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Differences in the sleep architecture of forager and young honeybees (*Apis mellifera*)

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SUMMARY

Honeybee (*Apis mellifera*) foragers are among the first invertebrates for which sleep behavior has been described. Foragers (typically older than 21 days) have strong circadian rhythms; they are active during the day, and sleep during the night. We explored whether young bees (~3 days of age), which are typically active around-the-clock with no circadian rhythms, also exhibit sleep behavior. We combined 24-hour video recordings, detailed behavioral observations, and analyses of response thresholds to a light pulse for individually housed bees in various arousal states. We characterized three sleep stages in foragers on the basis of differences in body posture, bout duration, antennae movements and response threshold. Young bees exhibited sleep behavior consisting of the same three stages as observed in foragers. Sleep was interrupted by brief awakenings, which were as frequent in young bees as in foragers. Beyond these similarities, we found differences in the sleep architecture of young bees and foragers. Young bees passed more frequently between the three sleep stages, and stayed longer in the lightest sleep stage than foragers. These differences in sleep architecture may represent developmental and/or environmentally induced variations in the neuronal network underlying sleep in honeybees. To the best of our knowledge, this is the first evidence for plasticity in sleep behavior in insects.

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Key words: *Apis mellifera*, sleep, response threshold, behavioral development, insect.

INTRODUCTION

Sleep is a behavioral state that is regulated by two main mechanisms: the circadian clock and sleep homeostasis. The circadian clock plays a crucial role in the timing and consolidation of wakefulness and sleep, whereas the homeostatic mechanism reflects the need for sleep that accumulates during periods of wakefulness and dissipates during sleep (Dijk et al., 1999; Tobler, 2005).

Accumulating evidence suggests that rest behavior in many invertebrates meets the criteria for defining it as ‘sleep’ (Tobler, 1983; Tobler and Stalder, 1988; Hendricks et al., 2000a; Shaw et al., 2000; Ramón et al., 2004; Stephenson et al., 2007). The best studied invertebrate model is the fruit fly *Drosophila melanogaster*, in which a combination of behavioral, neurophysiological and genetic analyses have linked molecular and neuronal processes to sleep behavior, demonstrating the usefulness of invertebrate models in the study of sleep biology (Greenspan et al., 2001; Hendricks and Sehgal, 2004; Shaw, 2003). Sleep in flies is similar to mammals in the following ways: (1) consolidated periods of immobility are homeostatically regulated, (2) the presence of an elevated arousal threshold (Hendricks et al., 2000b; Shaw et al., 2000; Huber et al., 2004), (3) characteristic brain electrical activity (Nitz et al., 2002; Andretic et al., 2005; van Swinderen et al., 2004), (4) a characteristic brain gene expression signature (Cirelli and Tononi, 1999; Cirelli et al., 2004; Cirelli et al., 2005; Zimmerman et al., 2006), and (5) sleep is increased by antihistamines and reduced by caffeine and other stimulants (Shaw et al., 2000; Andretic et al., 2005). In both mammals and flies, sleep persists in the absence of a functioning circadian clock, demonstrating the importance of non-circadian mechanisms in the homeostatic regulation of sleep (Mistlberger et al., 1983; Shaw et al., 2000). Furthermore, as in mammals (Tobler, 2005), sleep rebound in insects is not affected by levels of activity during sleep deprivation (Shaw et al., 2000; Sauer et al., 2004).

Honeybees (*Apis mellifera*) are among the first invertebrates for which sleep behavior has been described (Kaiser and Steiner-Kaiser, 1983). Honeybee foragers exhibit sleep, both in their natural hive environment, and when isolated individually in the lab. Foragers sleep in a posture characterized by a relaxation of the thorax, head and antennae. This characteristic posture is associated with a decrease in muscle tonus and body temperature, and an increase in response threshold, measured both neurophysiologically and behaviorally (Kaiser and Steiner-Kaiser, 1983; Kaiser, 1988). It was further suggested that deep sleep in foragers (determined as periods lacking antennal movements) is correlated with rhythmic electrophysiological activity in the brain, including the mushroom bodies (Schuppe, 1995). Foragers deprived of sleep for 12 h showed a rebound the next day; they increased the duration of antennal immobility, one of the characteristics of sleep in bees (Sauer et al., 2004). This suggests that sleep in honeybee foragers is homeostatically regulated, similar to sleep in mammals (Tobler, 2005), birds (Martinez-Gonzalez et al., 2008) and flies (Hendricks et al., 2000a; Shaw et al., 2000).

