The Role of Colony Temperature in the Entrainment of Circadian Rhythms of Honey Bee Foragers

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Abstract

Honey bees utilize their circadian rhythms to accurately predict the time of day. This ability allows foragers to remember the specific timing of food availability and its location for several days. Previous studies have provided strong evidence toward light/dark cycles being the primary Zeitgeber for honey bees. Recent work in our laboratory described large individual variation in the endogenous period length of honey bee foragers from the same colony and differences in the endogenous rhythms under different constant temperatures. In this study, we further this work by examining temperature inside the honey bee colony. By placing temperature and light data loggers at different locations inside the colony we uncovered that temperature oscillates with a 24-hour period at the periphery of the colony. We then simulated this temperature oscillation in the laboratory and found that using the temperature cycle as a Zeitgeber, foragers present large individual differences in the phase of locomotor rhythms with respect to temperature. Moreover, foragers successfully entrain to these simulated temperature cycles and advancing the cycle by six hours, resulted in changes in the phase of locomotor activity for the most foragers in the assay. The results shown in this study highlight the importance of temperature as a potential Zeitgeber in the field. Future studies will examine the possible functional and evolutionary role of the observed phase differences of circadian rhythms.

Introduction:

Circadian rhythms are biological clocks that synchronize endogenous processes to daily changes in environmental cues, such as light and temperature (Panda, Hogenesch, & Kay, 2002). While circadian rhythms were originally measured as daily behavioral patterns, such as locomotor activity, at the molecular level, circadian rhythms are driven by a set of proteins that generate negative feedback loops that regulate the transcription, translation and post translational modifications of a large number of genes (Blau, 2001; Cyran et al., 2003; Dunlap, 1999; Gardner, Hubbard, Hotta, Dodd, & Webb, 2006). These feedback loops can be measured by oscillations in the expression levels of the genes that make up the clock (Ko & Takahashi, 2006; Takahashi, 1999).Furthermore, changes in light/dark cycles, temperature, food and other factors that serve as Zeitgebers can alter the behavioral and molecular cycles (Hut & Beersma, 2011; Moore & Rankin, 1993; von Frisch, Aschoff, Frisch, & Aschoff, 1987).

In honey bees the circadian clock is associated with various complex behavioral processes that drive the survival and fitness of the colony. Drones and queens (the reproductive castes in the colony) mate at a specific time during the day (Galindo-Cardona et al., 2012). Foragers, who go out in search of different resources during daylight, learn the time and location of a food source and anticipate its availability on following days (Moore, 2001; Moore & Doherty, 2009; von Frisch, 1967). Also, foragers remember the sun's location throughout the day and are able to navigate on cloudy days in the absence of direct sunlight (Goodwin & Lewis, 1987; von Frisch, 1967). Although these and other processes rely on precise timing of the circadian clock, few studies have examined the potential environmental cues that entrain circadian rhythms in nature.

The first studies of honey bee circadian rhythms looked at foraging rhythms at the colony level (H. Buttel-Reepen, 1900; Forel, 1910; Wahl, 1932, 1933). Studies examining the potential Zeitgebers that entrain circadian rhythms in honey bee colonies and individuals came to the conclusion that light/dark cycles are the primary time giver (W Beier, 1968; Wilhelm Beier &

Lindauer, 1970; Detrain, Deneubourg, & Pasteels, 1999; Renner, 1960). At the group level, studies where groups of individuals or whole colonies trained to visit a specific food source were translocated to a different time zone (ex. New York to California) presented foraging activity at the time relative to their original light/dark cycles and would entrain over time to the new light/dark regiment (Renner, 1959). At the individual level studies examining the entrainment to light/dark cycles of foragers show that light successfully entrains the locomotor rhythms of foragers and that the lights-off transition determines the phase of activity in foragers (Moore & Rankin, 1985, 1993; Spangler, 1972). However, the fact that honey bee colonies and individuals entrain to light/dark cycles does not exclude the possibility to entrainment by other environmental cues.

