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The involvement of the antennae in mediating the brood influence on circadian rhythms in "nurse" honey bee (*Apis mellifera*) workers

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ABSTRACT

Age-related division of labor in honey bees is associated with plasticity in circadian rhythms. Forager bees that are typically older than 3 weeks of age show strong behavioral and molecular circadian rhythms with higher activity during the day. Younger bees that typically care for ("nurse") the brood are active around the clock with similar brain clock gene levels throughout the day. However, nurses that are caged on brood-less combs inside or outside the hive show robust circadian rhythms with higher activity during the day, suggesting that direct contact with the brood mediates the plasticity in the circadian system. The nature of the brood signals affecting the workers' circadian system and the modalities by which they are detected are unknown. Given that the antennae are pivotal sensory organs in bees, we hypothesized that they are involved in mediating the brood influence on the plasticity in circadian rhythms. The flagella of the antennae are densely covered with diverse sensory structures able to detect a wide range of signals. To test our hypothesis, we removed the flagella of nurses and observed their behavior in isolation and in free-foraging colonies. We found that individually-isolated flagella-less bees under constant laboratory conditions show robust circadian rhythms in locomotor activity. In observation hives, flagella-less bees cared for the brood, but were more active during the day. By contrast, sham-treated bees were active around the clock as typical of nurses. Detailed video recordings showed that the brood-tending behavior of flagella-less and sham-treated bees is similar. These observations suggest that the difference in the patterns of brood care activity is not because the flagella-less bees did not contact the brood. Our results suggest that nurses are able to find the brood in the dark environment of the hive without their flagella, perhaps by using other sensory organs. The higher activity of flagella-less bees during the day further suggests that the flagella are involved in mediating the brood signals modulating plasticity in the circadian system.

1. Introduction

Honey bee workers show age-related division of labor. Young bees (typically 2–12 days of age) typically care for ("nurse") the brood inside the hive, whereas older bees (typically ⩾ 20 days of age) forage for pollen and nectar outside the hive (Seeley, 1982; Winston, 1987). Nurses and foragers differ in their behavior, physiology, immune system, and tissue specific gene expression patterns (Ament et al., 2008; Ben-Shahar et al., 2003; Bloch et al., 2009; Durst et al., 1994; Fluri et al., 1982; Huang et al., 1991; Lockett et al., 2012; Moore et al., 1998; Schulz and Robinson, 1999; Toth and Robinson, 2005; Whitfield et al., 2003; Vance et al., 2009; Wilson-Rich et al., 2008; Winnington et al., 1996; Wolschin and Amdam, 2007). The nurse-to-forager transition can be accelerated, delayed or even reverted depending on the needs of the colony (Bloch and Robinson, 2001; Huang and Robinson, 1996; Robinson et al., 1992; Winston, 1987; Winston and Fergusson, 1985).

Nurse bees and foragers also differ in their pattern of activity. Nurses typically care for the brood around the clock with similar levels of clock gene transcripts throughout the day, whereas foragers exhibit strong circadian rhythms in behavior and brain clock gene expression (reviewed in Bloch, 2010). Circadian rhythmicity is context-dependent: foragers that reverted to nurse brood switched back to activity with attenuated molecular and behavioral circadian rhythms (Bloch et al., 2001; Bloch and Robinson, 2001); nurses showed circadian rhythms in locomotor activity and brain clock gene expression shortly after transfer to constant laboratory conditions (Shemesh et al., 2010, 2007). Nurses that were caged inside an observation hive on a brood-less comb or in brood-less cages outside the hive showed circadian rhythms in activity. By contrast, their same-age full sisters who could directly contact the brood were active around the clock as is typical of nurse bees (Shemesh et al., 2010). These observations suggest that direct contact with the brood modulates the plasticity in circadian

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rhythms, but the specifics of the brood signals and the sensory modalities involved in their detection remain elusive.

The honey bee antennae are pivotal sensory organs. The flagellum of the bee antenna is densely covered with numerous sensory structures that are used for detecting olfactory, gustatory and mechanical stimuli (Esslen and Kaissling, 1976; Goodman, 2003). The antennae are specifically important for cavity-dwelling insects such as honey bees that spend much of their time inside a dark hive in which visual information is limited. We therefore hypothesized that the antennae are involved in mediating the brood influence on circadian rhythms in nurse bees. To test this hypothesis we surgically removed the antennae flagella of nurse bees and monitored their behavior in the natural social context of the colony and in isolation under constant laboratory conditions.

2. Materials and methods

2.1. Bees

We kept honey bee colonies according to standard beekeeping techniques in the bee research facility at the Edmond J. Safra campus of the Hebrew University of Jerusalem, Givat-Ram, Jerusalem, Israel. The bees were derived from a mixture of races of *Apis mellifera* L. prevalent in Israel. The colonies used for this study were headed by naturally mated queens: Colonies H10-17 and H11-04 were used for the two trials of Experiment 1; colonies H10-03 and H11-10 were used for the two trials of Experiment 2, and colony H11-12 was used for Experiment 3.