Foragers are relatively old workers, have strong circadian rhythms, and sleep during the night. However, circadian rhythms are not typical to all worker bees; young bees typically perform various in-hive activities around-the-clock, with no circadian rhythms (Crailsheim et al., 1996; Moore et al., 1998). Young bees that are isolated individually, or kept in small groups in constant conditions, have no circadian rhythms in locomotor activity during their first 3–14 days (Moore, 2001; Meshi and Bloch, 2007; Bloch, 2008). Their around-the-clock pattern of activity raises the question

of whether young bees sleep as foragers do. It is possible that young honey bees do not sleep at all, which would make them an exception in the animal kingdom (Lyamin et al., 2005; Rattenborg et al., 2004). An alternative hypothesis is that young bees do sleep like foragers, but distribute their sleep throughout the day. A third hypothesis is that young bees sleep, but their sleep is essentially different from that of foragers.

In order to distinguish between these hypotheses, we characterized the sleep behavior of individually isolated young bees, and compared it to that of sister foragers. Our detailed behavioral observations and analyses of response thresholds lend weight to the third hypothesis. We show that young honeybees exhibit sleep behavior which is composed of the same stages observed in foragers, but that their sleep dynamics differ.

MATERIALS AND METHODS

Bees

We kept honeybee colonies according to standard beekeeping techniques in a 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 European races of *Apis mellifera* L. typical to this region. Two of the source colonies (colonies S23 and S25) were headed by a queen instrumentally inseminated with semen from a single (different) drone. Single-drone insemination helps reduce genetic variability between bees within each experiment [average coefficient of relatedness between workers=0.75 because of haplodiploidy (Page and Laidlaw, 1988)]. Colonies H3 and H12 were headed by a naturally mated queen (queens typically mate with 10–20 drones).

We identified foragers by the presence of pollen loads in their corbiculate. We only collected foragers with undamaged wings. To obtain 1-day-old bees, we removed honeycomb frames containing pupae (sealed in cells) from source colonies in the field. We transferred the frames immediately to a light-proof container, which we placed inside a dark incubator [32±0.5°C; relative humidity (RH)=55±5%; monitored with an Onset HOBO (Contoocook, NH, USA) H01-001-01 data logger]. We collected the newly emerging bees the next day, when they were 0–24 h old.

Video recording

We video recorded bees from three different source colonies. In the experiments with bees from colonies H3 and H12, we marked newly emerged bees with a paint-dot on their thorax, and introduced them to a foster colony that was housed in a two-frame observation hive (with transparent glass walls), placed in a constantly dark environmental chamber (29±1°C; RH 50±5%). We connected the observation hive to the outside by a clear plastic tube (length 60 cm, diameter 3 cm). After 48 h in the observation hive, we collected two marked callow bees, as well as two foragers from the same source colony (‘genotype’). In the experiment with colony H3, we collected the focal bees between 15:00 h and 17:00 h, whereas in the experiment with colony H12, we collected them between 7:30 h and 8:00 h. These time variations did not appear to influence the observed behavior, since the results from the two colonies were essentially similar. Each of the four bees was placed in an individual small cage (7.5×2.5×2.5 cm). The cages were made of transparent glass, and were padded on one wall with a panel of Palziv substrate. We provided each cage with a tube of sugar syrup (50%, w/w). We placed the cages in a dark environmental chamber (28±1°C; RH 55±5%), which was illuminated by dim red light that bees cannot see (von Frisch, 1967). Since some of the callows from colonies H3 and H12 atypically appeared to have a circadian rhythm, we monitored circadian rhythms in locomotor activity (see below) before performing sleep observations, in the last experiment with colony S25. Importantly, the callow bees from the three colonies were similar in age (3 days old). After monitoring the bees for 48 h, we transferred two foragers (with robust circadian rhythms), and two callows (that were active around-the-clock with no circadian rhythms) to a dark environmental chamber for video recording and sleep analysis. For the sleep analysis, we video recorded the bees using an infrared-sensitive camera (Sony TRV 75E), over successive 24 h periods. We started recording after the bees had acclimatized to the lab for 2 h. We video recorded 64 bees, eight groups of four bees (N=32 bees) from colony H12, and four groups of four bees (N=16 bees) from colonies H3 and S25, each.

