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RESEARCH ARTICLE

Nurse honeybee workers tend capped brood, which does not require feeding, around the clock

Moshe Nagari*, Yafit Brenner and Guy Bloch

ABSTRACT

'Nurse' honeybees tend brood around the clock with attenuated or no circadian rhythms, but the brood signals inducing this behavior remain elusive. We first tested the hypothesis that worker circadian rhythms are regulated by brood pheromones. We monitored locomotor activity of individually isolated nurse bees that were exposed to either various doses of larval extract or synthetic brood ester pheromone (BEP). Bees orally treated with larval extract showed attenuated circadian rhythms in one of four tested colonies; a similar but statistically non-significant trend was seen in two additional colonies. Nurse bees treated with synthetic BEP showed rhythm attenuation in one of three tested colonies. Next, we tested the hypothesis that capped brood, which does not require feeding, nevertheless induces around-the-clock activity in nurses. By combining a new protocol that enables brood care by individually isolated nurse bees, detailed behavioral observations and automatic high-resolution monitoring of locomotor activity, we found that isolated nurses tended capped brood around the clock with attenuated circadian rhythms. Bees individually isolated in similar cages but without brood showed strong circadian rhythms in locomotor activity and rest. This study shows for the first time that the need to feed hungry larvae is not the only factor accounting for around-the-clock activity in nurse bees. Our results further suggest that the transition between activity with and without circadian rhythms is not a simple switch triggered by brood pheromones. Around-the-clock tending may enhance brood development and health in multiple ways that include improved larval feeding, thermoregulation or hygienic behavior.

KEY WORDS: *Apis mellifera*, Social behavior, Division of labor, Circadian, Pheromones, Brood care, Plasticity, Hygienic behavior

INTRODUCTION

Social insect colonies exhibit an elaborate division of labor. Two of the major task specializations seen in insect societies are brood care ('nursing') and foraging for resources. In honeybees, the division of labor relates to worker age (Winston, 1987). Young worker bees (3–14 days of age) typically tend ('nurse') brood inside the dark and thermoregulated hive. Forager worker bees are typically older than 3 weeks of age and perform multiple foraging flights on a single day (Seeley, 1982). Nurses and foragers differ in their behavior, physiology and patterns of gene expression in various tissues (Lattorff and Moritz, 2013). One of the differences between nurses and foragers is in their activity rhythms (reviewed in Bloch, 2010; Eban-Rothschild and Bloch, 2012). Foragers, similar to other diurnal animals, show strong daily rhythms in their behavior – they are active during the daytime and show consolidated bouts of sleep during the night (Bloch, 2010; Eban-Rothschild and Bloch, 2012; Moore, 2001). Consistent with their behavioral rhythms, the abundance of 'clock gene' transcripts in the forager brain exhibits strong daily oscillations (e.g. Bloch et al., 2001, 2004; Rubin et al., 2006; Shemesh et al., 2007, 2010). Microarray analysis further revealed circadian oscillations in whole-brain levels of about 540 transcripts in foragers (Rodriguez-Zas et al., 2012). Foraging bees rely on circadian clocks to precisely time visits to flowers, for time-compensated sun-compass orientation and for waggle-dance communication, which also refers to the sun position (reviewed in Bloch, 2010; Moore, 2001). Nurse bees (and perhaps other nest bees) show a different daily pattern. They are active around the clock with overall similar levels of activity during the day and the night (Crailsheim et al., 1996; Moore et al., 1998; Shemesh et al., 2007, 2010). Despite their around-the-clock activity pattern, young bees and nurses do sleep, but their sleep is more fragmented and distributed throughout the day (Eban-Rothschild and Bloch, 2008, 2015; Klein et al., 2008, 2014). In contrast to foragers, their brain clock gene transcript abundance is similar throughout the day (Shemesh et al., 2007, 2010). In addition, the overall number of oscillating transcripts in their brain is significantly reduced (about 160) relative to that of foragers (Rodriguez-Zas et al., 2012).

Why are nurses active around the clock? Given that the main activity of nurse bees is brood tending, it is reasonable to assume that tending brood needs is important in determining their pattern of activity. Indeed, in colonies manipulated to have only foragers, some of the old foragers revert to nursing brood and switch to activity around the clock with no apparent circadian rhythms (Bloch and Robinson, 2001). Shemesh et al. (2010) further showed that the attenuated behavioral and brain gene-expression rhythms in nurses are induced by direct contact with larvae. The association between care for the young and around-the-clock activity is not limited to honeybee nurses (Bloch et al., 2013). For instance, bumblebee queens (mothers) and workers (nurses) switch to activity around the clock when they have brood (Eban-Rothschild et al., 2011; Yerushalmi et al., 2006). Moreover, the influence of care for the young on patterns of activity may not be specific to social insects. For example, in rodents, maternal physiology during pregnancy is associated with attenuation of circadian rhythms in clock and glucose homeostasis-related genes in the liver and with changes in the circadian patterns of glucocorticoid levels in the brain (Wharfe et al., 2016a,b).

A straightforward hypothesis is that around-the-clock activity in honeybee nurses is due to the need to recurrently feed larvae. The honeybee larva is extremely sensitive to starvation and is known to signal its nutritional status to nurse bees (He et al., 2016; Heimken et al., 2009; Huang and Otis, 1991a,b). However, honeybee workers

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also tend brood past the feeding stage. For example, worker bees cap the cells of 5th instar larvae that have passed the feeding stage (Winston, 1987), heat the capped brood through endothermy (Basile et al., 2008; Bujok et al., 2002; Kleinhenz et al., 2003; Kronenberg and Heller, 1982; Stabentheiner et al., 2010) and maintain brood-nest hygiene by inspecting and removing larvae and capped brood that are dead or diseased (an activity commonly termed 'hygienic behavior'; Evans and Spivak, 2010; Wilson-Rich et al., 2009). It is not known, however, whether these behaviors are performed around the clock.

The evidence that the brood can induce around-the-clock activity in nurses draws specific attention to the nature of the brood signals and cues that modulate plasticity in worker circadian rhythms. Given the pivotal role of chemical communication in the organization and coordination of honeybee societies (reviewed in Alaux et al., 2010; Le Conte and Hefetz, 2008; Trhlin and Rajchard, 2011), pheromones are immediate suspects. Pheromones that are found on the brood cuticle act in concert with the queen pheromones to influence hormonal titers and gene expression to suppress worker ovaries, develop their food-producing glands and affect the pace of their behavioral maturation (reviewed in Alaux et al., 2010). Brood cuticular extracts were shown to induce pollen foraging in honeybee colonies (Pankiw et al., 1998). Some brood chemical cues are volatile and can affect workers in the nest periphery, away from the area in which the brood develops (e.g. E-β-ocimene; He et al., 2016; Maisonnasse et al., 2009, 2010; Traynor et al., 2014, 2015). Others have low volatility and operate by close contact, like glyceryl-1,2-dioleate-3-palmitate (GDP), which induces worker clustering (Koeniger, 1978; Koeniger and Veith, 1983). We had previously shown that the antennae – the main olfaction sensory organs that serve for sensing both volatile and contact chemicals – are involved in mediating the brood effect on plasticity in circadian rhythms in honeybees (Nagari and Bloch, 2012), a finding that is in line with the premise that chemical cues are important.

