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RESEARCH ARTICLE

Cues for cavity nesters: investigating relevant zeitgebers for emerging leafcutting bees, *Megachile rotundata*

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ABSTRACT

Photoperiod is considered the universal zeitgeber, regulating physiological processes in numerous animals. However, for animals in light-restricted habitats (e.g. burrows or cavities), thermoperiod may be a more important cue. Our study tested this hypothesis in the alfalfa leafcutting bee, *Megachile rotundata*, which nests in cavities and undergoes development within a brood cell. We assessed the role of environmental cues (thermoperiod and photoperiod) on the process of adult emergence by examining: (1) whether those cues direct circadian rhythms, (2) which cue is more dominant and (3) how sensitive developing bees and emergence-ready adults are to cues. Although we found that 20% of light penetrates the brood cell, and bees respond to photoperiod by synchronizing emergence, thermoperiod is the dominant cue. When presented with a conflicting zeitgeber, bees entrained to the thermophase instead of the photophase. When temperature cues were removed, we observed free-running of emergence, indicating that underlying circadian mechanisms can be synchronized by daily fluctuations in temperature. We also found that emerging bees were highly sensitive to even small increases in temperature, entraining to a ramp speed of 0.33°C h^{-1}. The response and sensitivity to temperature cues suggest that *M. rotundata* evolved a temperature-mediated clock to time emergence from light-restricted cavities.

KEY WORDS: Circadian rhythm, Adult emergence, Periodism, Insect

INTRODUCTION

Circadian rhythms are ubiquitous among organisms and serve to synchronize their biological processes to daily fluctuations in the environment. Circadian systems require an input (stimulus) to regulate the timing of an output (behavior, physiology). Circadian rhythms are mediated by a cue or zeitgeber (which literally translates as 'time giver') that resets molecular feedback loops, referred to as clocks. Biological rhythms are considered to be under circadian control, if they: (1) are entrainable by a zeitgeber, (2) have a free-running period of approximately 24 h in constant conditions and (3) are temperature compensated. Photoperiod has been referred to as the 'universal zeitgeber' because of the role it plays in mediating circadian rhythms across many taxa (reviewed by Aschoff, 1965; Wehr, 2001; Saunders, 2012). However, organisms that develop or reside in light-restricted habitats may need to rely on other cues to synchronize their daily rhythms with the environment. For example, thermoperiods mediate 'spring awakening' or valve opening under the sediment in oysters, *Crassostrea virginica* (Comeau, 2014). The overall increasing temperatures of spring regulate circannual emergence in many turtle species that hibernate under the sediment (Costanzo et al., 2008; Crawford, 1991; Feaga and Haas, 2015). However, far less is known about the role of temperature compared with the role of light in regulating the timing of life history events.

A well-known phenomenon controlled by circadian rhythms in many insect species is the timing of eclosion, which is the emergence of the adult from the pupal cuticle (Miyazaki et al., 2016; Short et al., 2016; Kikukawa et al., 2013; Dolezel et al., 2008). Many studies have identified photoperiod as the critical cue in synchronizing eclosion (Pittendrigh et al., 1959; Pittendrigh and Minis, 1964; Smith, 1985; Kumar et al., 2007; Umadevi et al., 2009; Guo and Qin, 2010; Thöming and Saucke, 2011; Wu et al., 2014; Yadav et al., 2014). However, because insects pupate in diverse habitats, the sensitivity to a particular zeitgeber is expected to vary depending on where an insect pupates. For example, photoperiod may not be the best cue for synchronizing with the environment if an insect develops in a light-restricted environment. Some data support this hypothesis in insects that pupate below ground, where thermoperiod cues were shown to regulate emergence (Zdarek and Denlinger, 1995; Short et al., 2016; Miyazaki et al., 2011; Watari and Tanaka, 2014; Greenberg et al., 2006). Insects that pupate in other types of light-restricted habitats such as nests, natural or artificial cavities, and brood cells may also rely on thermoperiod cues. Many hymenopterans pupate in brood cells, structures that can be made out of many materials, including leaves, specific soils, and glandular secretions from the mother or developing larva (Klostermeyer and Gerber, 1969; Gupta et al., 2004). Thus, insects emerging from these environments may be more sensitive to cues other than photoperiod. In short, consideration of insect life history is important to understanding how sensitivity to different zeitgebers evolved.

Solitary bees nest in light-restricted habitats, in below- or above-ground cavities, where thermoperiod may be an important cue because of the lack of light. One thing that differs between Hymenoptera and other insect taxa is that adult emergence often occurs several days after eclosion (Danforth, 1999; Kemp and Bosch, 2000; Yocum et al., 2015; Reznik et al., 2008; Bertossa et al., 2010). For example, adult bees shed their pupal cuticle (eclosion) and then remain in place for several days before exiting the brood cell (emergence). Little is known about circadian regulation of adult emergence in comparison to studies on eclosion. The distinction between emergence and eclosion is important because these events can be differentially regulated by environmental cues. For instance, eclosion in parasitic wasps, *Trichogramma embrophagum*, is not rhythmic, but their emergence from the host is regulated by a circadian rhythm (Reznik et al., 2008). We hypothesize that other Hymenoptera may behave similarly to *T. embrophagum*.

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Emergence patterns of solitary bees have been studied over periods of days and months (Rust, 2007; Danforth, 1999; Vinchesi et al., 2013; White et al., 2009) but it is unclear what cues mediate more fine-scale hourly and daily rhythms. A previous study showed that small thermoperiods synchronized emergence from brood cells in the alfalfa leafcutting bee, *Megachile rotundata* (Yocum et al., 2015), although it is unknown whether the response to thermoperiod was under circadian control or whether it was a dominant zeitgeber. Furthermore, emerging *M. rotundata* did not respond to a pulse of light (Tweedy and Stephen, 1971), but it is unknown whether *M. rotundata* respond to photoperiod.

In this study, we used the alfalfa leafcutting bee, *M. rotundata*, to test the hypothesis that circadian regulation of emergence from light-restricted environments is regulated by thermoperiod cues. We chose *M. rotundata* to study these questions because they develop in a brood cell and are readily available because of their management for pollination services (Pitts-Singer and Cane, 2011). We measured fine-scale patterns of spring emergence using a custom-built automated recording device that allowed us to record the emergence of thousands of bees (Yocum et al., 2015). Using this method, we examined the roles of thermoperiod and photoperiod cues in circadian regulation of emergence and determined which is dominant. Furthermore, we examined the sensitivity of emergence-ready adult bees to environmental cues. We predicted that because of their cavity-nesting life history, light would be significantly buffered and bees would not respond to photoperiod cues. We predicted that emergence rhythms would free-run if the thermoperiod was removed and that thermoperiod is dominant over photoperiod cues, because they are likely to have temperature-mediated clocks.