Analysis of video records

We used Pinnacle Studio (version 9.1; Pinnacle Systems Inc., Mountain View, CA, USA) software to sample the video records to a computer. We omitted from our analysis records of bees that died during the experiment (N=2), were not visible throughout most of the experiment (N=4), were continually active (N=2), or repeatedly slipped along the glass wall during their rest period (N=8).

Table 1. Behavioral categories defining arousal/sleep stages in honeybees

Category	Abbreviation	Description
Active	A	The bee walked over a distance greater than twice her body size, during a 1 min period.
Immobile–active	IA	The bee moved her legs, made >20 antenna movements/min, or >5 head movements/min, but did not walk over a distance more than twice her body size (Fig. 1A).
Grooming	G	The bee cleaned her body parts or proboscis, by rubbing her legs over them, but did not walk over a distance more than twice her body size.
First sleep stage	FS	The bee stayed in the same location, without moving her legs. The abdomen and thorax were clearly raised above the substrate, and the antennae were extended at an angle of ~180° between the pedicle and the scape (Fig. 1B).
Second sleep stage	SS	Same as FS, but body posture was more relaxed, and the angle of the antennae was ~90° (Fig. 1C).
Third sleep stage	TS	Same as SS, but the abdomen and thorax were adjacent to the substrate (reduced muscle tonus), and the angle of the antennae was <90° (Fig. 1D).
Unknown sleep stage	US	The bee stayed in the same location, and was clearly in a sleep stage, but it was not possible to assign her to a specific stage (for example, when bees faced the camera with their ventral side, it was impossible to determine whether their abdomen and thorax were raised above the substrate).

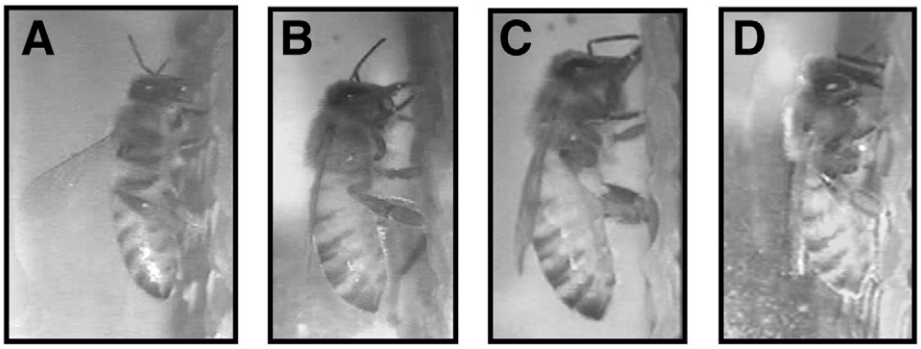

Fig. 1. Body posture of honeybee workers in various arousal states. Each photograph is a single frame taken from continuous 24 h video recordings. (A) Immobile–active state (IA) – the bee stays in the same place, the thorax, abdomen and head are clearly raised above the substrate. This bee is moving her wings. (B) First sleep stage (FS) – the abdomen and thorax are clearly raised above the substrate, and the antennae are extended at an angle of 90–180° between the pedicle and the scape. (C) Second sleep stage (SS) – the body is typically more adjacent to the substrate, and the antennae are extended at an angle of ~90° between the pedicle and the scape. (D) Third sleep stage (TS) – the muscle tonus is reduced, and the body is adjacent to the substrate. The angle between the pedicle and scape <90°, with the antennae tips typically touching the substrate. For more details, see Table 1.

