ($n = 34$) were included in the analysis.

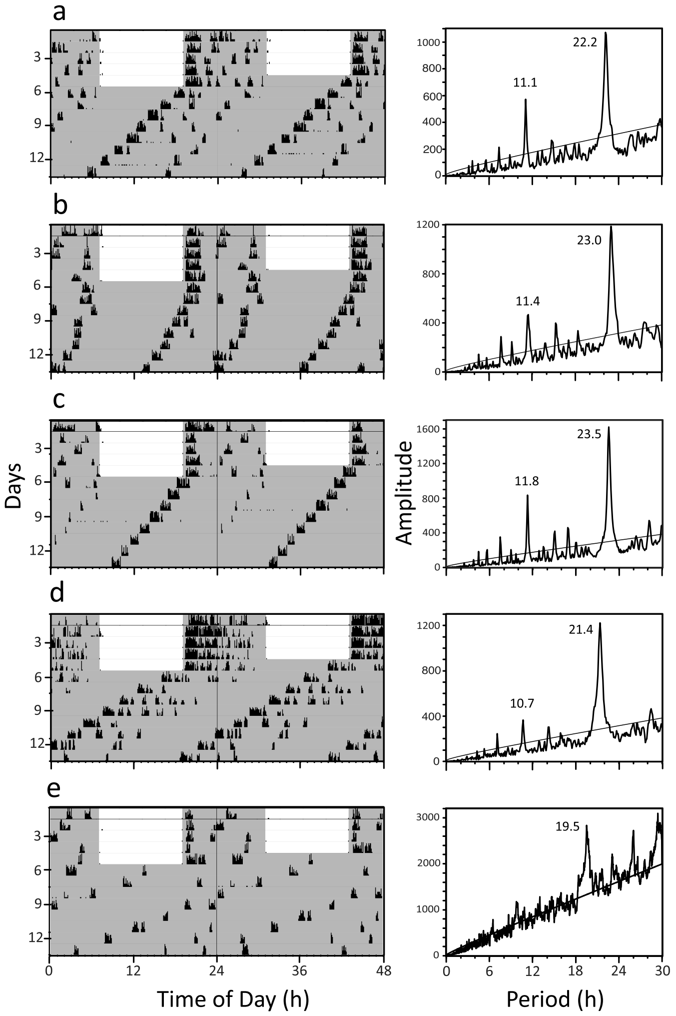

Figure 3.—Double-plotted actograms, each with its accompanying periodogram, illustrating entrainment and free-running locomotor activity in five different individuals of *Metazygia wittfeldae*. Actograms (left panels) depict the timing of activity for five consecutive days under a LD 12:12 h cycle, followed by constant dark (DD) conditions thereafter. Dark periods are represented by gray background. Chi-square periodograms (right panels) indicate significant periodicities ($P < 0.001$) of locomotor activity under DD (FRPs in hours are indicated adjacent to significant peaks). Individuals a-c show particularly strong second components of activity at about half the period of the main component under DD, while in d, the second component is less pronounced, but still significant. Individual e is an example of a short-period outlier.

Lycosidae and Pisauridae (Suter & Benson 2014), in which seven species were predominantly nocturnal and one predominantly diurnal but, in all species, activity was distributed throughout the LD cycle (the most extreme mean DiNoc ratio being -0.528). The nocturnal LD 12:12 activity patterns we found for *M. wittfeldae* were more similar to another araneid spider, *C. turbinata,* which has a mean DiNoc ratio of -0.79 (Moore et al. 2016).

Metazygia wittfeldae builds its web after dusk and forages only during the night (Levi 1977), while *C. turbinata* replaces its web pre-dawn and forages throughout the day and night (Moore et al. 2016). It is curious, therefore, that both species show predominantly nocturnal patterns of locomotor activity. Moreover, there is a question as to what laboratory locomotor activity represents in orb-weaving spiders which, in natural conditions, remain motionless unless capturing prey or working on their webs. Generally, in the circadian literature, locomotor activity is interpreted as an indication of underlying neurological arousal (Brady 1981). This interpretation is compelling for animals such as rodents which do, in fact, move around their environment during periods of arousal. It also seems to fit the natural behavior of wandering spiders which move about their environment when foraging, such as the lycosids and pisaurids studied by Suter & Benson (2014). It is clear from the distinctly non-random entrainment and free-running patterns that locomotor activity represents something biologically real in araneid spiders (Moore et al. 2016; this study). The idea that locomotor activity represents general neurological arousal at night in these spiders is supported by the observation that *C. turbinata* is more likely to attack a prey stimulus at night than during the day (Watts et al. 2014) and that *Larinioides cornutus* (Clerck, 1757) is generally bolder at night than in the day (Jones et al. 2011). From our own observations of several araneid species refusing to build webs in large cages (which are presumably more natural than activity monitor tubes), we speculate that what we measure as locomotor activity corresponds to exploratory behavior which is observed almost exclusively at night.