An important aspect of hive maintenance is that honey bees socially regulate colony temperature keeping it optimally at 35˚C (Heinrich, 1980). At the behavioral level this is achieved by fanning (ventilation) and water evaporation inside the colony (Kronenberg & Heller, 1982; Seeley, 1974). Researchers examining the mechanisms underlying this thermoregulation found that individual variation at the genetic level is associated with differences in worker's fanning response threshold to temperature (Jones, Myerscough, Graham, & Oldroyd, 2004). This variation results in different patrilines fanning at different temperatures and, therefore, promoting the stability of brood nest temperature (Jones et al., 2004). Although one of the main challenges to colony thermoregulation is heat associated with the sun, which changes throughout the day, whether individual variation in circadian rhythms play a role in thermoregulation remains unknown.

Thermoregulation of honey bee colonies is essential for colony performance and survival (Heinrich , 1981; Heinrich, 1993). Experiments examining the effects of low temperatures (28-30˚C) on brood development revealed that at these temperatures can result in shriveled wings or other malformations, while brood kept at high temperatures (38˚C-40˚C) present high mortality rates ((Heinrich, 1993; Himmer, 1927). Experiments following up on this work, show that pupal development at 32˚C, which is only 3˚C less than the optimal core temperature, results in

significant deficits on waggle dance behavior and learning and memory assays, compared to bees raised at 36˚C (Tautz, Maier, Groh, Rossler, & Brockmann, 2003). Nonetheless, little is known about the effects of colony temperature on the physiological regulation of the circadian rhythms of honey bees.

Researchers explored the entrainment of circadian rhythms by square-wave temperature cycles and found that cycles with amplitudes greater than 9˚C could successfully entrain circadian rhythms of foragers in the laboratory (Moore & Rankin, 1993). However, whether temperature cycles strong enough to entrain circadian rhythms exist inside the colony has not been thoroughly studied. Changes in environmental temperature in the laboratory have been shown to have strong effects on the endogenous period length of foragers (Fuchikawa & Shimizu, 2007; Giannoni-Guzmán et al., 2014). Furthermore, recent work has revealed that there is a large degree of individual differences in the endogenous period length of the circadian clock of foragers (Giannoni-Guzmán et al., 2014). This large variation in the endogenous period length could be adaptive at the colony level and could result in individual differences in the phase of the circadian clock to time givers.

The main goal of this study was to ascertain if temperature changed on a daily basis inside honey bee colonies and if these changes (temperature cycles) were capable of entraining the circadian rhythms of honey bee foragers. In order to do this, we measured light and temperature at various locations inside of the colony to examine whether temperature and light present daily oscillations inside honey bee colonies. We then explored the effects of temperature cycles observed in the colony on the circadian rhythms of honey bees in the laboratory. To determine if these cycles were capable of entraining the circadian rhythms we advanced the temperature cycle after 6 days of locomotor activity measurements and observed its effects on the phase of the locomotor rhythms.

Material and Methods

100 **Colony Light and Temperature Measurements:**

101 Light and temperature measurements were carried out using HOBO® pendant data
102 loggers (UA-002-64). Four loggers were placed inside the colony at the center, entrance,
103 periphery and top, while one was placed outside the hive as shown (Figure 1A). The colony in
104 which we collected these measurements was a two story colony in good health with a naturally
105 mated queen laying eggs. With the exception of 30 minutes during July 11, 2013, when data was
106 uploaded from the pendants and the batteries were replaced, temperature and light
107 measurements were continuously collected in 30-minute intervals from June 13, 2012 until
108 September 2, 2014. Temperature and light data presented was averaged as 48 hours for
109 comparison with double plotted actograms (Figure 1).