We defined seven behavioral states that we used for analyzing the remaining 48 records. Three characterized awake bees, and the other four sleeping bees. We assigned a single prevailing behavioral category (see Table 1 and Fig. 1 for definitions of behavioral states) for every minute using the following heuristic. If the bee showed 'active' (A) behavior during any part of the minute, we labeled the entire minute as 'A'. Otherwise, if the bee showed 'immobile–active' (IA) and/or 'grooming' (G) behavior, we labeled the minute as 'IA' or 'G' respectively, according to the predominant behavior in that minute (even if the bee also exhibited sleep behavior during this minute). In minutes in which the bees did not show any of the awake categories, we assigned the most prevailing sleep stage. In addition, we counted the number of antenna movements for each minute for sleeping bees. We defined a 'bout' as a continuous episode in the same behavioral state.

Analysis of response threshold

We determined the response threshold of bees to light. We placed each focal bee in a small cage that was placed in a separate dark chamber (23×6×20 cm), in an experimental room (28.5±0.5°C; RH=50±5%). This enabled us to expose the focal bees to light without disturbing bees in neighboring chambers. We started each experiment by calibrating the light intensity. We placed the light source (an optic glass fiber; Schott-Fostec, LLC, Elmsford, NY, USA) 2 cm away from a light meter (LI-185A, Li-Cor, Lincoln, NE, USA), measured the light intensity of each illumination level three times, and calculated the mean value. After this calibration, we tested the response to light of the focal bees at various arousal states. We illuminated the lateral part of the bee's head from a distance of 2 cm (as in the calibration of the light intensities), for a period of exactly 10 s. We increased the light intensity at intervals of 5 s between light stimuli, and video recorded the bee throughout the entire procedure. We used 20 discrete levels of light intensity. We defined a response as the bee turned toward the light source, and/or moved her head more than twice during, or immediately after (<1 s) the stimulus. The response threshold for each bee was the lowest light intensity that triggered a response.

We limited our analysis of response threshold to 3-day-old bees with no circadian rhythms, and foragers with robust circadian rhythms. In order to determine circadian rhythms, we monitored bee locomotor activity during the 2 days preceding the analysis (see below). In each experiment, we tested 20 foragers and 20 callows, out of 30 bees for which we monitored locomotor activity. We conducted seven trials with bees from colony S23 (N=58 bees tested), and 12 trials with bees from colony S25 (N=107 bees tested). Each trial started approximately 4 h after sunset, and lasted about 6 h. The response threshold analysis for foragers and callows at the different arousal states was carried out at approximately the same time of day. Thus, variation in circadian time cannot account for the observed variation in response threshold.

Locomotor activity

We placed each bee in a separate glass cage (as described above) in an environmental chamber (28±1°C; RH= 45±5%), and monitored locomotor activity with the ClockLab data acquisition system (Actimetrics Co., Wilmette, IL, USA). We used a high-quality monochrome image acquisition board (IMAQ 1409, National Instruments Co., Austin, TX, USA), and a light-sensitive black and white Panasonic WV-BP334, 0.08 lux CCD camera. The system collected the data continuously, at a frequency of 1 Hz, as described by Yerushalmi et al. (Yerushalmi et al., 2006). Circadian rhythms in activity were assessed with the ClockLab software.

Statistical analyses

In order to test whether the sleep stages differed in bout duration and amount of antenna movement, we carried out a separate statistical test on the data set of each individual bee (we included only bees with N>10 samples for each sleep stage; foragers, N=17; callows, N=24). We used non-parametric tests, since these variables were not normally distributed [Kruskal–Wallis analysis with a correction for ties, followed by multiple comparisons (Siegel and Castellan, 1988)]. In addition to the individual analyses, we ran three-way ANOVAs to determine the influence of colony, age (callow *vs* foragers) and sleep stage on bout duration and antenna movement. For these analyses we used the average values calculated for each individual bee, and used a data set that included the values of all individuals. We carried out complementary t-tests for each sleep stage to determine whether antenna movement and bout duration differed between callows and foragers. We used non-parametric analyses to determine

whether the response thresholds differed between arousal states (Kruskal–Wallis test), and between foragers and callows for each arousal state (Mann–Whitney test).