To obtain newly emerged bees, we transferred honeycombs with sealed brood and without adult bees to an incubator (~33 °C, RH ≈ 60%). On the next day, we marked all the newly-emerged bees (age, 0–24 h) on their thorax with a paint dot and reintroduced them into their mother colonies. For our experiments, we collected nurse-age bees (age, 5–7 days) from a honeycomb containing unsealed brood. Nurse bees at this age typically care for the brood around the clock while in the hive, but show circadian rhythms in locomotor activity and clock gene expression shortly after transfer to a constant laboratory environment (Shemesh et al., 2007, 2010).

2.2. Treatments

Nurse bees were subjected to one of three treatments: (1) *Flagella-less* (F-): we collected nurse bees from the brood area of a field colony and put them in modified 50 ml plastic test tubes (Miniplast Ein-Shemer) with perforated caps for ventilation. Four bees were placed in each tube and provisioned with 50% (w/w) sucrose solution. Before the surgical procedure, the tubes were embedded in shredded ice until the bees had stopped moving. The flagella were amputated at their base with scissors (Fig. 1D, left panel). (2) *Sham-treated*: we collected and chilled the bees as in treatment (1). When the bees were cold-anesthetized, we touched the flagella with the scissors, but did not cut or damage the tissue (Fig. 1D, right panel). (3) *Control*: the bees were collected and handled as in treatment (1) but were not chilled or manipulated.

2.3. Experiment 1. The influence of flagella removal on circadian rhythms in locomotor activity for individually isolated bees in constant laboratory conditions

We collected nurse-age bees (age, 5–6 days) from the brood area of a brood comb in a field colony and subjected them to one of the 3 treatments described in Section 2.2 (Trial 1: Flagella-less, $N = 33$; sham-treated, $N = 7$; control, $N = 10$; Trial 2: Flagella-less, $N = 60$; sham-treated, $N = 30$; control, $N = 25$). Following the treatments, each bee was placed individually in a cage made of a modified Petri dish provisioned with a tube containing 50% (w/w) sucrose solution. All the caged bees were transferred to an environmental chamber (~30 °C, RH ≈ 60%) illuminated with dim red light. Locomotor activity was monitored automatically for 5 or 6 days (Trial 1 and Trial 2, respectively) with the ClockLab data acquisition system (Actimetrics Inc., Evanston, IL, USA) with light-sensitive black and white Panasonic WV-BP334, 0.08 lux CCD cameras (4 cameras, each recording the activity from 30 cages), and a high-quality monochrome image acquisition board (IMAQ 1409, National Instruments; as in Shemesh et al., 2007; Yerushalmi et al., 2006). Locomotor activity was monitored continuously at a frequency of 1 Hz. Circadian rhythmicity was determined for the first 4 days in the laboratory, starting at 16:00 on day one (bees were introduced to the environmental chamber at around 14:00). A χ^2 periodogram analysis with 10 min bins (ClockLab circadian analyses software, Actimetrics) was used for the analyses of circadian rhythms. The power of rhythmicity (the height of the periodogram peak above the $P = 0.01$ significance threshold) was determined for each bee with a statistically significant circadian rhythm ($P < 0.01$) as described in Yerushalmi et al. (2006). Given the small sample size in some of the groups, we used the non-parametric two-tailed Kruskal–Wallis test ($\alpha = 0.05$) for determining the influence of the treatments on the power of circadian rhythms in locomotor activity. To obtain data comparable to that in the hive (see below), we performed an additional analysis in which we compared the level of locomotor activity during the subjective day and the subjective night (data recorded from 04:00 on day two, to 04:00 on day three, see Fig. 1A). The hours of the subjective day and night were determined according to the times of sunrise and sunset in Jerusalem as reported in the http://www.timeanddate.com website. Sunrise and sunset were 6:07 and 19:14, respectively, on Trial 1 (August 23, 2010), and 5:42 and 19:47, respectively, on Trial 2 (July 11, 2011). Accordingly, we set day hours to be 6:00–19:00 for Trial 1 and 6:00–20:00 for Trial 2. The two-tailed Wilcoxon Signed Ranks test ($\alpha = 0.01$) was used to compare the levels of activity during the subjective day and the subjective night.

2.4. Experiment 2. The influence of flagella removal on brood care and overall activity in an observation hive

We placed a small colony, consisting of a queen and ~4000 workers in a two-frame observation hive with transparent walls. The upper honeycomb frame consisted of empty cells for the queen to lay eggs, and some pollen cells. The lower frame was filled with pollen and honey. The workers and the queen were taken from the same source colony. The observation hive was housed in an environmental chamber (~30 °C, RH ≈ 60%) illuminated with dim red light, and was connected to the outside with a 1.5 m "S"-shaped plastic tube covered with aluminum foil. The shaping of the entrance tube allowed the bees to exit the hive and freely forage for food, but prevented direct exposure of the inner parts of the observation hive to sunlight. The bee population was composed of a queen and three cohorts of workers: ~1000 nurses, ~1500 foragers and ~1500 newly-emerged (0–24 h of age) bees. Triple-cohort-colonies of this kind have worker population demography with caste and age structure analogous to that in typical field colonies. The workers in triple-cohort-colonies show normal behavioral development from in-nest to foraging activities (Giray and Robinson, 1994). We restricted the queen to the upper frame by placing a small metal queen-excluder between the two frames. The excluder enabled workers, but not the queen, to move between the two frames. Thus, queen egg-laying and brood rearing, were limited to the upper frame, facilitating the observations on brood care behavior. After approximately 2 weeks, during which the bees