The best-studied brood pheromone is the brood ester pheromone (BEP) – a contact pheromone, secreted by both larvae and pupae honeybees, which consists of a mixture of 10 fatty methyl or ethyl esters (Le Conte et al., 1990; Trouiller et al., 1991). BEP has profound effects on worker behavior, physiology and gene expression (reviewed in Alaux et al., 2010; Le Conte and Hefetz, 2008). The composition and amount of BEP secreted by the brood signal its sex, developmental status and perhaps level of infection by parasites (Mondet et al., 2016; Trouiller et al., 1991, 1992), enabling nurses to adjust their behavior according to the brood needs (Le Conte et al., 1994). Some of the BEP influences on workers include regulating task-related behavioral development (Le Conte et al., 2001), inhibiting ovarian development (Mohammedi et al., 1998) and stimulating the worker hypopharyngeal glands to produce protein-rich brood food ('jelly'; Pankiw et al., 2004). The BEP blend found on the cuticle of 5th instar larvae induces nurse bees to cap larvae cells (Le Conte et al., 1990, 1994). Given this evidence, BEP is a good candidate for mediating the brood influence on the circadian rhythmicity of brood-tending bees. Its low volatility (Le Conte et al., 1990) fits the findings that close contact with the brood is necessary for inducing attenuated circadian rhythms in nurse bees (Shemesh et al., 2010). A commercially available stabilized synthetic BEP blend (SuperBoost, Contech Inc., Victoria, BC, Canada), which contains approximate proportions of the esters found on the cuticles of old larvae (Pankiw and Page, 2001), was shown to induce pollen foraging and to increase brood rearing in honeybee colonies (Pankiw et al., 2011). Thus, we used brood extracts as well as the commercial BEP blend to test the hypothesis that BEP is involved in mediating the brood effect on circadian rhythms in worker bees. Next, we asked whether only larvae that can benefit from frequent feeding induce around-the-clock activity in nurses. To test this hypothesis, we developed a high-throughput system for monitoring individually isolated nurses and tested how the presence of capped brood, which is not fed, influences activity rhythms in nurse bees.

MATERIALS AND METHODS

General design

We performed the experiments at the Bee Research Facility in the Edmond J. Safra campus of the Hebrew University of Jerusalem, Givat Ram, Jerusalem, Israel. The honeybees (*Apis mellifera* Linnaeus 1758) used in the study represent a mixture of strains typical to Israel. We kept the colonies using standard beekeeping techniques.

To obtain nurse bees, we removed combs with mature capped brood from a field colony and placed them in an incubator (~35°C, ~60% relative humidity, RH). The next day, we collected the newly emerged callow bees (0–24 h of age) and marked each with a dot of paint on the dorsal parts of both their thorax and abdomen. Once the paint had dried, we reintroduced the marked bees into their mother colony. When these reintroduced bees reached an age typical of nurses (3–5 days, see below), we removed a larvae-containing comb from the colony and carefully observed the behavior of paint-marked bees. Nurse bees in our studies were defined as painted bees observed with their head inserted into a larva-containing cell.

For the experiments described below, we randomly assigned focal nurses to treatments. Each focal nurse was collected and immediately placed individually in a cage made of a modified Petri dish (diameter 90 mm) that was either provisioned with *ad libitum* sucrose solution (50% w/w) or sugar candy and tap water (experiment 2, see below). In experiments 2–4, we also provisioned each cage with *ad libitum* pollen.

Monitoring locomotor activity

For experiments 1–3, we placed all the cages with the individually isolated nurses in an environmental chamber (29±1°C, RH 53–69%). The chamber was illuminated with dim red light [Edison Federal EFEF 1AE1 Far (Cherry) Red LED; maximum and minimum wavelengths 750 and 730 nm, respectively]. Data were recorded at a frequency of 1 Hz with four CCD cameras (Panasonic WV-BP334) and an image acquisition board (IMAQ 1409, National Instruments, Austin, TX, USA). In experiments 1 and 2, the monitoring cages were set on horizontal trays facing up and the cameras were placed above them. In experiment 3, the monitoring cages were set vertically and were attached to their holding trays with magnetic tape. The holding trays were fixed to the chamber wall and the orientation of the cameras was adjusted accordingly. Locomotor activity was monitored continuously over 6 successive days with the ClockLab data acquisition system as previously described (Shemesh et al., 2007; Yerushalmi et al., 2006). We used the ClockLab circadian analyses software (Actimetrics, Wilmette, IL, USA) to generate actograms and for χ^2 periodogram analyses. The χ^2 periodograms were applied to the activity data collected on days 2–6 in the laboratory (4 days), using 10 min bins with periods ranging between 20 and 28 h. We used the 'Power' obtained from the periodogram as a proxy for the strength of circadian rhythmicity (Yerushalmi et al., 2006). Briefly, power was calculated as the height of the periodogram peak above the P=0.01 significance threshold line. Bees with periodograms below the threshold line were assigned a zero power value. We analyzed

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the sleep and overall daily activity of individual bees using a custom-made algorithm (Eban-Rothschild and Bloch, 2015). We defined a bout of 5 min or more with no movement as 'sleep'. This sleep proxy is based on detailed video analyses of sleep-like behavior of individually isolated honeybees (Eban-Rothschild and Bloch, 2008) and is similar to that used in studies of sleep in *Drosophila melanogaster* (e.g. Shaw et al., 2000).

Experiment 1: the influence of larval extract on circadian rhythms of individually isolated nurses

To prepare larval extract, we removed a brood comb from a field colony and collected 2–4 day old larvae into 10 ml glass containers, each containing 1 ml *n*-pentane (Sigma-Aldrich, St Louis, MO, USA). We placed 50 larvae in each container, sealed the containers and left them for 1 h in an incubator at 20°C. Then, we used a glass pipette to transfer the extract to a larger glass stock container (100 ml). We washed the larvae three additional times with 1 ml *n*-pentane, and added the washes to the stock container. After the third wash, we poured all the solvent with the extract into the stock container through a sieve, leaving the larvae bodies out of the stock solution. We evaporated (inside a chemical hood) or added *n*-pentane to adjust the stock solution to a final concentration of 50 larval equivalents (Leq) ml^{-1} and stored it at −20°C until use.

In order to confirm that our brood extracts were biologically active, we applied 880 Leqs of extract to a piece of glass with a surface area of 25×10 cm and introduced it into a field colony. The number of foragers returning to the hive with pollen (but not without pollen) during the 3 h after introduction was higher in the colony treated with brood extract than in colonies to which we introduced a piece of glass (of a similar size) treated with only the solvent (data not shown). These results are consistent with the findings of Pankiw et al. (1998) and suggest that our brood extract was biologically active. We performed an additional set of preliminary experiments in which we applied 10 or 20 Leq of extract to the cage floor (with or without paraffin wax) of individually isolated nurse bees. As neither of these two trials had a significant influence on circadian rhythms (data not shown), we next applied the larval extract to the bee food. To do this, we mixed the larval extract with sugar solution (Extract+ treatment). First, we poured various amounts of *n*-pentane with larval extract onto granulated sucrose (6 g sucrose per 10 bees). We then placed the mixture inside a chemical hood and waited until the solvent had completely evaporated from the sucrose. Next, we diluted the sucrose–extract mixture with water while mixing the solution with a magnetic stirrer to obtain a 50% (w/w) solution. As controls, we used a similar sucrose solution treated in the same way with clean *n*-pentane solvent (S+) or sucrose solution that was not treated with the solvent (S−; not done in trial 3). For the behavioral analyses, we collected 3–5 day old nurses from a field colony and placed each of them individually in a monitoring cage. Each cage was provisioned with 1 ml sucrose solution subjected to one of the three treatments described above. We then transferred the cages to the laboratory and monitored the locomotor activity of the caged bees over 6 successive days in isolation, as described above. In trials 1–3, we tested extracts containing 10 Leq per bee (trial 1), 20 Leq per bee (trial 2) or 20 or 50 Leq per bee (trial 3). Trials 1 and 3 were each performed with nurses from a single source colony (S84 and H10-02, respectively); in trial 2, we used bees from colonies H10 and H10-01. We tested the effect of the treatment on the power of circadian rhythms in locomotor activity using one-way ANOVA in trials 1 and 3, or two-way mixed-model ANOVA in trial 2 (with the source colony added as a random factor). We further compared selected pairs of treatment groups using orthogonal planned pair-wise comparisons (contrasts). In contrast with unplanned (*post hoc*) pair-wise comparisons, which compare all possible pairs of treatments, planned contrasts are performed on selected pairs of treatments and each comparison tests a distinct hypothesis. Therefore, there is no need to correct the significance level (α) for increased Type I error rate using this method (Quinn and Keough, 2002; Sokal and Rohlf, 1995). All analyses were performed with SPSS version 21.0.