The average free-running period of *M. wittfeldae* was 22.7 h, suggesting that this species has a 'typical' circadian clock resonating adaptively with the 24-h day (Ouyang et al. 1998; Johnson & Kondo 2001; Woelfle et al. 2004; Spoelstra et al. 2016). These data from an araneid spider, and previous studies on non-araneid species (Seyfarth 1980; Suter 1993; Ortega-Escobar 2002), support the hypothesis that the short-period clock (mean 18.5 h) of *C. turbinata* is, in fact, unusual. *Metazygia wittfeldae* now is the closest relative to *Cyclosa* Menge, 1866 for which an FRP is known, but as yet, this does

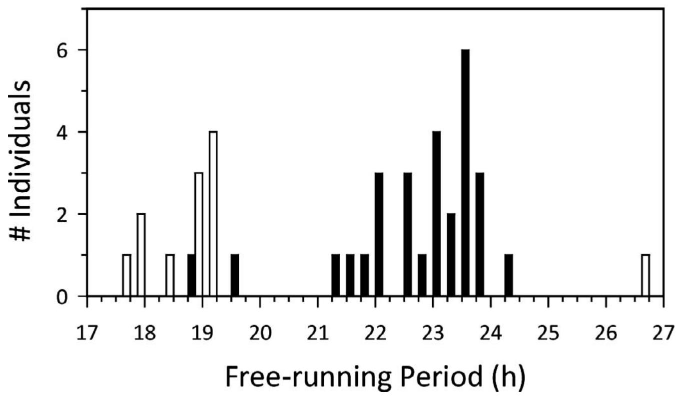

Figure 4.—Frequency distribution of circadian free-running periods of locomotor activity under DD conditions for two species of araneids: *Metazygia wittfeldae* (filled bars) and *Cylosa turbinata* (open bars; Moore et al. 2016).

not give much insight as to where, in the phylogeny of Araneidae, a short-period clock evolved. In an older morphological phylogeny (Scharff & Coddington 1997), *Metazygia* F. O. Pickard-Cambridge, 1904 and *Cyclosa* appear fairly closely related within Araneidae, but nevertheless are in a clade of 18 genera. Unfortunately for this work, *Metazygia* is not included in more recent molecular phylogenies of Araneidae (Dimitrov et al. 2016; Wheeler et al. 2016).

Two aspects of the free-running locomotor activity in *M. wittfeldae* were notable. First was the precise regularity of the onset of activity under constant conditions (Fig. 3). This, coupled with the precise delay of the onset of activity in LD (Figs. 1 & 2), suggests that locomotor activity is tightly regulated by the circadian clock in this species. Second was the presence of a second component of free-running activity, the periodicity of which appeared to be precisely half that of the primary FRP. This second component (appearing in 18 of the 28 individuals for which significant FRPs were obtained) suggests a single oscillator with two active phases or perhaps two separate but tightly coupled oscillators. A similar bimodality in locomotor activity, in which the components maintain a 180° phase difference under constant conditions, occurs in the cockroach *Leucophaea maderae* (Fabricius, 1781). Because the two components free-ran with the same period, they were interpreted as outputs of a single circadian oscillator (Wiedenmann 1980). Despite the fact that the second component in *M. wittfeldae* extrapolated back to a phase position in late scotophase, we did not detect any corresponding peak in locomotor activity at this phase under LD 12:12. This is in contrast to what was found in *C. turbinata*, in which there was a second peak of locomotor activity under LD 12:12 which corresponded to the timing of web-replacement behavior (Moore et al. 2016). Interestingly, however, *C. turbinata* did not have a second component of activity in the free-runs. Future work will look for correlations with the second component in the natural behavior and boldness of *M. wittfeldae*.

The primary objective of this study was to examine the entrainment and free-running patterns of locomotor behavior, with a particular interest of providing a comparison with *C. turbinata* and its short-period circadian clock. Thus, it is remarkable that, while most individuals of *M. wittfeldae* had typical FRPs close to 24 h, two outliers had short FRPs similar to those of *C. turbinata* (Fig. 4). However, the variation in FRPs does not appear continuous, in that the short-period outliers in *M. wittfeldae*, and the distribution of FRPs in *C. turbinata*, are distinctly separate from the main FRP distribution of *M. wittfeldae* (Fig. 4). This is inconsistent with a quantitative trait, and may indicate that there is an associated mutation such as those leading to short circadian clocks in the *per*^{s} mutant in *Drosophila* (Konopka & Benzer 1971), and the *tau* and super duper mutants in hamsters (Ralph & Menaker 1988; Monecke et al. 2011). Future studies will focus on the evolution of circadian clocks in Araneidae, and the molecular mechanisms underlying variation in FRPs.

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