MATERIALS AND METHODS

Animals and rearing conditions

Bees were purchased from JWM Leafcutters, Inc. (Nampa, ID, USA) as loose brood cells in 2014 and 2016. Thermoperiod removal experiments were conducted in 2014. Thermoperiod switch experiments and ramp speed experiments were conducted in 2016. In all experiments, prepupae were kept at a constant 6°C in darkness for approximately 6 months until development was initiated by placing bees at 29°C. This temperature results in the highest numbers of emerging adult bees and is standard management practice for *M. rotundata* (Kemp and Bosch, 2000; Pitts-Singer and Cane, 2011). Temperature regimes were administered in an environmental chamber (Percival models LT-36VL and I30BLL, Percival Scientific, Perry, IA, USA). Chambers contained Philips 700 full-spectrum fluorescent tubes, rated for 4100 K temperature color output. Light intensity for experiments using photoperiod had an average illuminance of 5880.66±159.43 lx within the environmental chambers.

The Δ4°C thermoperiod had a mean temperature of 29°C and consisted of a cryophase (11 h at 27°C) and thermophase (11 h at 31°C) and two separate 1 h temperature ramping periods (4°C h^{-1} ramp speed). The cryophase ran from 07:00 h to 18:00 h and the thermophase ran from 19:00 h until 06:00 h. The Δ8°C thermoperiod retained a mean temperature of 29°C and consisted of a cryophase (11 h at 25°C) and a thermophase (11 h at 33°C) with 1 h temperature ramping periods (8°C h^{-1} ramp speed). The thermophase ran from 07:00 h to 18:00 h and the cryophase ran from 19:00 h until 06:00 h. Both thermoperiods were administered under complete darkness, except for the dominant zeitgeber experiment. In all experiments using photoperiod, we measured the increase in temperature due to light and programmed the incubator to compensate for this increase and reduce the possibility of temperature fluctuations. Furthermore, we used Percival model LT-36VL, which has fluorescent bulbs on the external sides of the incubator, to reduce heat production from the lights. Together, these measures ensured that any responses we observed were not due to a temperature increase when the lights were turned on.

Monitoring emergence

Emergence was monitored using a modified Watari apparatus (Watari and Tanaka, 2010; Yocum et al., 2016), positioned inside an environmental chamber (models PCG-105 and I30BLL, Percival Scientific). A single loose brood cell containing a prepupa was placed in a 0.5 ml microcentrifuge tube (Fisher Scientific, Pittsburgh, PA, USA) with the cap cut off. The microcentrifuge tubes were held in place by plastic racks which were designed using SketchUp® (Trimble Inc., Sunnyvale, CA, USA) software and 3D printed (Lulzbot, Aleph Objects, CO, USA). On top of the brood cell, a 6 mm plastic ball (Softair, Grapevine, TX, USA) and a 4.5 mm steel ball (Copperhead, Crosman, NY, USA) were loaded into the tubes. A cover was placed over the loaded tubes, with holes sized so that the plastic ball would block the escape of the bee. When a bee emerged, it pushed the plastic ball, which in turn pushed the steel ball forward, rolling free from the tube racks down a runway. The steel ball passed through a 5 mm infrared emitter and detector pair (Lite-on Electronics, Inc., Milpitas, CA, USA), recording the date and time of emergence. The apparatus was controlled by an Arduino Nano board (Sparkfun Electronics, Boulder, CO, USA). The temperature (±2°C) and humidity were recorded every 60 s using a DHT11 sensor (Adafruit, New York, NY, USA).

Light penetrance of brood cell

To determine whether light can penetrate the brood cell, we used a MK350 spectrometer (UPRtek, Ikan Corporation, Houston, TX, USA) to measure light intensity (±5%) outside versus inside the brood cell. Because *M. rotundata* nest inside a cavity (Fig. 1), it was difficult to measure light inside the nest; therefore, we deconstructed nest cells to measure how much light penetrates a single brood cell. Isolated wavelengths were administered using ultraviolet (400 nm), blue (470 nm), green (525 nm), yellow (588 nm) and red (630 nm) LEDs (Super Bright LEDs Inc.,

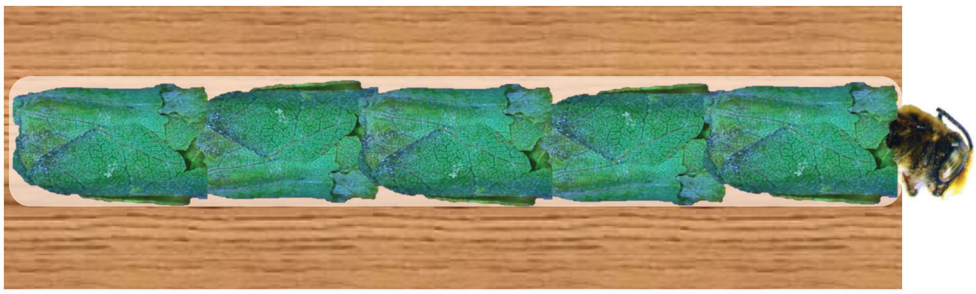

Fig. 1. A cartoon depiction of a male *Megachile rotundata* emerging from a nest inside a cavity. Brood cells are inside a wooden cavity and are made from leaves.

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St Louis, MO, USA). Light penetrance measurements were carried out inside a dark walk-in incubator to eliminate external light. An adapter made from a 6 ml syringe wrapped in black electrical tape was fitted around the aperture of the spectrometer. The back end of the brood cell (not the cap) was cut to fit snugly over the syringe head (adapter). Brood cells were haphazardly chosen from a 24-well plate and used for LED measurements. Any that were damaged while removing them from the adapter were discarded from the analysis. The average illuminance (lux) was measured for each brood cell at each wavelength of light, before and after the brood cell was placed on the adapter. The percent light intensity was calculated from the mean difference before and after the brood cell was placed on the adapter.

Circadian experiments

Photoperiod removal

To determine whether photoperiod affected the circadian regulation of emergence, a long-day photoperiod of 16 h light:8 h dark was applied until approximately 100–200 bees emerged, after which they were exposed to constant 29°C in complete darkness for the remainder of emergence. During the days of emergence with a photoperiod, lights were turned on from 07:00 h until 23:00 h.

Thermoperiod removal

To determine whether thermoperiod can regulate emergence via circadian rhythm, bees were exposed to a Δ4°C or Δ8°C thermoperiod until approximately 100–200 bees emerged, then the thermoperiod was removed, and bees were exposed to constant 29°C for the remainder of emergence.

Conflicting (dominant) zeitgeber

To determine whether photoperiod or thermoperiod was dominant, cues were decoupled (Pittendrigh and Minis, 1964), which is generally referred to as a conflicting zeitgeber experiment (Watari and Tanaka, 2010; Short et al., 2016). Bees were exposed to a Δ4°C thermoperiod, although the lights were turned on during the cryophase and turned off during the thermophase. The photoperiod was a 12 h light:12 h dark cycle with the lights turned on from 07:00 h until 19:00 h.