Experiment 2: the influence of synthetic BEP on circadian rhythms of individually isolated nurses

We conducted three trials with nurses collected from colonies 11-14, 11-01 and 13-18. Sugar candy was prepared by mixing sucrose sugar powder with 2 ml of inverted sugar (a mixture of glucose+fructose) until the total mixture weighed 10 g and had a dough-like texture. We used a commercially available BEP blend (SuperBoost). In trials 2 and 3, we applied and mixed different amounts of BEP directly into the sugar candy. We then prepared pea-sized pieces (∼0.3 g each) of the sugar candy–BEP mixture, weighed each piece and placed it in a monitoring cage. In trial 1, we applied the BEP in a 50% sucrose solution or in sugar candy. To mix the pheromone with either food type, we first dissolved the required amount in *n*-pentane. Then, we poured the solution on weighed amounts of either granulated sucrose or sugar candy and allowed the solvent to evaporate for 35 min inside a chemical hood. Next, we mixed the sugar candy and BEP. The BEP-treated granulated sucrose was dissolved with water. In trial 3, we also tested the influence of BEP mixed with paraffin, in addition to BEP mixed with sugar candy. We melted the paraffin at 58°C and added the appropriate amount of BEP. We cast the melted paraffin into molds and created wax lures approximately 7 mm in length, 5 mm in diameter and ∼0.1 g in mass. We cut rectangular pieces of honeycomb, containing approximately 15 comb cells on each side of the comb, and inserted BEP-treated lures into five cells in each comb. The honeycomb pieces with the lures were then introduced into the monitoring cages. The BEP concentrations and the medium by which it was provided in each trial are detailed in Table 1. The age of the nurses used for this experiment was 3–4 days (trials 1 and 2) or 4–5 days (trial 3). In trial 1, we used two-way ANOVA to test the influence of both BEP concentration and food medium (sucrose solution or sugar candy) on the strength of the circadian rhythm. In trials 2 and 3, the effect of the treatment was tested using one-way ANOVA. We compared selected treatment groups with orthogonal planned contrasts. We also tested the influence of the treatment on the daily amount of candy consumed by each bee (one-way ANOVA).

Experiment 3: the influence of capped brood on the circadian rhythms in locomotor activity of nurses

We next examined the influence of capped brood on the locomotor activity rhythms of individually isolated nurses in the laboratory. We performed two trials with bees from colonies 15–22 and 15–15. For the experimental treatments (Empty, Brood− and Brood+), we used small round pieces of comb fixed inside small Petri dishes (diameter ∼3.5 cm). The surface of one side in each comb piece covered ∼30 hexagonal wax cells. In the Brood+ and Brood− treatment cages, one side of the comb had capped brood (i.e. up to 30 capped broods per cage); in the Brood− treatment cages, we removed all the brood from the comb, leaving the cells empty. Empty treatment cages received empty pieces of wax comb, which did not contain brood at all, but were left overnight in a colony prior to the experiment to allow exposure to colony odors. At the

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Table 1. The effect of brood ester pheromone (BEP) on survival and food consumption in experiment 2

Trial	BEP medium	BEP (µl g^{-1})	% Survival	Chi-squared *P*-value	*N*	Sugar candy consumption (mg day^{-1})	ANOVA *P*-value
1	50% sucrose solution (w/w)	0	71	<0.001*	10	n.d.	n.d.
		1	93		14		
		10	100		15		
		100	8		1		
	Sugar candy	0	79	<0.001*	11	10.0±1.08	0.013
		1	93		14	11.7±1.15	
		10	93		14	11.1±1.26	
		100	43		6	5.1±0.57	
2	Sugar candy	0	97	0.4*	26	7.2±0.8	0.31
		0.1	97		28	7.1±0.7	
		1	90		28	7.6±0.9	
		10	100		28	5.8±0.5	
3	Sugar candy+paraffin lures	0	93	0.36‡	28	12.1±2.2	0.4
		1+§ 1:1000 (w/w)	80		24	9.9±0.7	
		10+§ 1:100 (w/w)	83		24	13.1±1.2	

N is the number of bees that survived. Sugar candy consumption values are means±s.e.m. n.d., not determined.
*4×2 Chi-square test. ‡3×2 Chi-square test. §BEP dilution in the paraffin.

beginning of the experiments, we opened two cells in each piece of brood-containing comb and determined the developmental stage of the brood. The brood developmental stage in the Brood+ and Brood− cages ranged from pre-pupae to pink-eyed pupae in trial 1, and pre-pupae to black-eyed pupae in trial 2. The small dishes with the embedded combs were fixed to the cage floor with magnetic tape. We introduced focal nurses to the experimental cages at age 4–5 days in trial 1, and 3–4 days in trial 2. The cages with the bees were transferred to the environmental chamber for locomotor activity monitoring. At the end of the experiment, we opened a sample of brood cells from each cage and confirmed that the pupae were alive and looked healthy. We used Kruskal–Wallis tests for testing the effect of treatment on the strength of circadian rhythms, the percentage of daily sleep time and the daily amount of locomotor activity. This non-parametric method was used because of the small sample size in the Brood+ treatment group in trial 2 (N=7). Next, we used two-way ANOVA followed by planned contrasts to test the influence of treatment and source colony on the strength of the circadian rhythm. For the Brood+ treatment, we used step-backward multiple linear-regression to test the effects of trial and the number of capped brood cells per cage on the strength of the circadian rhythm. Given that this regression analysis revealed a significant negative association between the number of brood cells and the power of circadian rhythms, in later analyses we used only Brood+ bees that were placed with at least 20 brood cells per cage (see Results). We repeated the Kruskal–Wallis tests separately for each trial using this cut-off, followed by planned contrasts using Mann–Whitney tests, as recommended by Ruxton and Beauchamp (2008).

Experiment 4: detailed observations on the behavior of nurse bees housed with or without brood