We used a first-order Markov chain to model the likelihood of transitions between behavioral states. A behavioral transition was defined as a change in the behavioral state displayed between two consecutive minutes. We constructed a separate transition matrix for each bee, in which each row represents transitions originating from one behavioral state (X) to all other states. Each cell represents the proportion of transitions to behavior Y, out of all transitions originating from behavior X. In order to examine whether the transition pattern of callows and foragers differed, we conducted a 'leave-one-out cross-validation' (LOOCV) analysis. We removed the data of one bee, and computed two separate transition matrices ($T_{X,Y}$) for the remaining foragers and callows (denoted as the 'foragers' transition model', and the 'callows' transition model', respectively). These models were based on the average transition matrices of each group member. We calculated the likelihood that the transition pattern of the removed bee originated from each model using the following formula:

$$L = \sum_{X,Y} \log(T_{X,Y}) \times B_{X,Y} ,$$

where $T_{X,Y}$ is the transition model (of callows or foragers), and $B_{X,Y}$ is the actual number of transitions from X to Y observed in the bee we removed. The removed bee was assigned to the group that yielded the higher likelihood value (L). We repeated this procedure for each bee, and summarized the number of individuals correctly assigned to their group (e.g. foragers assigned to the group of foragers). We determined the statistical significance of this analysis by calculating the probability distribution of correct assignments based on randomly divided groups (similar in size to the groups of callows and foragers in the analysis above). We repeated the LOOCV procedure 100 000 times, and recorded, for each trial, the number of correctly assigned bees (see Fig. S1 in supplementary material). The P-value is the