Sensitivity experiments

Emergence-ready bees: photoperiod response

To determine whether emergence-ready bees respond to light, bees were exposed to constant 29°C in complete darkness until approximately 100–200 bees emerged, then a long-day photoperiod of 16 h light:8 h dark was applied for the remainder of emergence. During the days of emergence with a photoperiod, lights were turned on from 07:00 h until 23:00 h.

Emergence-ready bees: thermoperiod response

To determine whether emergence-ready bees respond to thermoperiod, bees were exposed to 29°C until approximately 100–200 bees emerged, and then were switched to the Δ4°C thermoperiod for the remainder of the emergence period. The constant 29°C represents the control for comparison of the mean time of emergence with that for the Δ4°C thermoperiod.

Emergence-ready bees: thermoperiod sensitivity

To determine whether emergence-ready bees were sensitive to a change in thermoperiod amplitude, we exposed bees to a Δ4°C thermoperiod until approximately 200 bees emerged, then switched to a Δ8°C thermoperiod for the remainder of emergence.

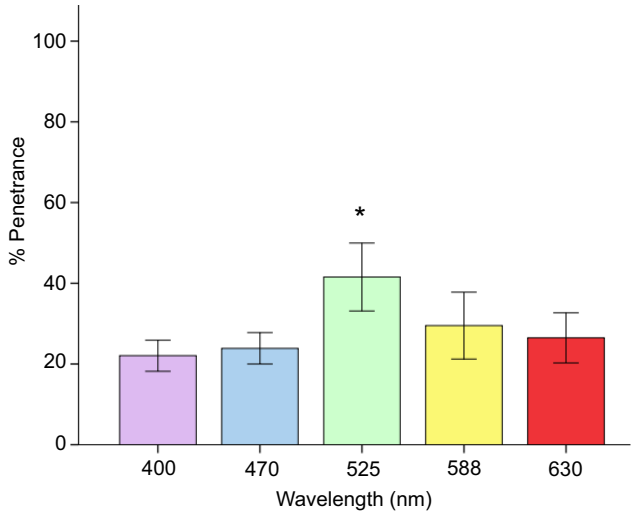

Fig. 2. Percent penetrance of light wavelengths through the brood cell with cocoon intact. Wavelengths (indicated by colored bars) were: ultraviolet (400 nm), blue (470 nm), green (525 nm), yellow (588 nm) and red (630 nm). *Significant difference from penetrance of other wavelengths (P<0.05).

Sensitivity to ramp speed

The slow ramp speed experiment had a ramp speed of 0.33°C h^{-1} over 12 h. The ramp to the thermophase started at 07:00 h and reached peak temperature (31°C) at 19:00 h, then immediately ramped down to the cryophase (27°C) at 20:00 h. The fast ramp speed experiment had a steep ramp speed of 4°C h^{-1}. The ramp to the thermophase started at 07:00 h and reached peak temperature (31°C) by 08:00 h. After reaching 31°C, the temperature was decreased by 0.33°C h^{-1} until it reached the cryophase temperature of 27°C at 20:00 h.

Statistical analysis

Circular statistics were used to determine whether emergence was synchronous or distributed uniformly around the clock. Emergence times collected on a 24 h clock (h:min:s), were first converted to angular measurements. To obtain meaningful descriptive statistics for circular data, angular data were transformed to rectangular polar coordinates. This allows calculation of the circular mean, which yields better representation of the data. For example, the circular mean of 359 deg (just before midnight) and 1 deg (just after midnight) is 0 deg (midnight), rather than 180 deg (noon), which would be the simple arithmetic mean. Circular ANOVA was used to determine whether the mean time of emergence was different before and after zeitgebers were switched in an experiment.

We tested the hypothesis of uniformly distributed circular data using Rayleigh's test for uniformity. This test is based on the mean resultant vector, rbar, which ranges from 0 to 1. When data are

Table 1. Descriptive statistics for light penetrance of the brood cell

		Illuminance (lx)		
Wavelength	n	Ambient	Brood cell	% Penetrance
Ultraviolet, 400 nm	8	20.5±0.83	4.5±0.428	25.52±1.932
Blue, 470 nm	16	261.86±4.14	61.6±4.73	23.52±1.896
Green, 525 nm	10	41.9±1.15	17.2±1.5	41.05±4.209
Yellow, 588 nm	14	110.8±1.34	32.33±4.3	29.18±4.147
Red, 630 nm	16	120.81±1.73	32.125±3.8	26.59±3.110

Illuminance and penetrance data are means±s.e.m.

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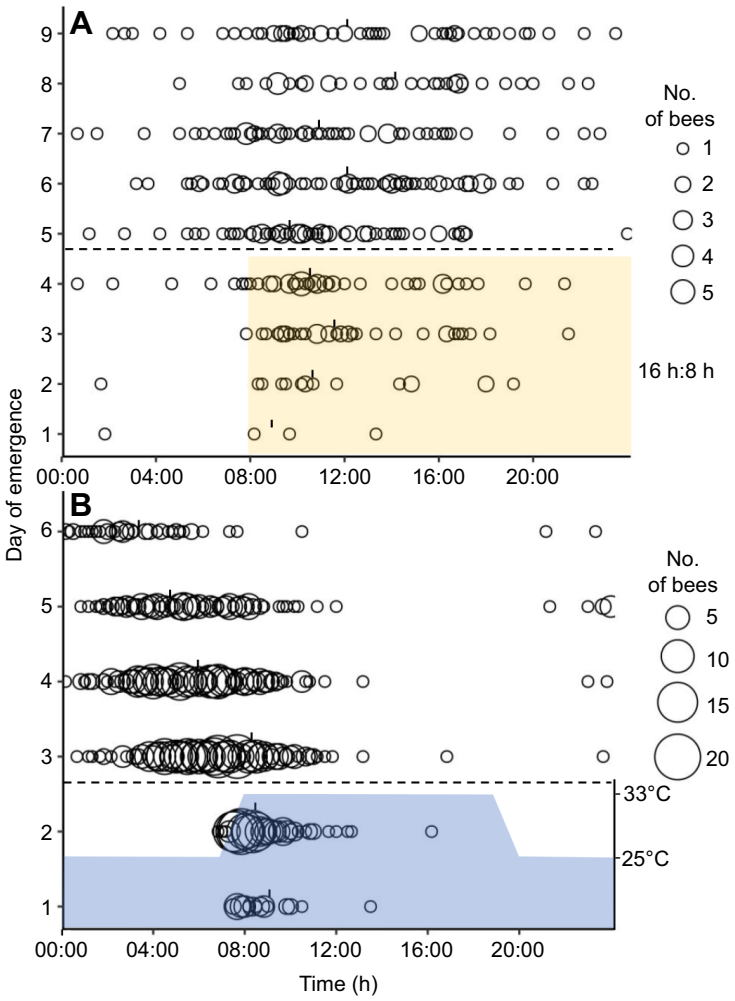