To more rigorously study the behavior of individually isolated nurse bees in the presence of capped brood, we conducted an additional experiment in which we observed isolated nurses with brood. We placed wax sheets (control treatment) or brood combs (Brood+ treatment) in the cage center. We introduced the control wax sheets into the brood nest of the experimental colony about 20 h before the experiment began, to allow it to absorb the colony odors. We covered the remaining cage surface with commercial honeybee wax sheets (not introduced into the colony). This was done in order to facilitate walking on the cage surface. On the first day of the experiment, we collected focal nurses that were 3–4 days of age, placed them in individual cages, and housed them in an environmental chamber (31.8±0.5°C, RH 57.5±10%) illuminated with dim red light (Edison Federal EFEE 1AE1 Deep Red LEDs; maximum and minimum wavelengths 670 and 650 nm, respectively). The cages were placed in vertical trays on the chamber wall, as in experiment 3. On each day of the experiment, we photographed and recorded the brood status in all the cages under standard 30 W LED 'white' light (Alpina SF-700-220 LED/30 W) at 10:00 h–12:00 h. On days 5–6, when the bees were 8–10 days of age, we conducted four, 1 h observations: two during the day (09:00 h, 13:00 h) and two at night (21:00 h, 00:00 h). Observations were conducted under dim red light. Each observation was divided into 10, 6 min scans. For each scan, we observed all of the focal bees and recorded their behavior and location. We defined the cage 'center' as the surface of the central small dish and its contents (brood comb or control wax sheet), including the outer dish wall. A bee was recorded to be in the 'center' in a particular scan if she was observed there for at least 5 consecutive seconds. Otherwise, her location was recorded as 'periphery'. Additionally, in each scan we assigned each bee to one of nine behaviors that were pooled into three categories: (1) rest, (2) activity and (3) brood care (Table 2). For the comparison of overall rest and activity (see below), brood care was counted as activity. When more than one behavior was observed in the same scan, the higher-ranked behavior was recorded (see ranking in the left column of Table 2). Rest behaviors were assigned with the lowest ranks, followed by activity and brood-care behaviors. We continued to monitor the status of the brood and bees on subsequent days until callow bees emerged in all cages. For each bee, we compared the number of scans in which she was observed in the 'periphery' versus the 'center' and the day versus night activity (including brood care) using two-sided binomial tests. We further used Mann–Whitney tests to compare the percentage of scans observed in the center, and the percentage of day activity between the treatments. Finally, for each scored behavior, we compared the percentage occurrence during day and night. All the analyses in experiment 4 were performed with Matlab, version 2013b. At the end of the observations, we made a daily recording of the number of newly emerging callow bees over a period of additional 14 days.

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Table 2. Classification and definitions of the behaviors recorded in experiment 4

Priority rank	Behavioral category	Behavior	Definition
9	Rest	Stand	Stand motionless throughout the scan
8		Auto-groom	Stand and auto-groom body parts
7		Stay in empty cell	Stay inside the same empty cell for the entire scan duration
6	Activity	Eat pollen	–
5		Walk	Move at least twice the body length for at least 3 consecutive seconds
4		Tend empty comb	Visit empty comb cells or tend their wax walls for at least 5 consecutive seconds
3		Tend wax sheet	Tend wax-sheet surface with mouthparts and antennae for at least 5 consecutive seconds
2	Brood care	Tend capped brood	Tend brood caps with mouthparts and antennae for at least 5 consecutive seconds
1		Tend opened brood	Tend a brood cell for which the cap was opened for at least 5 consecutive seconds

RESULTS

Experiment 1: the influence of larval extract on circadian rhythms of individually isolated nurses

Given that previous studies indicated that contact with live larvae induced attenuated circadian rhythms in the locomotor activity of nurse bees (Shemesh et al., 2010), we tested whether larval extract that was expected to contain most of the brood pheromones was sufficient to evoke attenuation of circadian rhythms in individually isolated nurse bees. We performed three trials using various concentrations (larvae equivalents). In all three trials, the strength of circadian rhythms was not affected by treating the sugar syrup with the pure solvent (i.e. no difference between the S− and S+ treatments; planned comparisons, P=0.65, P=0.99 and P=0.67 for trials 1–3, respectively). In the first trial, the effect of treatment was not significant (one-way ANOVA, P=0.18; Fig. 1A), although there was a trend of weaker circadian rhythms in bees treated with food containing 10 Leqs of extracts compared with the pooled sample of the two controls (planned comparison, P=0.082). In the second trial, in which we tested bees from two different colonies, we increased the amount of larval extracts to 20 Leqs. In one-way ANOVA, we found a significant effect of treatment in colony H10 (P=0.02; dark gray bars in Fig. 1B), but not in colony H10-01, although it showed a similar trend (P=0.25, light gray bars in Fig. 1B). Despite these similar trends, a mixed-model two-way ANOVA did not produce statistically significant results (P=0.25, P=0.15 and P=0.22 for the treatment, colony, and treatment×colony interaction effects). However, the planned comparisons analysis revealed that bees fed with larval extract had significantly weaker circadian rhythms than the pooled sample of the two control treatments (P=0.003; Fig. 1B). In the third trial, in which we tested an additional higher dose of 50 Leq, there was no effect of the brood extract either in one-way ANOVA (Fig. 1C; P=0.81) or in the planned comparisons (pooled controls compared with the pooled extract treatments, P=0.71; 20 Leq compared with 50 Leq treatments, P=0.39). In all three trials, the larval extract did not affect the free-running period (data not shown). The significant attenuation of circadian rhythms in bees exposed to larval extract in trial 2 is consistent with the hypothesis that brood pheromones influence circadian rhythmicity in nurse bees. However, a similar statistically significant effect was not observed in the other two trials. Given that some of the inconsistency across trials could stem from technical variation related to the extraction protocol or from biological variability among the brood extracted in the different trials, we next used a complementary approach testing the influence of commercial brood pheromone, which can be better standardized across trials.

Experiment 2: the influence of synthetic BEP on circadian rhythms of individually isolated nurses

We used several methods to apply synthetic BEP to individually isolated nurse honeybees (see Fig. 2A,B for an example of the experimental setup from trial 3). In trial 1, we tested three BEP concentrations applied either to sucrose syrup or to sugar candy (Table 1). Bees that were provisioned with 100 µl g^{-1} BEP in their food showed reduced sugar candy consumption (tested only in the BEP–sugar candy group) and survival (in both the BEP–sugar candy and BEP–sucrose groups) compared with bees fed with lower BEP concentrations (Table 1). Thus, we did not use a 100 µl g^{-1} dose in subsequent trials. Neither survival nor food consumption was affected by the lower BEP concentrations (0.1–10 µl g^{-1}; Table 1). The effects of BEP concentration and food type on the strength of circadian rhythm were not statistically significant in a

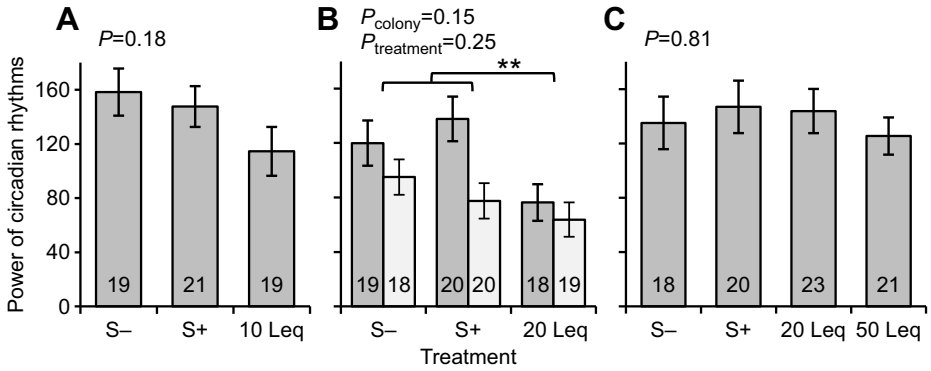

Fig. 1. The influence of larval extract on the strength of circadian rhythms in the locomotor activity of individually isolated nurse bees. (A) Trial 1; (B) trial 2: light gray bars indicate bees from colony H10; dark gray bars are bees from colony H10-01; (C) trial 3. Means±s.e.m., sample sizes are shown within bars. S−, plain sugar syrup; S+, sugar syrup treated with only solvent (*n*-pentane); 10, 20 and 50 Leq, sugar syrup treated with solvent extract made of 10, 20 or 50 larval equivalents per bee, respectively. Power (*y*-axis) serves as an index of the strength of circadian rhythms. *P*-values and asterisks indicate the results of one-way ANOVA (trials 1, 3) or two-way ANOVA (trial 2) followed by planned comparisons (**P<0.01).