110 **Forager Collection:**

111 All of the bees utilized in our experiments came from colonies in good health that had naturally
112 mated queens and were laying eggs at the time of the experiments. For each experiment, foragers
113 were collected returning to the colony by blocking the entrance with 8-mesh wire screen and
114 capturing them in tubes as previously described (Giray, Galindo-Cardona, & Oskay, 2007).
115 Collected bees were provided with food and water during transportation to UPR Rio Piedras
116 campus (30-40min car ride). Once in the lab bees were anesthetized and placed in locomotor
117 activity monitors as previously described (Giannoni-Guzmán et al., 2014).

118 **Experiment 1: Simulating temperature cycles of the colony in the laboratory**

119 Locomotor activity recordings were performed inside an environmental chamber (Percival,
120 I-30BLL), where temperature was programed to oscillate with an amplitude of 9.2˚C, as observed
121 in the periphery of the colony (Figure 1C). To insure that the temperature was oscillating in the
122 same manner (or as close as possible) as the observed oscillation in nature, the incubator was
123 set up and calibrated 2 weeks prior to the experiment. Age was controlled by paint marking

individuals 1-day old workers and returning them to the colony on October 7th 2014. Locomotor activity recordings began at 21 days of age and took place from October 28th until November 7th 2014. Phase analysis of locomotor activity was performed on days 6-11 of locomotor recording, to allow 5-6 days needed for for bees to acclimate to square wave temperature cycles (Moore & Rankin, 1993).

Experiment 2: 6-hour phase advance of simulated temperature cycles

Locomotor activity assays were conducted using the the same environmental conditions as experiment 1 for the first 6 days of the experiment. On the 7th day the environmental temperature cycles were advanced by 6 hours and were kept this way until the end of the experiment (Figure 3A).

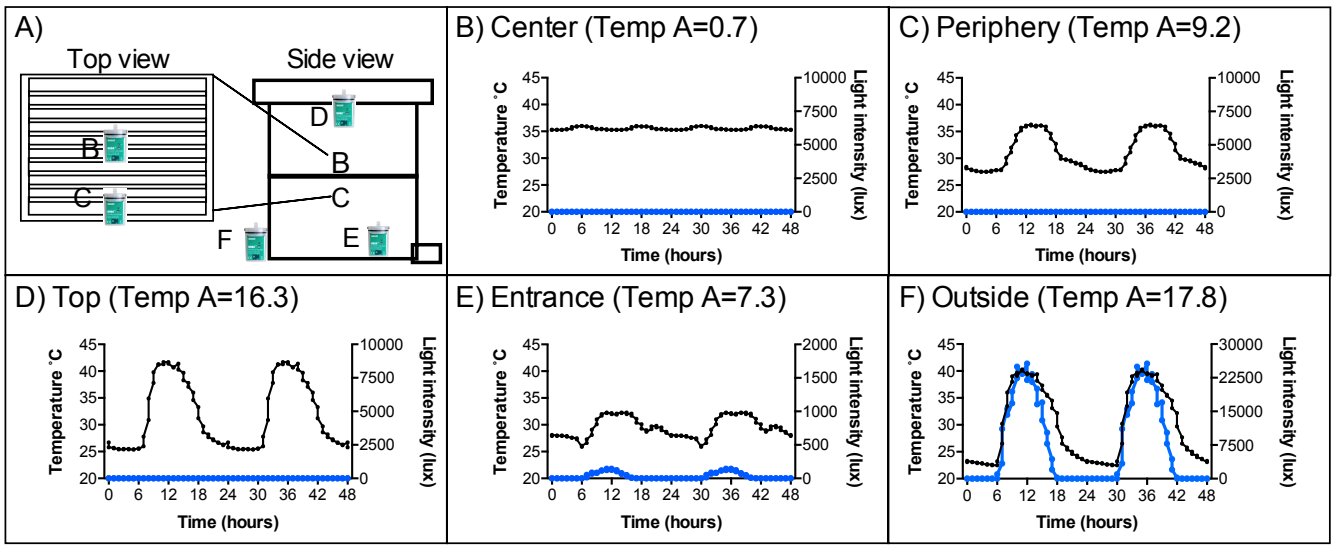
Data analysis:

Locomotor activity data was processed using MatLab® toolboxes developed in Jeffrey Hall's laboratory (Levine et al., 2002). Period length and rhythm strength were calculated using autocorrelation analysis. The locomotor activity was then compared to the environmental cycles to which bees were exposed using cross correlation analysis. Changes in the phase of locomotor rhythms after temperature phase advance were analyzed via Repeated Measures MANOVA.