Fig. 3. Number of emerging bees for the photoperiod removal experiment and Δ8°C thermoperiod removal experiment. (A) For the photoperiod removal experiment, emergence patterns are displayed for a 16 h light:8 h dark photoperiod and constant 29°C (yellow area, below dashed line) and after photoperiod removal, in constant conditions (above dashed line). (B) Emergence patterns of *M. rotundata* with the Δ8°C thermoperiod (below dashed line) and after thermoperiod removal, in constant conditions (above dashed line). The blue shaded area is the Δ8°C thermoperiod, with temperature marked on the secondary *y*-axis, which was administered each day but is shown here to indicate the timing of the temperature ramps. The vertical bars display the circular mean time of emergence for each day. The size of the bubbles indicates the number of bees emerging during 15 min time intervals.

uniformly distributed, the mean resultant vector is expected to be close to zero, and when the data are strongly unimodal, rbar will be close to 1. rbar was converted to Rayleigh's z ($z=n\times\text{rbar}^2$), which follows a χ^2 distribution and yields P-values for the test of uniformity. Because rbar has a standard range and is more interpretable by itself, we have provided rbar as the test statistic for Rayleigh's test with the P-value coming from Rayleigh's z (Fisher, 1993). For several of the experiments where we were interested in testing the null hypothesis of common directional means, we used the high-concentration F-test (Mardia and Jupp, 2000). We used the circSASv1 SAS macros to calculate all circular statistics (http://statweb.calpoly.edu/ulund).

Table 2. Circular test statistics for zeitgeber experiments

Temperature treatment	Time (h:min:s)			*R*-value	Rayleigh test (rbar), *P*-value
	Circular mean	s.d.	Median		
Circadian experiments					
Long day, 29°C constant	11:22:39	03:47:20	10:41:59	39.18	0.6114, $P<0.0001$
29°C constant	11:12:38	04:26:40	10:54:31	53.57	0.5086, $P<0.0001$
4°C thermoperiod	08:56:28	01:09:51	08:50:33	0.82	0.95463, $P<0.0001$
29°C constant	06:47:35	02:29:52	06:51:30	13.97	0.80752, $P<0.0001$
8°C thermoperiod	08:27:44	01:08:49	08:12:38	0.47	0.95592, $P<0.0001$
29°C constant	05:41:03	02:29:12	05:48:12	11.78	0.80905, $P<0.0001$
Conflicting zeitgeber	20:43:59	01:59:18	20:31:04	6.73	0.87330, $P<0.0001$
Sensitivity experiments					
4°C thermoperiod	08:09:14	01:13:20	08:02:14	2.00	0.95009, $P<0.0001$
8°C thermoperiod	08:29:11	01:13:23	08:07:44	1.35	0.95003, $P<0.0001$
29°C constant	21:09:02	08:50:08	13:18:47	153.48	0.1753, $P>0.05$
Long day, 29°C constant	06:28:09	03:13:27	07:06:29	46.75	0.7003, $P<0.0001$
29°C constant	02:21:25	08:57:29	13:51:05	131.81	0.06393, $P>0.05$
4°C thermoperiod	07:14:28	02:29:41	07:41:15	30.46	0.80794, $P<0.0001$
Slow ramp speed	06:27:51	03:44:03	08:02:41	35.30	0.62012, $P<0.0001$
Fast ramp speed	09:00:33	01:49:53	08:53:43	6.04	0.89143, $P<0.0001$

Long-day conditions were 16 h light:8 h dark.

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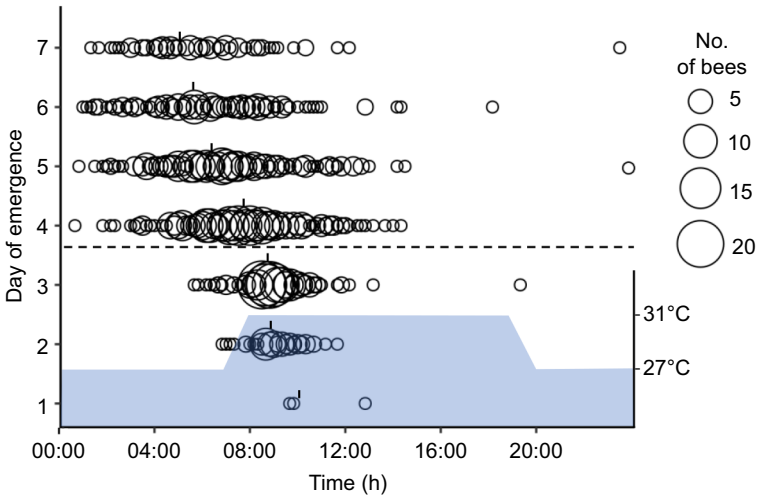

Fig. 4. Number of emerging bees and mean time of emergence for Δ4°C thermoperiod removal experiment. Emergence patterns of *M. rotundata* with the Δ4°C thermoperiod (below dashed line) and after thermoperiod removal, in constant 29°C conditions (above dashed line). The blue shaded area is the Δ4°C thermoperiod, displayed to show temperature marked on the secondary *y*-axis, which was administered each day but is shown here to indicate the timing of temperature ramps. The size of the bubbles indicates the number of bees emerging during 15 min time intervals.

In addition to the Rayleigh test for uniformity, the parameter R was calculated to measure the degree of rhythmicity in emergence (Winfree, 1970; Watari and Tanaka, 2010; Short et al., 2016). The parameter R is a scalar statistic that identifies whether emergence is rhythmic or arrhythmic by calculating the highest number of emerging adults in an 8 h gate then dividing this number by the number of adults emerging outside the 8 h gate, multiplied by 100. All individuals that emerged were pooled to calculate the number of emerging adults for each hour of the day. The theoretical range of parameter R is from 0, if all emergence occurs within the gate, to 200, if emergence is distributed uniformly throughout the day (Winfree, 1970). $R<60$ is considered to show rhythmic emergence, $60<R<90$ is weakly rhythmic and $R>90$ is arrhythmic; $R>150$ indicates uniform distribution of emergence (Winfree, 1970).

RESULTS

How much light penetrates the brood cell?

Green wavelength penetrance was significantly different from that of all other wavelengths (Fig. 2; ANOVA $F_{4,66}=4.433$, $P<0.05$). Just over 40% of green light passed through the brood cell, while only 26% of light from other wavelengths passed through (Fig. 2, Table 1).

Circadian experiments

Does photoperiod interact with the circadian system?