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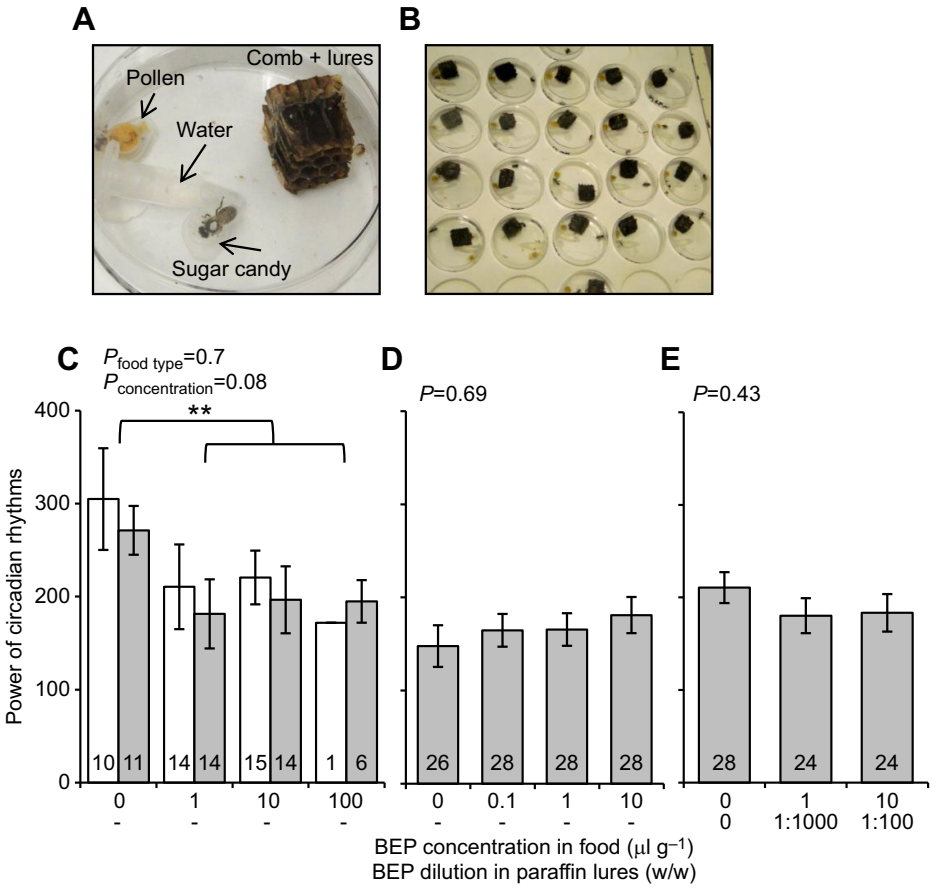

Fig. 2. The influence of synthetic brood ester pheromone (BEP) on the strength of circadian rhythms in locomotor activity. (A) A close look at a cage from trial 3 provisioned with *ad libitum* sugar candy (used in all trials) and a piece of comb with paraffin lures (used only in trial 3). Each cage was supplemented with *ad libitum* pollen and water. (B) Cages from trial 3 set on a horizontal tray before the beginning of locomotor activity monitoring. The cameras were set above the trays (not shown). (C–E) The power of circadian rhythms in locomotor activity in trials 1–3, respectively (means±s.e.m., sample sizes are shown within bars). The upper *x*-axis shows the BEP concentration applied to the bee food, and the lower row shows the dilution of BEP in the paraffin lures (used only in trial 3). White bars represent BEP mixed with 50% (w/w) sucrose solution; gray bars represent BEP mixed with sugar candy. *P*-values and asterisks indicate the results of two-way ANOVA (trial 1) or one-way ANOVA (trials 2 and 3) followed by planned comparisons (**P<0.01).

two-way ANOVA for bees in trial 1 (*P*-values are shown in Fig. 2C). However, the planned comparisons showed that the circadian rhythms for the control bees (0 BEP) were significantly stronger compared with those in the pooled sample of bees from the three BEP treatments (P=0.019; Fig. 2C), with no differences between the three BEP concentrations (1 versus 10 µl g^{-1}, P=0.72; 10 versus 100 µl g^{-1}, P=0.73). The BEP treatment had no effect in trials 2 and 3 either in one-way ANOVA (Fig. 2D,E) or in the planned comparisons analysis (0 BEP versus pooled BEP treatments: trial 2, P=0.37 and trial 3, P=0.19; in trial 2, comparison of 0.1 versus 1 µl g^{-1} and 1 versus 10 µl g^{-1} gave P=0.97 and P=0.56, respectively; in trial 3, comparison of 1 versus 10 µl g^{-1} gave P=0.91). It should be noted, however, that the control treatments of trials 2 and 3 had very weak circadian rhythms (power of 147.7±22 and 210.6±17, respectively, means±s.e.m.) compared with the control treatments of trial 1 (287.7±27.5) and the two trials of experiment 4 (see below; 366.6±21.5 and 254.7±24.1 in trials 1 and 2, respectively). The treatment did not affect the free-running period or the daily amount of locomotor activity in any of the trials (data not shown).

Experiment 3: the influence of capped brood on circadian rhythms in locomotor activity of nurses

The experimental brood cages and set-up are shown in Fig. 3A,B. Survival did not differ between bees placed individually with empty wax combs (Empty), capped brood combs (Brood+) or capped brood combs from which we removed the brood (Brood−) (in trial 1, survival was 100%, 92% and 76%, respectively, 3×2 Chi square test, P=0.08; in trial 2, all bees survived). In trial 2, we could not use the data from 21 of the 28 Brood+ cages because callow bees had eclosed prior to the end of the monitoring session. Nurse bees with brood showed attenuated circadian rhythm but the effect was statistically significant only in trial 2 (Kruskal–Wallis test, P=0.21 and P=0.01 for trials 1 and 2, respectively). In a complementary two-way ANOVA that included both trials, the *P*-value for the treatment effect was P=0.082 (the power in trial 1 was significantly higher than that in trial 2, P=0.035). The bees with brood, however, had significantly weaker circadian rhythms compared with the pooled controls (planned comparisons, P=0.002), with no differences between the Empty and Brood− control treatments (P=0.23). A multiple linear regression analysis for a pooled sample of bees from the two trials revealed that the circadian rhythm strength decreased with an increasing number of brood cells (Fig. 3C; step-backward multiple linear regression, R^2=0.45; trial, P<0.01; brood number, P=0.04; interaction, P=0.77). Given that these findings predict that a small number of brood has little effect on the nurse strength of circadian rhythms, we performed an additional analysis in which we did not include the data of bees from cages with 20 or fewer brood cells (Fig. 3D,E). We chose 20 cells as the cut-off because below this number most nurses showed relatively strong circadian rhythms (the mean power recorded for Brood+ bees placed with 20 or fewer brood cells was even slightly higher compared with that for the pooled control bees: 347.47±43.4 compared with 300.66±14, respectively; independent *t*-test, P=0.33). Using only bees placed with 20 brood cells or more, we found that the power of circadian rhythms significantly varied between treatments in both trials even when analyzed independently (Kruskal–Wallis test, *P*-values shown in Fig. 3D,E). Brood+ bees had significantly weaker rhythms compared with the pooled sample of the controls (planned comparisons; P=0.03 and P=0.001 for