Results:

Previous work has shown that temperature is a successful Zeitgeber for the circadian clock of honey bees in the laboratory (Moore & Rankin, 1993). However, whether temperature cycles capable of entraining the circadian rhythm of honey bees are present inside the colony was unknown. To examine if temperature cycles are present in honey bee colonies, we placed 5 data loggers at different locations of the colony (center, entrance, top, periphery and outside) and recorded light and temperature measurements in 30-minute intervals (Figure 1A). Our results revealed that temperature oscillates inside honey bee colonies in a daily manner, with amplitudes

148 greater than 7 degrees (Figure 1). Specifically, we observed that at the center of the colony
149 temperature was maintained within 35±0.7 ˚C, while daily temperature cycles at the entrance
150 (amplitude=7.3˚C), periphery (amplitude=9.2˚C) and top (amplitude= 16.3˚C) of the colony were
151 detected. The control data logger that was placed outside the colony detected strong temperature
152 and light cycles with greater amplitude than those detected in the colony (Figure 1E). In addition,
153 the presence of light inside the colony was only detected by the logger at the entrance and was
154 200 times less at its highest peak than that detected by the logger outside the hive (Figure 1E,
155 F).



156
157 **Figure 1. Temperature oscillates with a 24h period in the periphery of the colony. A)** Top and
158 side view of a two story honey bee colony presenting the positions of the 5 sensors used to measure
159 environmental temperature and light, inside and outside the hive. Average environmental data logged
160 at the **B)** center, **C)** periphery, **D)** top, **E)** entrance and **F)** outside the colony. Temperature was plotted
161 on the left y-axis (black line), while light is plotted on the right Y-axis (blue line). As sensors move
162 further away from the center of the colony the amplitude of temperature oscillations increases from
163 0.7˚C at the center of the colony up to 16.3˚C in the top of the colony.

164

165

166 Given the previously reported effects of environmental temperature on the endogenous
167 rhythm of honey bees (Fuchikawa & Shimizu, 2007; Giannoni-Guzmán et al., 2014), we examined
168 the effect of the observed temperature cycles on the locomotor rhythms of foragers. In order to

169 examine the possible effects of temperature cycles on the circadian rhythm of foragers, we
170 simulated the temperature cycle of the periphery of the colony in the laboratory and measured
171 the locomotor rhythms of foragers. This particular temperature oscillation was chosen due to its
172 amplitude (9.2˚C), since square-wave cycles with amplitudes less than 9˚C were unable to entrain
173 foragers in a previous study (Moore & Rankin, 1993). We hypothesized that exposing foragers to
174 simulated temperature cycles would result in their locomotor activity rhythms syncing with the
175 phase of the temperature cycle. We were surprised to uncover that honey bee foragers exposed
176 to the simulated cycles presented individual differences in the phase of their locomotor rhythms
177 with respect to the temperature cycles (ψ) (Figure 2). While there were differences in the phase
178 of the clock with respect to temperature, most individuals in our sample were phase advanced
179 (Figure 2A), while some presented a delayed phase (Figure 2B). Phase quantification for each
180 individual was performed via cross-correlation of the locomotor activity and the temperature cycle
181 (Figure 2, panel (iii)). Frequency distribution of the phase of individuals revealed that more than
182 60 percent of foragers are phase advanced to the temperature cycle (Figure 2C). In addition,
183 period length of foragers was on average 24 hours with little variation (0.06h SEM) (Figure 2D).
184 This small variation of period length is significantly smaller to that of foragers under constant
185 conditions (23.8±0.19 h), and suggests that these temperature cycles may be entraining the
186 circadian rhythm of foragers.