Bees were exposed to a long-day photoperiod (16 h light:8 h dark) at constant 29°C for the first 4 days of emergence ($n=105$), then the photoperiod was removed and bees were in constant darkness for the remainder of emergence ($n=302$). Emergence was rhythmic ($R=39.18$, rbar=0.6114, $P<0.0001$) during the photoperiod and when photoperiod was removed, emergence remained rhythmic (Fig. 3A; $R=53.57$, rbar=0.5086, $P<0.0001$). The mean time of emergence during the photoperiod was not statistically different from the mean time of emergence when the photoperiod was removed (Table 2; circular ANOVA, $F_{1,406}=0.0454$, $P=0.80855$).

Does thermoperiod interact with the circadian system

In the Δ8°C thermoperiod-removal experiment, emerging bees were exposed to the Δ8°C thermoperiod for the first 3 days of emergence

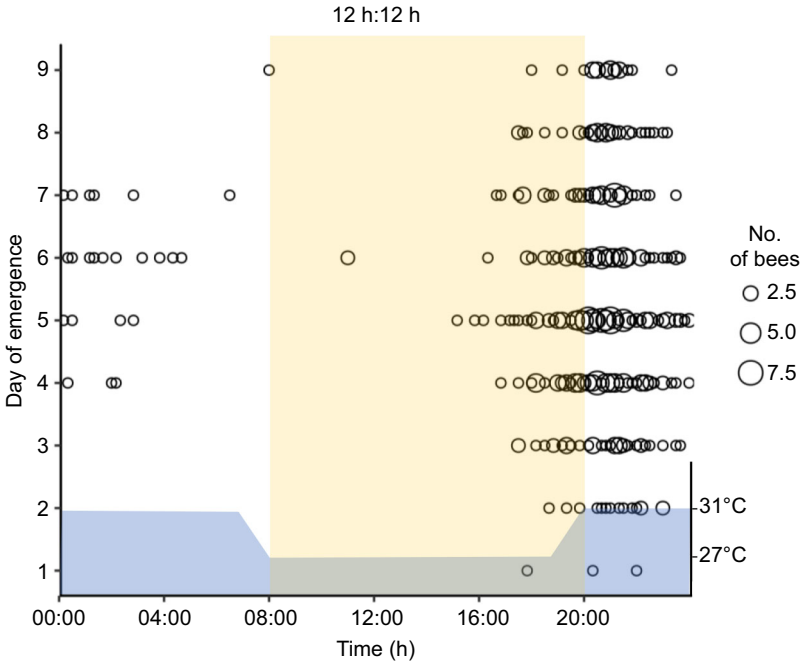

Fig. 5. Number of emerging bees for the conflicting zeitgeber experiment. The thermoperiod ramps are shown by the blue shaded area, with a cryophase ramp from 07:00 h to 08:00 h and a thermophase ramp from 19:00 h to 20:00 h. The photoperiod was a 12 h light:12 h dark cycle where the lights were turned on at 07:00 h and turned off at 19:00 h, as shown by the yellow area. The size of the bubbles indicates the number of bees emerging during 15 min time intervals.

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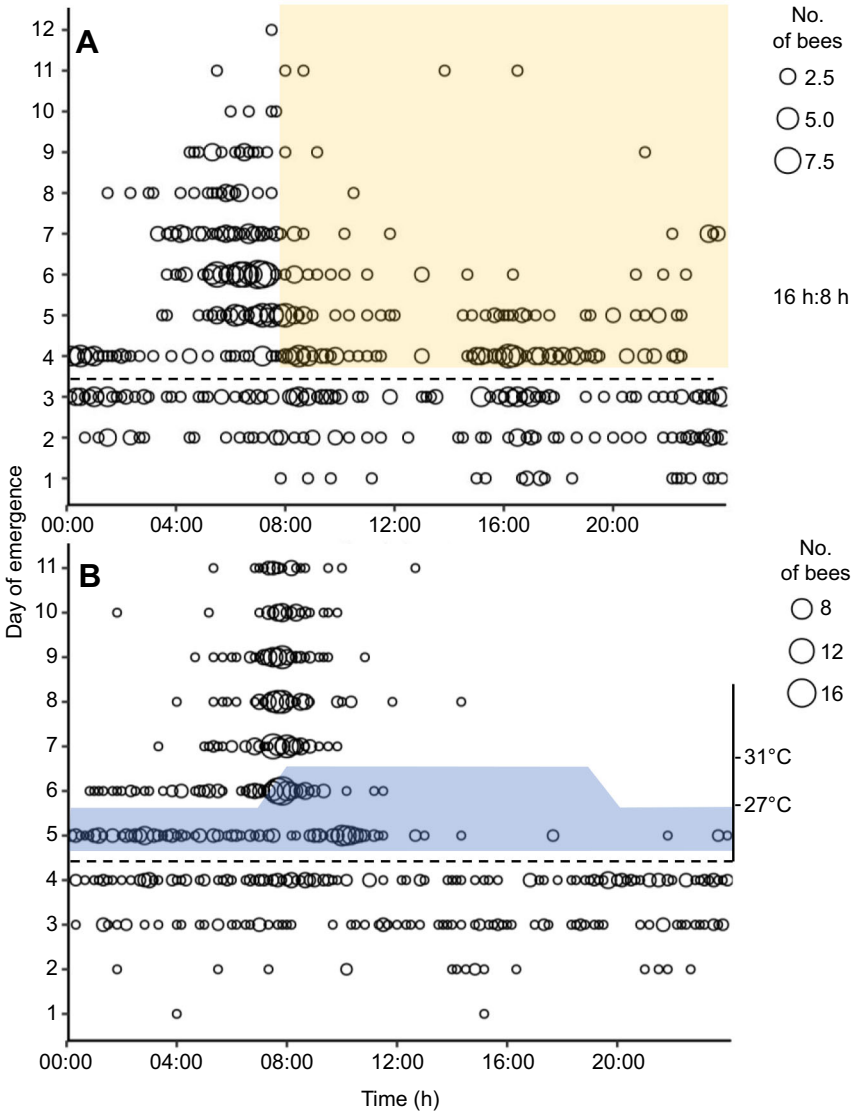

Fig. 6. Number of emergence-ready bees responding to photoperiod and thermoperiod. (A) For the photoperiod response experiment, emergence patterns are displayed under constant dark conditions (below dashed line) and after photoperiod initiation (16 h light:8 h dark) and constant 29°C (yellow area, above dashed line). (B) Emergence patterns of *M. rotundata* under constant 29°C conditions (below dashed line) and after thermoperiod initiation (Δ4°C thermoperiod, above dashed line). The blue shaded area is the Δ4°C thermoperiod, displayed to show temperature marked on the secondary *y*-axis, which was administered each day but is shown here to indicate the timing of the temperature ramps. The vertical bars display the circular mean time of emergence for each day. The size of the bubbles indicates the number of bees emerging during 15 min time intervals.