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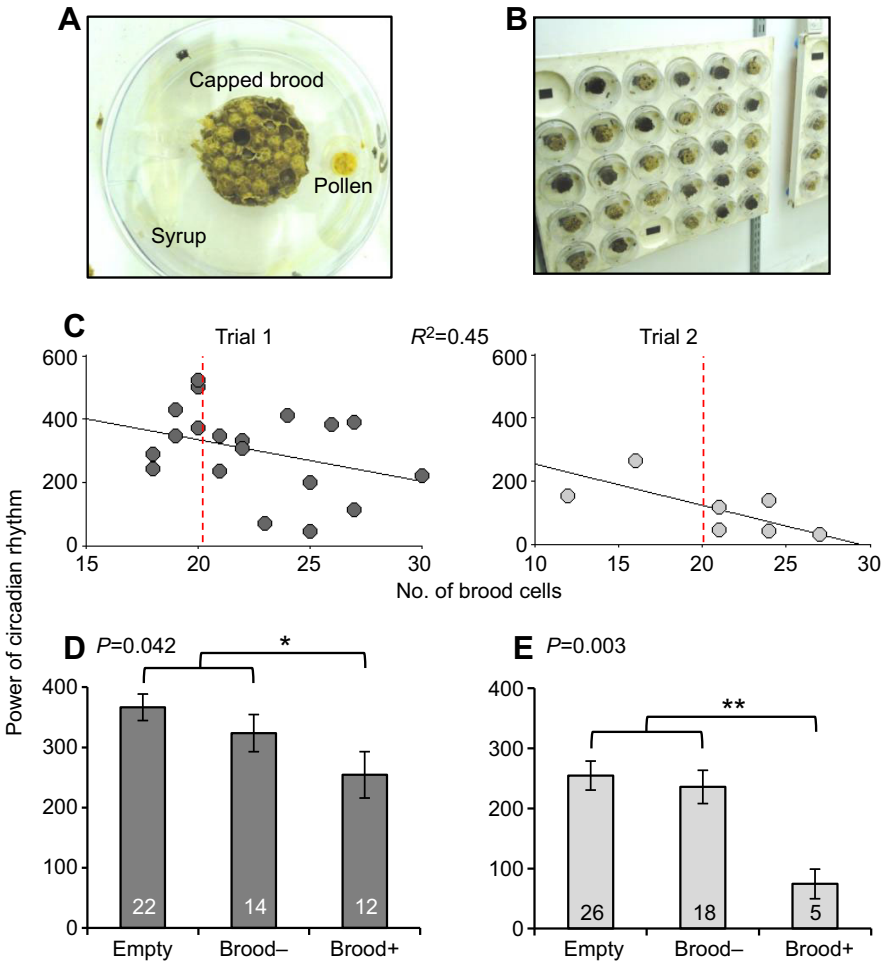

Fig. 3. The influence of capped brood on the strength of circadian rhythms. (A) A cage containing a brood comb (Brood+ treatment). (B) Cages from experiment 3 fixed to a vertical tray. (C) The relationship between the power of circadian rhythms in locomotor activity and the number of capped brood cells in the cage, for Brood+ bees in trial 1 (left) and trial 2 (right). Each circle shows the power for one individually isolated bee. The black lines depict the fit of the multiple linear regression model. The dashed red lines depict the cut-off point of 20 brood cells. (D,E) The power of the circadian rhythm (means±s.e.m., sample sizes are shown within bars) in trials 1 and 2, respectively, when using only Brood+ cages with 20 or more brood cells. Empty, nurse bees housed with an empty wax comb; Brood–, nurse bees housed with a sealed brood comb from which the brood was removed; Brood+, nurse bees housed with sealed brood comb. *P*-values and asterisks were obtained from Kruskal–Wallis tests followed by planned comparisons (Mann–Whitney tests, **P*<0.05, ***P*<0.01).

trials 1 and 2, respectively) and the Brood– and Empty controls did not significantly differ from each other (trial 1: *P*=0.16; trial 2: *P*=0.66). The capped brood treatment did not have significant effects on the amount of sleep or overall levels of activity (in both the Kruskal–Wallis and planned comparisons analyses; data not shown). These results suggest that nurses tend capped brood, which does not require feeding, around the clock with attenuated circadian rhythms.

Experiment 4: detailed observations on the behavior of nurse bees housed with or without brood

The evidence above suggesting that capped brood induces attenuation of circadian rhythms in nurse bees (Fig. 3) prompted us to look more closely at the influence of capped brood on the behavior of young bees. Therefore, we performed detailed observations on the behavior of nurse bees placed individually in cages with or without capped brood (Fig. S1A,B). All the bees were alive at the end of the monitoring period and 90±4.5% (mean± s.e.m.) of the brood eclosed in the Brood+ cages within 14 days of the beginning of the experiment. Only one cage was omitted from our analyses because callow bees emerged from the capped brood before the end of observations (bee no. 3).

The bees in nine of the 10 cages with brood were observed tending the brood (as defined in Table 2). They typically stayed on the brood comb at the center of the cage and constantly mandibulated and antennated the wax caps of the brood cells, or the exposed brood cells that were opened by the experimenter (Movie 1, right). By contrast, bees in cages with no brood typically walked around the cage perimeter (Movie 1, left). Occasionally these bees were observed eating pollen or mandibulating the wax sheet that was placed on the cage floor. Eight of the bees housed with brood partially (two) or fully (six) recapped cells that were opened by the experimenter (e.g. bee no. 5 in Fig. S1C,D and bee no. 15 in Movie 2). One of the bees killed four pupae, including the ones in cells that were opened by the experimenter. Nevertheless, after killing these pupae, she was seen tending brood, i.e. mandibulating and antennating the remaining capped brood cells. Three of the bees were also seen opening wax caps – an activity that is typical of honeybee hygienic behavior (e.g. Fig. S1E,F).

The day and night observations allowed us to study the influence of the brood on the temporal organization of behavior in individually isolated nurse bees. Eleven of the 13 broodless Control bees that we observed were overall more active during the day, and the differences were statistically significant for five of them in a binomial test (Fig. 4A; Fig. S2). By contrast, nurses in cages with capped brood were typically similarly active during day and night observations (Fig. 4B; Fig. S2). The only bee with brood for which activity was significantly higher during the day observations was bee no. 13 (Fig. S2). This bee was atypical because she did not tend the brood, as opposed to all other Brood+ nurses (Fig. S2), and was never recorded on the brood comb (not shown). Given that bee no. 13 was such a clear outlier, it was excluded from later analyses. A pooled analysis revealed that the percentage of overall activity (including both general activity and brood-care behaviors; Table 2)

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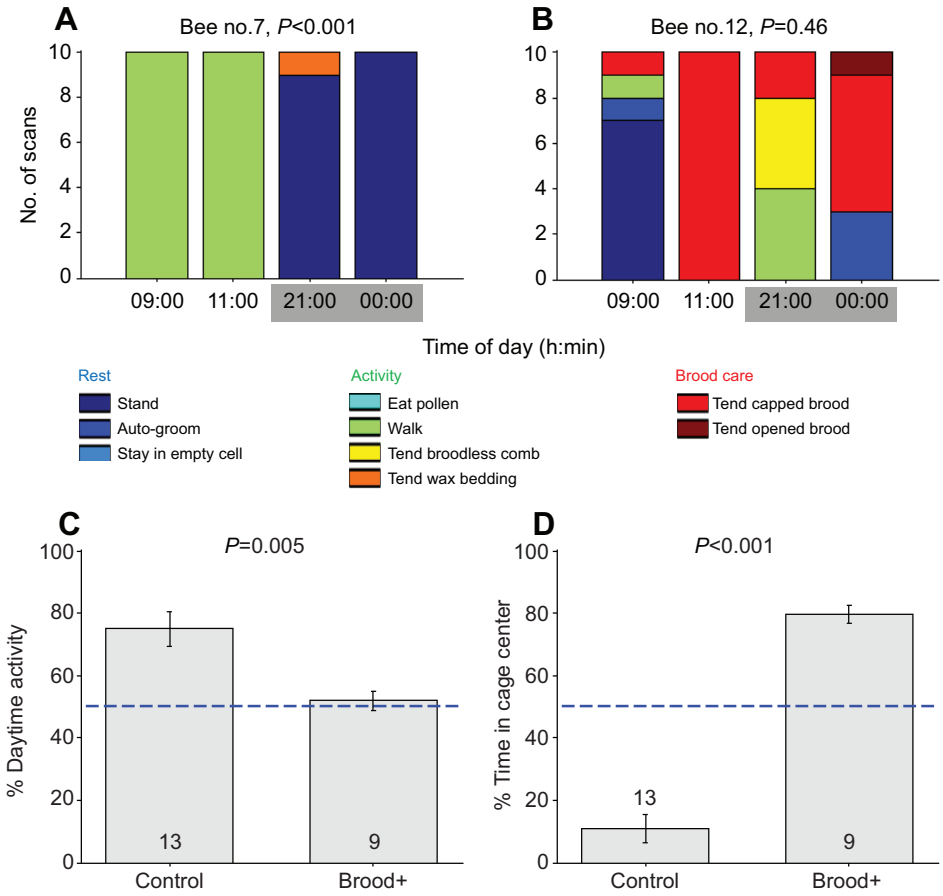