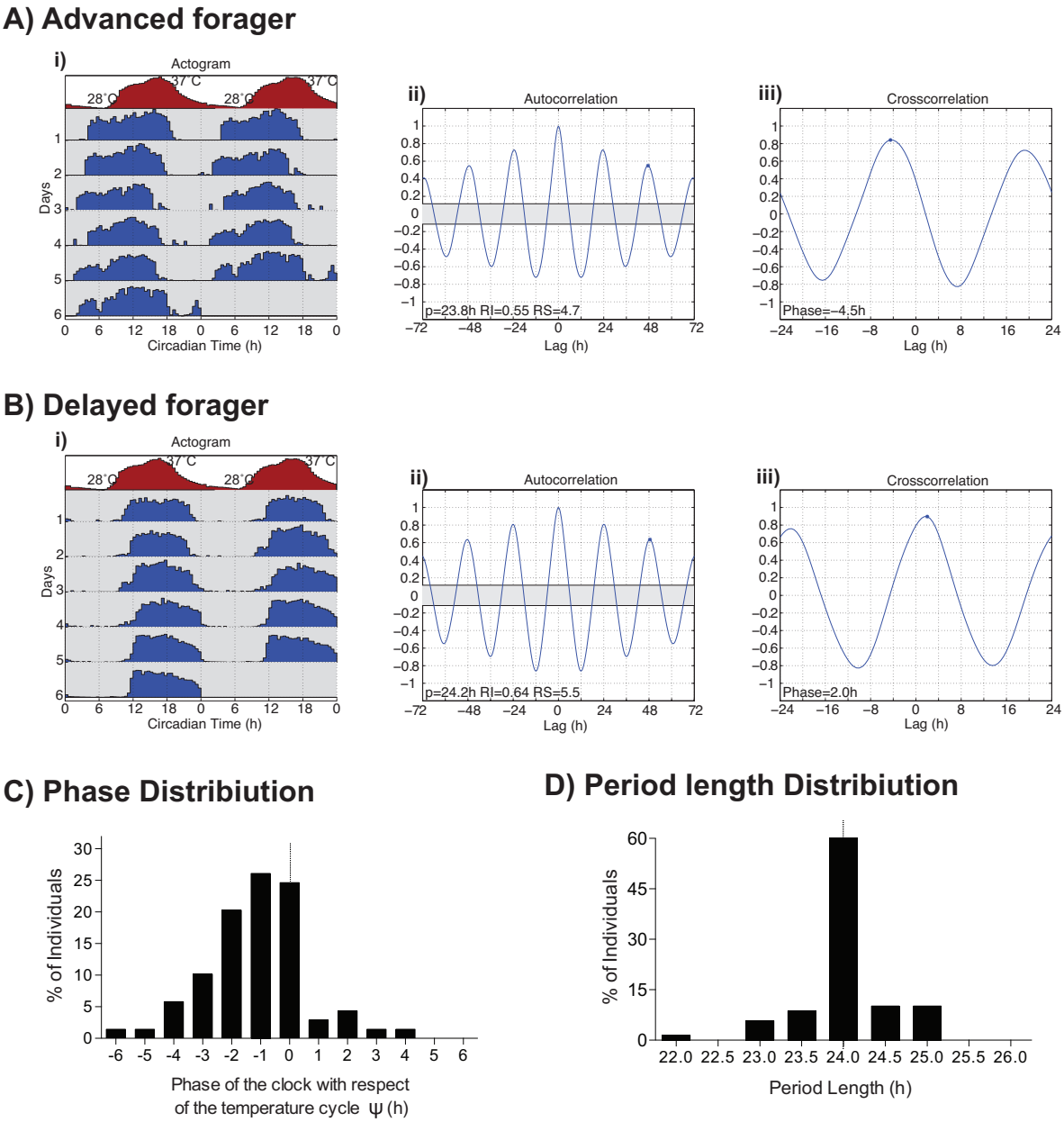


Figure 2. Simulating peripheral temperature oscillations is associated with strong rhythmicity and large individual differences in circadian phase. Representative examples of foragers that presented **A)** phase advance or **B)** phase delay with respect to the simulated temperature cycles in controlled laboratory conditions. **i)** Double plotted actogram of the simulated temperature cycle (red histograms) at the top and locomotor activity patterns below (blue histograms). **ii)** Autocorrelation analysis was utilized to determine period length and overall rhythm strength of locomotor rhythms (Levine et al., 2002). **iii)** Phase of the locomotor rhythms with respect to the temperature cycles (ψ) was quantified using cross-correlation analysis, which compares the distribution of locomotor activity with the temperature cycle and yields the phase as the lag (h). **C)** Frequency distribution of the phase of locomotor activity with respect to temperature cycle. **D)** Frequency distribution of period length of foragers exposed to simulated temperature cycles.

To test if the observed temperature cycles were capable of entraining the locomotor rhythms of foragers, we performed a 6-hour phase advance to the temperature cycle on the 7th day of locomotor activity measurements (Figure 3A). We hypothesized that if the temperature cycles observed in the colony were capable of entraining circadian rhythms, then shifting the temperature cycle would result in changes in the phase of locomotor activity of bees exposed to this change. Consistent with our hypothesis we observed that some individuals shifted their locomotor rhythms as temperature was shifted (Figure 3B), while some foragers seemed to be unaffected by the temperature shift (Figure 3C). Repeated measures analysis of the phase before and after the temperature advance resulted in average locomotor activity phase advance of 2 hours (Repeated Measures MANOVA, $F=1.62$, $df=1$, $p<<0.01$, $n=144$) (Figure 3D). Frequency distribution of the differences in the phase before and after the 6hr advance, revealed differences in the response of locomotor rhythms to temperature (Figure 3E). Taken together, these results suggest that temperature cycles observed in the colony are capable of entraining locomotor rhythms of honey bee foragers, but individual differences in the response to temperature cycle changes suggest differences in the sensitivity of the clock to changes in temperature.