(*n*=210), then the thermoperiod was removed, and remaining bees were exposed to a constant 29°C (*n*=854). Emerging bees maintained synchronicity after the Δ8°C thermoperiod was removed (Fig. 3B). The Rayleigh tests indicated directional distribution during the Δ8°C thermoperiod (*R*=0.478, rbar=0.9559, *P*<0.0001) and when the thermoperiod was removed (*R*=11.78, rbar=0.8091, *P*<0.0001). The mean time of emergence was statistically different between constant 29°C and Δ8°C thermoperiod temperature regimes (circular ANOVA $F_{1,1063}$=247.351, *P*<0.0001). The mean time of emergence differed significantly by day after the switch (Table 2; circular ANOVA, $F_{10,1063}$=46.5802, *P*<0.0001).

Bees were exposed to a Δ4°C thermoperiod for the first 3 days (*n*=243), at which point the thermoperiod was removed and the remaining bees were exposed to constant 29°C (*n*=791). The Rayleigh test indicated synchronous emergence during the Δ4°C thermoperiod (Fig. 4; *R*=0.8290, rbar=0.9546, *P*<0.0001), which remained when the thermoperiod was removed at constant 29°C (*R*=13.97, rbar=0.8075, *P*<0.0001). Bees exposed to the Δ4°C thermoperiod emerged earlier than when the thermoperiod was removed (circular ANOVA, $F_{1,1033}$=168.207, *P*<0.0001). The mean time of emergence significantly differed by day after the switch (Table 2; circular ANOVA, $F_{12,1033}$=22.404, *P*<0.0001).

Which cue is the dominant zeitgeber?

Bees were exposed to a Δ4°C thermoperiod and a long-day photoperiod, with the thermophase occurring during the start of the dark phase. Emergence was rhythmic, coinciding with the approximate start of the thermophase (Fig. 5, Table 2; *R*=6.73, rbar=0.8733, *P*<0.0001).

Sensitivity experiments

Do emergence-ready bees respond to photoperiod?

Bees were exposed to constant 29°C in darkness for the first 4 days (*n*=218), at which point a long-day (16 h light:8 h dark) photoperiod was initiated (*n*=443). Emergence was uniform in darkness (Fig. 6A; *R*=153.48, rbar=0.1753, *P*>0.05), but after the photoperiod was initiated, emergence was rhythmic (*R*=46.75, rbar=0.7003, *P*<0.0001). Mean emergence time was significantly different before and after bees were exposed to a photoperiod (Table 2; circular ANOVA, $F_{1,660}$=67.25, *P*<0.0001). Once the photoperiod was initiated, the circular mean time of emergence shifted earlier to 06:28:09±03:13:27 h:min:s (median 07:06:29 h:min:s).

Do emergence-ready bees respond to thermoperiod?

Emerging bees that initially had no thermoperiod were allowed to emerge for 3 days (constant 29°C; *n*=102) and then the remaining

emerging bees were exposed to a Δ4°C thermoperiod (n=668). Emergence was uniform (R=131.81, rbar=0.0639, P>0.05) at constant 29°C. Once the Δ4°C thermoperiod was initiated, emergence was synchronous (Fig. 6B; R=30.46, rbar=0.8079, P<0.01). The R-value on the day after the thermoperiod was initiated was <60 (R=58.62), indicating synchronous emergence when first exposed to the zeitgeber. Bees exposed to the Δ4°C thermoperiod emerged earlier in the day (mean emergence time) than bees exposed to constant 29°C (Table 2; circular ANOVA, $F_{1,769}$=25.4330, P<0.0001).

Are emergence-ready bees sensitive to a thermoperiod switch?

To determine whether bees distinguish between slight variations in thermoperiod, emerging bees were exposed to a Δ4°C thermoperiod for 3 days (n=225), then switched to a Δ8°C thermoperiod for the remainder of emergence (n=559). Emerging bees entrained to both thermoperiods throughout adult emergence. The Rayleigh test supported directional distribution (R=2.00, rbar=0.9501, P<0.01) during the Δ4°C thermoperiod, indicating synchronous emergence. During the Δ8°C thermoperiod, emergence was synchronous (Fig. 7; R=1.35, rbar=0.9500, P<0.0001). Bees in the Δ8°C thermoperiod emerged later than bees exposed to the Δ4°C thermoperiod (Table 2; circular ANOVA, $F_{1,733}$=11.2264, P<0.0001).

How sensitive are developing bees to temperature ramps?

To determine how sensitive emerging bees are to changes in temperature, pupating bees were exposed to slow or fast ramps in the thermophase (0.33°C h^{-1} or 4°C h^{-1}). In the fast ramp experiments (n=686), emergence was synchronous (Fig. 8A; R=6.04, rbar=0.8914, P<0.0001). Mean time of emergence was 09:00:33 h:min:s (s.d., 01:49:53 h:min:s), which coincides with the end of the ramp period of the thermophase (Table 2). During the slow ramp experiment (n=536), emergence was synchronous (Fig. 8A; R=35.30, rbar=0.6201, P<0.0001). Mean time of emergence was 06:27:51 h min s (s.d., 03:44:03 h:min:s), coinciding with just before the ramp period of the thermophase.

DISCUSSION

Our data strongly support the hypothesis that thermoperiod is an important environmental cue for synchronizing emergence of adult *M. rotundata*. Because we observed free running of emergence rhythms in constant conditions after exposure to a zeitgeber, we have shown for the first time that thermoperiod regulates circadian rhythm of emergence in *M. rotundata*. We also showed for the first time that the brood cell buffered many wavelengths of light by approximately 80%, suggesting that the brood cell is an important modulator of environmental cues. Even though light is buffered, *M. rotundata* clearly responded to photoperiod cues as emergence-ready adults. Interestingly, they may not be as sensitive to photoperiod cues as they are to thermoperiod cues, because we observed no evidence of free running after photoperiod removal. Furthermore, when exposed to a conflicting zeitgeber, emerging bees entrained to the thermophase instead of the photophase, indicating that temperature cues are dominant to light cues. Notably, we observed entrainment to the slow ramp speed to the thermophase of 0.33°C h^{-1}, alluding to the sensitivity of temperature-mediated clocks in *M. rotundata*. These data support the hypothesis that insects that develop in light-restricted environments may rely on other cues for timing of development and emergence.

Circadian rhythms and zeitgebers

One way to identify circadian regulation of a process is to expose organisms to a stimulus and then remove it to observe the presence of free-running periods (Saunders, 2012, 2013). We observed evidence of free running when we removed the thermoperiod, but not the photoperiod. This suggests that photoperiod may be a weaker cue than thermoperiod. Our finding that thermoperiod entrained emergence is consistent with other studies. For example, free-running eclosion rhythms occur in the flesh fly *Sarcophaga crassipalpis* (Miyazaki et al., 2011) and in the onion fly, *Delia antiqua* (Miyazaki et al., 2016). In the current study, the Δ4°C and Δ8°C thermoperiod removal experiments showed free-running periods. This is strong evidence that temperature-mediated clocks are involved in regulating the emergence of *M. rotundata*.