Fig. 4. The presence of capped brood influences the temporal organization of behavior of individually isolated nurse bees. (A,B) Representative summary of behavioral observations for bees housed with (A) or without (B) capped brood. The *x*-axis shows observation time; night observations are indicated by a gray background. The *y*-axis summarizes the number of 6 min scans in which the bees performed each behavior during the 1 h observation sessions. Each color depicts a different behavior and behavioral category, as shown in the key. *P*-values were obtained from a two-sided binomial test on the amount of total activity (including brood care) during day versus night observations. (C,D) Summary of the percentage of daytime activity (C) and of time spent in the cage center (D) in bees with or without brood (means±s.e.m., sample sizes are shown within bars). *P*-values were obtained from Mann–Whitney tests.

observed during the day was lower in bees with brood that showed similar levels of activity during day and night (Control: 74.9±5%; Brood+: 51.9±3%, means±s.e.m.; Mann–Whitney test, P=0.005; Fig. 4C). These detailed behavioral observations are consistent with the continuous automatic recording in experiment 3 (Fig. 3).

The presence of capped brood also influenced the location of the nurse bees (Fig. 4D). Bees caged without brood spent more time in the cage periphery than in its center (the difference was significant in a binomial test for 11 of the 13 bees; data not shown). In contrast, 8 of 10 bees in cages with brood spent significantly more time on the brood comb at the cage center (P=0.08 for an additional bee). Only bee no. 13, which did not tend the brood, was observed more (only) at the cage periphery. These differences were significant in a pooled analysis (from which bee no. 13 was excluded) that compared the percentage of scans in which the bees were observed in the cage center (Mann–Whitney test, P<0.001; Fig. 4D).

We next analyzed the occurrence of specific behaviors. Our detailed observations revealed that bees without brood were observed walking more often during the day (65.4±8% and 25.8±5% for the day and night scans, respectively; Wilcoxon signed-rank test, P<0.001; Fig. 5A), and standing motionless more often during the night (Fig. 5A; 58.46±7.8% and 24.2±6.6%, respectively, P=0.002). In contrast, most of the behaviors performed by bees that were housed with brood occurred at similar levels during the day and night (Fig. 5B). Importantly, this included tending capped brood, which was the most frequent behavior of the Brood+ bees (31.66±7.9% of day scans and 30.56±8.73% of night scans; P=0.73). Some behaviors were biased towards the day or the night. For instance, walking was significantly higher during the day (30.6±6% compared with 16.1±3% at night; P=0.047; Fig. 5B). There was also a statistically non-significant trend for tending empty comb to occur more frequently at night (23.3±11% compared with 5±3% during the day; P=0.063) and for tending opened brood cells to be more frequent during the day (8.3±3% compared with 1.1±0.8% at night; P=0.094). These detailed observations show that individually isolated nurse bees tend capped brood with similar levels of activity during the day and night. This temporal pattern is similar to the behavior of nurses in typical field colonies.

DISCUSSION

Some species of social bees and ants naturally switch between activity with and without circadian rhythms. In honeybees, this remarkable chronobiological plasticity is modulated by contact with the brood. It is assumed that around-the-clock brood tending improves brood development, but it is not clear how the brood induces nurse bees to tend it around the clock and how this activity pattern contributes to brood development. We tested two major hypotheses accounting for task-related plasticity in circadian rhythms. The first states that the brood communicates its need for continuous care by means of pheromones, which modulate the worker activity rhythm. The second is that the need to feed larvae causes nurses to tend brood around the clock. We found that bees exposed to brood extract or BEP showed inconsistent and weak attenuation of circadian rhythms in locomotor activity. These results do not lend strong support to the first hypothesis (but neither do they reject it). We further found that capped brood, which is not fed, was tended around the clock by individually isolated nurse bees. The brood-care behaviors of isolated nurse bees were similar to those performed by nurses in typical colonies. The finding that the strength of the nurse rhythm diminished with the number of capped

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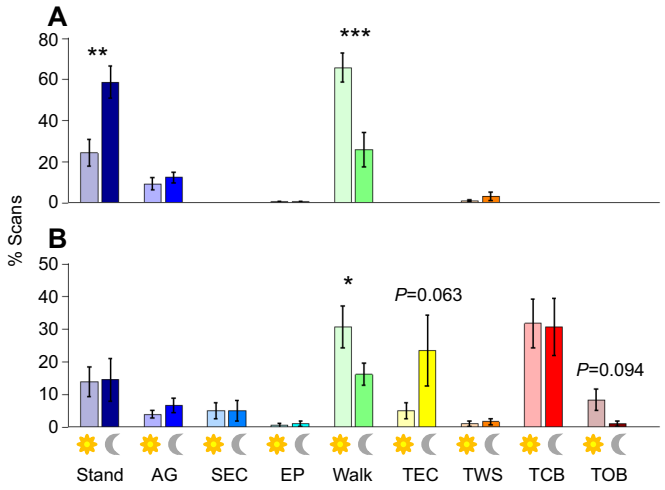

Fig. 5. The influence of capped brood on the day versus night performance of behaviors by individually isolated nurse bees. The bars indicate the percentage of scans (means±s.e.m.) in which each behavior was scored during the day (sun symbol, pale bars) or night (moon symbol, dark bars) observations. (A) Bees without brood (Control; *n*=13). (B) Bees with brood (Brood+; *n*=9). Asterisks indicate significant *P*-values (**P*<0.05, ***P*<0.01, ****P*<0.001), obtained from a Wilcoxon signed-rank test. *P*-values of 0.1>*P*>0.05 are shown above the bars. The color code for the bars follows that used in Fig. 4. Stand, auto-groom (AG), stay in empty cell (SEC), eat pollen (EP), walk, tend empty comb (TEC), tend wax sheet (TWS), tend capped brood (TCB) and tend opened brood (TOB).

brood cells to which she was exposed further supports the capped-brood effect. Taken together with previous studies, our results suggest that task-related plasticity in circadian rhythms does not serve a single brood need (such as feeding), but rather that multiple factors that may be related to various brood needs can induce nurses to be active around the clock with attenuated circadian rhythms.