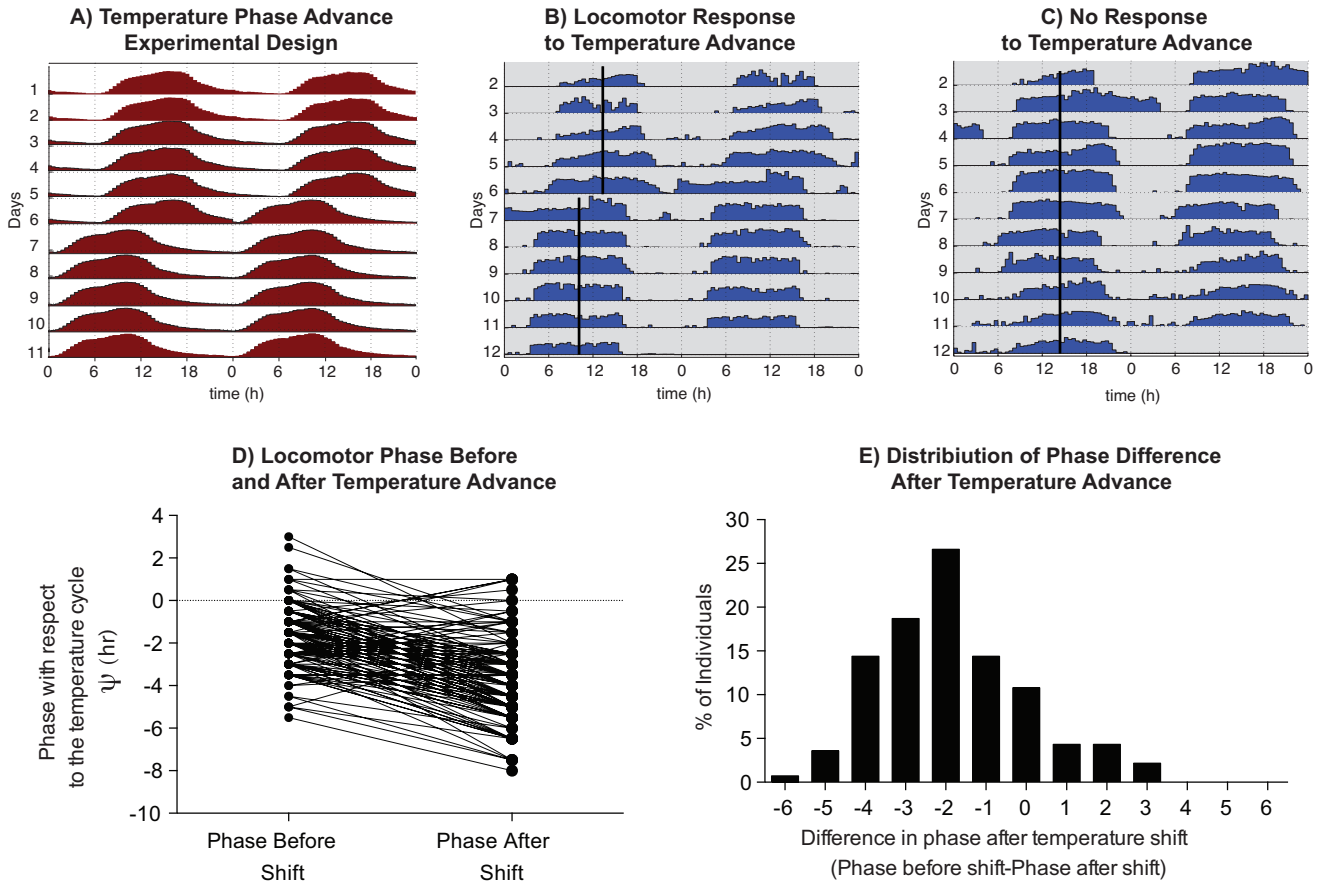


Figure 3. Simulated peripheral temperature serves as a Zeitgeber to the circadian rhythm of foragers. A) Double plotted simulated temperature cycles. From day 7 onward temperature cycle inside the incubator were advanced by 6 hours. This resulted in some individuals displaying changes in their locomotor activity patterns **B)**, while some individuals presented no response to the change in the environmental temperature cycle **C)**. **D)** Repeated measures MANOVA utilized to analyze the effects of the 6hr temperature advance on the phase of locomotor rhythms, revealed significant differences between the phase before the advance and the phase after said advance ($F=1.62$, $df=1$, $p<<0.01$, $n=144$). **E)** Frequency distribution of the change in phase locomotor activity rhythms after 6-hour temperature phase advance.

Discussion:

Here we show that at the periphery of the colony, where foragers spend time, temperature significantly oscillates when a 24hrs period (Figure 1). Simulating this temperature signal in the laboratory is sufficient to entrain the circadian clock of foragers, suggesting that temperature could be an important Zeitgeber in the colony (Figure 2, 3). Interestingly, we found that there are large individual differences in the phase of locomotor rhythms with respect to the temperature cycles.

These differences may provide the basis for a shift strategy suggested by a previous study (Kraus, Gerecke, & Moritz, 2011). Taken together, temperature is an important environmental cue inside the colony and could possibly entrain the circadian rhythms of foragers in nature.