Two general models have been proposed to describe the mechanisms underlying circadian rhythms for emergence in insects: the single-oscillator model and the two-oscillator model (reviewed in Saunders, 2012). The two-oscillator model (morning

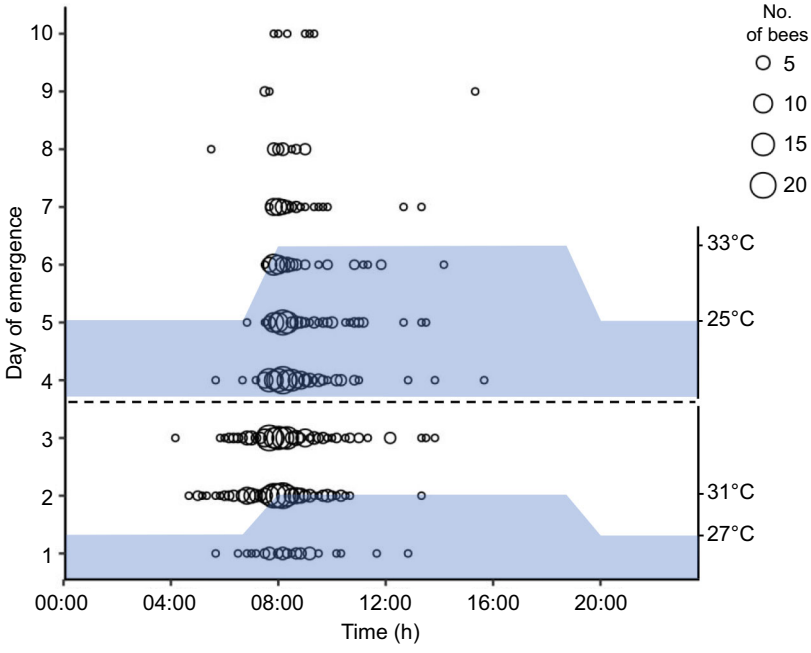

Fig. 7. Number of emerging bees and mean time of emergence for the Δ8°C thermoperiod switch experiment. Emergence patterns of *M. rotundata* under the Δ4°C thermoperiod (below dashed line) and after the switch to the Δ8°C thermoperiod (above dashed line). The blue shaded areas are the temperature treatments, which were administered each day but are displayed here to show the timing of temperature ramps. Temperatures are shown on the secondary *y*-axis. The size of the bubbles indicates the number of bees emerging during 15 min time intervals.

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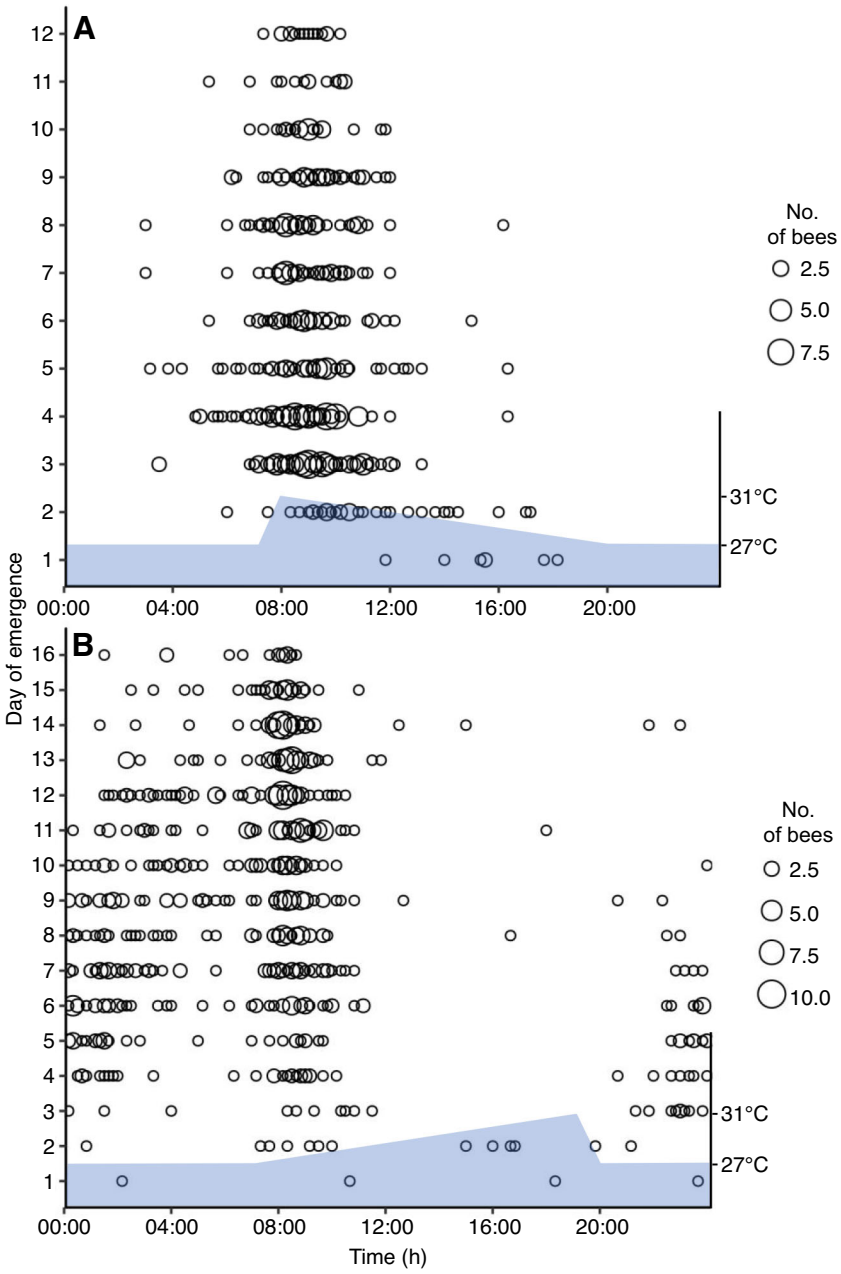

Fig. 8. Number of emerging bees and mean time of emergence for the ramp speed experiments. Emergence patterns from (A) the fast ramp speed (4°C h^{-1}) experiment and (B) the slow ramp speed (0.33°C h^{-1}) experiment. The blue shaded areas indicate the temperature treatment administered each day but displayed here to show the timing of temperature ramps. The size of the bubbles is relative to the number of bees emerging during the 15 min time intervals.

and evening oscillators) is proposed for organisms that use both temperature and light cues to mediate emergence, such as in the onion fly, *D. antiqua*, in which eclosion rhythm is affected by the interacting effect of light and temperature (Watari and Tanaka, 2010). Single oscillators are reset by one zeitgeber, irrespective of other cues. It is worth noting that a single-oscillator model includes multiple oscillators – they are just so tightly coupled that they act as a single unit (Wirz-Justice et al., 2003). We found that thermoperiod overrides the photoperiod cue in *M. rotundata*, supporting the single-oscillator model. More experiments need to be conducted to determine the underlying mechanisms mediating the timing of emergence in *M. rotundata*.