Brood pheromones are major regulators of honeybee behavior and are therefore good candidates for research on the modulation of brood-related plasticity in circadian rhythms. Studies with larval extract and BEP, with doses similar to those used in our study, showed effects on honeybee worker behavior and physiology (Le Conte et al., 1989, 1990, 1994, 2001; Pankiw et al., 1998; Trouiller et al., 1991). Yet, the individually isolated nurses in our study did not exhibit consistent rhythm attenuation. It is understandably difficult (and sometimes impossible) to reconstitute pheromonal regulation of complex social behavior in a simple laboratory setup. The brood extracts and synthetic pheromones that we used may not contain the correct amounts of some important components. Indeed, the influence of BEP on behavior was reported to be affected by subtle modifications in the chemical composition of the pheromone (Le Conte et al., 1994). It is also possible that the influence of brood pheromones is context dependent and is not fully shown by a nurse bee individually isolated in the laboratory. For example, the brood-care behavior of nurse bees may be influenced by their interactions with other adult bees. It is also notable that the power of circadian rhythms measured for the control groups in all trials of experiment 1 and in trials 2 and 3 of experiment 2 was low relative to that of the controls in experiment 3. We do not know whether the weak rhythms of these control bees stem from genetic or environmental variability. However, the relatively weak rhythms in the controls could make it difficult to detect rhythm attenuation in these experiments. With these constrains in mind, it is notable that in some trials with food treated with either brood extract (Fig. 1) or synthetic BEP (Fig. 2) there was a clear trend of rhythm attenuation in the treated bees. Thus, although we invested great effort in these experiments and used multiple approaches, the absence of proof is not a proof of absence; tests of additional brood pheromones or extraction protocols are needed to unequivocally reject the hypothesis that brood pheromones are important modulators of circadian rhythm plasticity in worker bees. For example, young honeybee larvae are very sensitive to starvation periods as short as 1 h (He et al., 2016). The larva signals its nutritional state and the nurses respond to these signals by adjusting their visitation rate (He et al., 2016; Heimken et al., 2009; Huang and Otis, 1991a,b). A volatile brood pheromone, E-β-ocimene was recently suggested to function as such a larval hunger signal (He et al., 2016), making it an obvious candidate for future studies.

Our results show that the presence of capped brood (pupae and pre-pupae) causes nurse bees to be active around the clock with attenuated circadian rhythms (Figs 3, 4, 5; Fig. S2). Our detailed observations further indicate that individually isolated nurse bees tend capped brood in a similar way overall to nurses in typical colonies: they were attracted to the brood comb, inspected and tended the brood caps, and most of them recapped opened brood cells. Some nurses also opened capped cells, which is reminiscent of the hygienic behavior seen in typical colonies. Nurses with capped brood were similarly active during the day and the night. By contrast, their sister bees of a similar age that were housed without brood spent most of their time in the cage periphery and showed a clear diurnal activity pattern. Thus, the modulation of circadian rhythms in these individually housed bees is accounted for by the presence of capped brood and not by other factors such as age, previous experience or the environment. Importantly, these results clearly show that brood, which does not need to be fed, is nevertheless tended around the clock by nurse bees.

But why does capped brood need to be tended around the clock? One hypothesis is that frequent brood tending is necessary for proper thermoregulation. There is, indeed, evidence that even slight fluctuations in the ambient temperature experienced by the pupae can have severe effects on its development (Groh et al., 2004; Tan et al., 2005; Tautz et al., 2003). For example, waggle-dance precision and learning and memory performance of the emerging bees may be compromised (Tautz et al., 2003). Even slight deviations of only 1°C from the optimal pupal developmental temperature (34.5°C) can cause a significant reduction in the number of microglumeruli in the mushroom bodies, which are neuroanatomical structures crucial for learning, memory and complex behaviors in insects (Groh et al., 2004). To regulate the brood temperature, workers typically cluster around the capped brood in response to a temperature decrease, during both day and night (Kronenberg and Heller, 1982). Worker bees performing various tasks, including foragers, may participate in thermoregulation during the daytime (Stabentheiner et al., 2010). However, older workers, such as foragers, typically sleep at night (Klein et al., 2014; Moore et al., 1998) and one may speculate that nurses need to contribute more to brood thermoregulation during this period. There is some evidence that the clustering behavior of workers is mediated by brood pheromones (i.e. GDP; Koeniger; 1978; Koeniger and Veith, 1983).

An additional, not mutually exclusive, hypothesis is that around-the-clock tending of capped brood is necessary for preventing the spread of pathogens and parasites. The identification and removal of diseased or dead brood (‘hygienic behavior’) is part of what is commonly termed the colony ‘social immunity’, which is a mechanism to reduce the spread of pathogens and parasites (reviewed in Evans and Spivak, 2010; Wilson-Rich et al., 2009).

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Our detailed observations revealed that individually isolated nurse bees constantly inspected capped brood with their antennae and mouthparts; some were additionally observed opening capped brood cells, which is similar to an important behavioral component of hygienic behavior seen in typical colonies (Arathi et al., 2000). Although hygienic behavior is typically performed by middle-aged workers (Arathi et al., 2000), there is evidence that it may be performed by nurse bees as well. Bees performing hygienic behavior commonly interact with the brood, similar to nurses, and there is evidence that some types of hygienic behavior are regulated by BEP (e.g. *Varroa*-sensitive hygiene, VSH; Mondet et al., 2016). Further, there is an overlap in age and antennal gene-expression patterns between nurse bees and VSH workers (Le Conte et al., 2011; Mondet et al., 2015). Perhaps around-the-clock tending of capped brood is important for rapidly removing weak or infected brood, which may help to reduce pathogen spread (Evans and Spivak, 2010). This hypothesis is supported by our findings that capped brood induced nurse bees to tend them around the clock and that the nurses seen opening capped brood cells were active around the clock as well. However, additional studies are needed to explicitly test the hypotheses that around-the-clock activity with attenuated circadian rhythms serves for improved brood thermoregulation or hygiene.

Although the amount of overall activity in nurses housed with capped brood was similar during day and night, our observations suggest that they are not completely arrhythmic. For example, they walked more frequently during the day, which is consistent with power values >0 measured for bees with brood that were monitored with the automatic data acquisition system (Fig. 3D,E). Additionally, there was a strong, nearly statistically significant, trend for nurses with brood to tend empty comb cells more often during the night. Thus, our study suggests that some nurse behaviors may be biased to specific times of the day. The premise that some processes in around-the-clock active nurses nevertheless show circadian regulation is consistent with a microarray study which showed that some transcripts oscillate in the nurse brain in a different, or even opposite, phase from that of foragers (Rodriguez-Zas et al., 2012). A recent study further suggests that the capacity of the nurse antennae to track pulses of odorant stimuli is higher at night, in almost anti-phase with their peak of locomotor activity rhythms when removed from the hive (Nagari et al., 2017). These findings suggest complex patterns of circadian regulation in nurses: biological processes that are under circadian regulation in foragers may not be under clock regulation in nurses or, alternatively, they may be regulated with phases similar to or different from those of foragers.

To sum, our data suggest that around-the-clock brood tending can improve various aspects of brood care, in addition to feeding, and that the regulation of task-related plasticity in circadian rhythms is more complex than previously thought. The transition between activity with and without circadian rhythms is not a simple switch-like mechanism that is triggered by brood pheromones. Rather, multiple signals that are assumed to reflect various brood needs may be integrated to regulate temporal patterns of worker behavior. It is interesting to note that there is evidence suggesting that around-the-clock sibling care between offspring in social insects evolved from maternal care (Eban-Rothschild et al., 2011), and that maternal biology in mammals is associated with attenuated circadian rhythms as well (Lyamin et al., 2005; Nishihara et al., 2002; Wharfe et al., 2016a,b). Around-the-clock tending may improve growth rate, survival and health of young stages. Thus, plasticity in circadian rhythms that is regulated by relevant social signals may be evolutionarily beneficial for diverse social and solitary animals (Bloch et al., 2013).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.N., G.B.; Methodology: M.N., Y.B., G.B.; Formal analysis: M. N., Y.B.; Writing - original draft: M.N., G.B.; Writing - review & editing: M.N., Y.B., G. B.; Visualization: M.N.; Supervision: G.B.

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