Before this study, the circadian clock of honey bees was thought to be entrained mainly by light/dark cycles (Moore & Rankin, 1985; Renner, 1959, 1960). This idea of light as the main Zeitgeber of honey bee foragers results from the combination of field observations of foraging, translocation experiments where a colony was moved from the east to the west coast of the United States, and locomotor activity measurements performed in the laboratory (Renner, 1959). However, these experiments do not take into account temperature changes that occur within the colony. Our discovery that temperature cycles strong enough to entrain circadian rhythms of foragers are present inside the colony (Figure 1) opens a novel avenue of research where temperature could potentially influence the rhythms of bees in a natural setting.

Consistent with this theory Moore and Rankin proposed that light and temperature may have a synergistic effect on the circadian clock system in honey bees (Moore and Rankin 1993). At this point it is unknown which of the environmental Zeitgebers (temperature or light) plays a more significant role in the entrainment of circadian rhythms in the hive. Experiments where both light and temperature cycles show that some individuals respond strongly to light, while others present locomotor activity during times when light is on and temperature high (Moore & Rankin, 1993). Since foragers have been shown to stay inside the colony after visiting their food source (Körner, 1940; Moore, Siegfried, Wilson, & Rankin, 1989; Seeley, 1995; H. B. von Buttel-Reepen, 1903; von Frisch, 1940), it is possible that while inside the colony foragers rely on temperature cycles to accurately tell time and use light input as a Zeitgeber foraging. This hypothesis stems from the lack of light inside most of the colony. In order to test this hypothesis, further studies exploring the functional role of temperature cycles in the colony as well as the mechanisms of circadian entrainment in honey bees are needed.

256 Consistent with the results from the Moore and Rankin (1993) study, we observed that
257 honey bee foragers entrain to temperature cycles. To our knowledge, this is the first time that
258 gradual temperature cycles, simulating those observed in the field, have been utilized in the
259 laboratory. In addition, by simulating the peripheral temperature cycles we found that foragers
260 present a large degree of individual variation in the phase of locomotor rhythms with respect to
261 temperature (Figure 2). Although further studies are needed to understand the functional and
262 evolutionary role of this variation, we can speculate that phase differences of the individual honey
263 bee foragers may result in differences in the temporal allocation of tasks. This hypothesis would
264 be in line with the results of a recent study that suggests the existence of shift work in honey bees
265 and its genetic component (Kraus et al., 2011). Alternatively, the observed differences in the
266 phase of circadian rhythms could be the result of previous entrainment to an outside stimulus,
267 such as nectar and pollen. This can be evidenced by the ability of foragers to successfully be
268 trained for several days to a food source (von Frisch et al., 1987).

269 When performing a 6-hour advance on the temperature cycle we observed that some
270 individual's locomotor activity was altered with an average change in phase of -2 hours, while in
271 other cases it remained unchanged (Figure 3). This variation in the change of locomotor activity
272 patterns may reflect differences in the perception of temperature by the circadian system of
273 foragers. Although the mechanisms for light and temperature input to the clock of honey bees
274 remain to be elucidated, a study in *Drosophila melanogaster* larvae shows that light and
275 temperature signaling occurs via alternate mechanisms (Picot, Klarsfeld, Chélot, Malpel, &
276 Rouyer, 2009). Further studies examining the pathways of light and temperature to the circadian
277 rhythms in honey bees may provide insight into the observed differences in the response to
278 temperature shifts and possible roles of temperature entrainment in nature.

279 In conclusion, temperature oscillations as observed in the colony serve as a Zeitgeber for
280 the honey bee circadian rhythms. Future studies will explore the importance of temperature as a

time giver in the wild. Individual differences in the phase of circadian rhythms are loosely reminiscent to those of circadian chronotypes in human populations and with further studies, honey bees could be a potential model for these differences in human populations. Research into the functional relevance of these phase differences in the colony may provide a better understanding of the evolution of circadian rhythms and honey bees as a eusocial species. Taken together, temperature plays several important roles in the honey bee colony that range a developmental to the entrainment of circadian rhythms. Future experiments should not ignore temperature conditions since as shown in this and previous studies, environmental temperature can have major consequences on honey bee behavior.

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