To test the relative strength of zeitgebers on circadian rhythms, one must decouple the phases of the cue (Pittendrigh and Minis, 1964). These types of experiments are called 'conflicting zeitgebers', and they can reveal which zeitgeber is more dominant (Sharma and Chandrashekaran, 2005; Short et al., 2016; Watari and Tanaka, 2010). Conflicting zeitgebers occur when cues have different phases, such as the photophase occurring during the cryophase of a thermoperiod. When we exposed *M. rotundata* to these conditions, our hypothesis was supported because they entrained to the thermophase instead of the photophase. This result is evidence that thermoperiod may be the more dominant cue than photoperiod. Our results are comparable to a study on the flesh fly *S. crassipalpis*, which pupates under the soil, where it entrains to the thermophase of the thermoperiod instead of the photophase of the photoperiod (Short et al., 2016). Complex interactions can exist between the relative timing of photoperiod and thermoperiod phases in mediating insect emergence. For example, the timing and amplitude of a thermoperiod affects whether onion flies, *D. antiqua*, entrain to a thermoperiod or photoperiod zeitgeber (Watari and Tanaka, 2010). Thus, we are interested in further investigating the interactions between light and temperature cues for mediating emergence of *M. rotundata*.

Sensitivity to zeitgebers

Sensitivity to photic stimuli depends on the developmental stage when the signal is received (Joplin and Moore, 1999; Yadav et al., 2012; Miyazaki et al., 2011; Kumar et al., 2007). For example, *Drosophila* development rate after the third instar has been shown to be affected by wavelengths of green (500 nm), violet (420 nm) and ultraviolet (380 nm) (Warrick et al., 1999). Interestingly, honey bees, *Apis mellifera*, do not exhibit circadian rhythms in clock gene expression until after adult emergence (reviewed by Moore, 2001). Because the hive environment is kept relatively constant by the colony, and newly emerged adults do not leave the hive, there may not be selection to synchronize development with the environment. Previous work showed that adult *M. rotundata* emergence was unresponsive to light cues if exposed to a light pulse during the pupal and emergence-ready adult stages (Tweedy and Stephen, 1970). However, that study only used a single pulse of light, which may not have been a strong enough cue to synchronize emergence. We showed that emergence-ready adult *M. rotundata* were sensitive to light, a time when they would most likely be receiving light cues in the field. In a nest, emergence-ready adults could receive light cues when the sibling in the nearest nest cell emerges, clearing the way for more light to enter the cavity. However, it is unknown how much light can enter the cavity and whether *M. rotundata* would be sensitive to lower intensities of light than used in this study. Sensitivity to environmental cues may change across the lifetime of *M. rotundata* because they undergo development in a cavity and forage during the daytime.

What is intriguing about temperature-mediated clocks is that thermoperiod is presumably a much more variable cue than photoperiod. Thermoperiod can vary by ramp speed, amplitude and duration of the temperature pulse (Rensing and Ruoff, 2002). Variation in these characteristics can affect sensitivity or responses of insect emergence. For example, thermoperiod amplitude can affect peak eclosion time in some insects (Kikukawa et al., 2013; Miyazaki et al., 2016). We found in the thermoperiod switch experiment that the mean time of emergence was significantly different when switched from a Δ4°C to a Δ8°C thermoperiod. Furthermore, studies have shown that the thermophase is an important characteristic of a thermoperiod for entrainment of insect emergence (Watari and Tanaka, 2010; Yocum et al., 2016). Similar to these studies, we found that emerging bees entrained to the ramp or the beginning of thermophase versus the cryophase across all experiments. In the slow thermophase ramp experiment, which had a 0.33°C h^{-1} ramp speed, adult emergence was synchronized to the start of the thermophase (ca. 07:00 h). This conflicted with our prediction that bees could not entrain to a slow ramp speed and provides evidence that bees are very sensitive to temperature. These results are comparable to those with the tsetse fly *Glossina morsitans*, for which 0.4°C variations in temperature can synchronize eclosion rhythm (Zdarek and Denlinger, 1995). It would be interesting to determine the smallest temperature increase that could synchronize emergence. We found that mean time of emergence changed when switching between different amplitudes of thermoperiods. Examining the effects of natural variation in temperature on circadian regulation of insect emergence is understudied. It is unknown how random temperature variability or natural conditions can affect temperature-mediated emergence of insects.

Why would emergence be synchronized? One possibility for synchronous emergence is to increase fitness. We predict that synchronization in the morning could aid in optimizing the location of resources or mating opportunities. Mating success may rely on entrainment to a thermoperiod cue, synchronizing bees in a population to emerge during the same windows of time. Newly emerged adult *M. rotundata* are immediately in search of food and mates, making it important for them to synchronize with the environment. In none of our experiments did we observe synchronization to the cryophase. This makes sense because temperatures increase in the morning and *M. rotundata* are diurnal, foraging during the daylight. Our results suggest emergence-ready adults are more sensitive to temperature than light cues, but this does not mean that photoperiod-mediated clocks do not exist in *M. rotundata.* Such clocks could be more sensitive at other stages of development or for other biological processes.

This study magnified patterns in emergence using automated data collection to better understand the circadian responses and sensitivity to environmental cues. This tool, and our large sample sizes, allowed us to analyze patterns in emergence with more accuracy. Our data support the fact that temperature-mediated clocks play a role in the emergence of *M. rotundata*. Testing this hypothesis in other hymenopterans and other insect species will be important to determine whether this is a general response of insects that pupate in light-restricted habitats or something specific to this taxon of cavity-nesting bees. Future studies should examine the relationship between light intensity and thermoperiod further. Perhaps a lower mean temperature or brighter light would affect which cue is more dominant for emerging *M. rotundata*. Insects relying on temperature cues may be susceptible to temperature variability due to climate change. Understanding what cues drive circadian rhythms and how these may change for animals living in different habitats will be important for predicting how climate change may affect the life cycle events of not only insects but also the plants they pollinate.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.M.B., J.P.R.; Methodology: M.M.B., J.P.R., G.D.Y., K.J.G.; Software: M.M.B.; Formal analysis: M.M.B., G.D.Y., C.D.; Investigation: M.M.B., J.P.R., K.J.G.; Resources: M.M.B., J.P.R.; Data curation: M.M.B.; Writing - original draft: M.M.B.; Writing - review & editing: M.M.B., J.P.R., G.D.Y., C.D., K.J.G.; Visualization: M.M.B., G.D.Y.; Supervision: J.P.R., G.D.Y., K.J.G.; Project administration: J.P.R., K.J.G.; Funding acquisition: J.P.R., K.J.G.

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