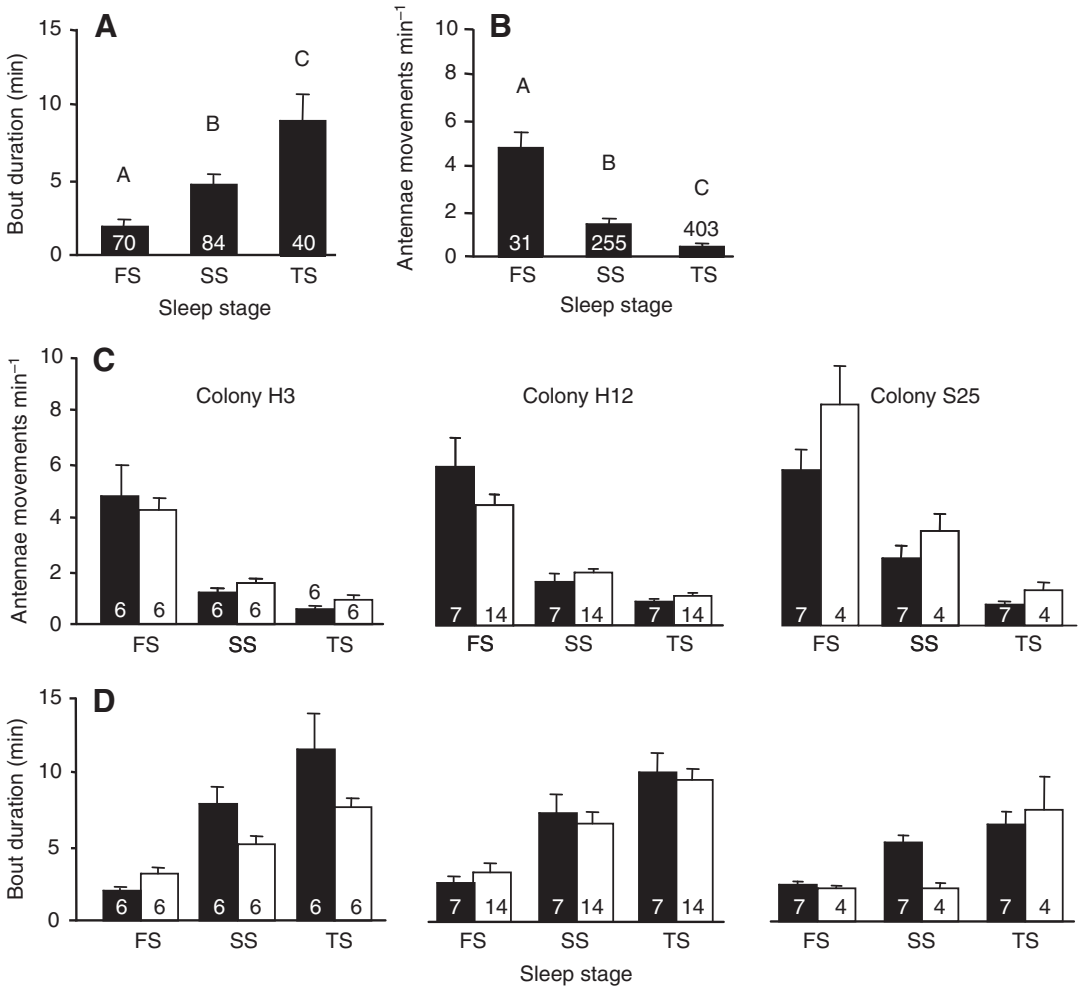

Fig. 2. Ethological characterization of sleep stages in honeybees. (A) Bout duration at different sleep stages (mean ± s.e.m.), for a representative forager. Similar results were obtained for 15 additional foragers (N=21–194 bouts/bee). (B) Antennae movements at different sleep stages (mean ± s.e.m.), for a representative forager. Similar results were obtained for 16 additional foragers (N=131–906 min/bee). Different capital letters indicate statistically significant differences. (C) Group summary of antennae movement data for all foragers and callows. There was no significant difference between foragers and callows (see supplementary material Table S1). (D) Summary of bout duration data for all foragers and callows. Bout duration differed between foragers and callows (see supplementary material Table S2). Numbers within or above bars are the sample sizes. Filled bars, foragers; open bars, callows; left panels, colony H3; middle panels, colony H12; right panels, colony S25. FS, first sleep stage; SS, second sleep stage; TS, third sleep stage.

probability of having at least the number of correct assignments obtained in our analysis of callows and foragers.

In order to find which transitions contributed most to the observed differences between foragers and callows (see Results), we performed six separate additional LOOCV analyses, each one based on transitions originating from one behavioral state (one row in the transition matrix; N=6). We used a Bonferroni correction for multiple comparisons to correct our P-values.

RESULTS

Sleep behavior of forager and callow bees

We carried out detailed video analyses of the sleep behavior of 20 foragers and 24 callows from three source colonies. We found that during consolidated periods of immobility, foragers exhibited sleep behavior which was similar to that described in previous studies (Kaiser, 1988; Sauer et al., 2003; Sauer et al., 2004). Our experimental protocol, in which the bees could move freely inside their cages, allowed us to identify and characterize three distinct sleep stages differing in body posture and antennae position (Fig. 1; see Table 1 for details). These three sleep stages were termed 'first sleep stage' (FS), 'second sleep stage' (SS) and 'third sleep stage' (TS). Bout duration differed between the three sleep stages, and was typically shortest for the first sleep stage, and longest for the third sleep stage (Kruskal–Wallis tests, N=31–194 bouts/bee; P<0.05 in 16 out of 20 foragers from three different colonies; Fig. 2A). The three sleep stages also differed in the number of antenna movements per minute. The highest level of antenna activity was observed in first sleep stage, and the lowest in third sleep stage (Kruskal–Wallis tests, N=131–906 min/bee, P<0.05 in all 20 foragers; Fig. 2B).

Callow bees exhibited the same three sleep stages as described above for foragers. Again as in foragers, the three sleep stages differed in their bout duration (N=36–237 bouts, P<0.05 in 19 out of 24 bees, the P-value was 0.052 for an additional bee; a similar trend was observed for the remaining four bees; Fig. 2C), and number of antenna movements per minute (Kruskal–Wallis tests, N=95–1094 min/bee, P<0.05 in all 24 callows; Fig. 2D).

Foragers and callows in the same sleep stage did not differ in the number of antenna movements [three-way ANOVA, P=0.5 for the comparison of foragers and callows ('age'); supplementary material Table S1; Fig. 2C]. In the analysis of bout duration we found significant differences between foragers and callows, and a significant interaction between age and sleep stage (three-way ANOVA, age effect: P=0.037; 'age × sleep stage' effect: P=0.035; supplementary material Table S2; Fig. 2D). In order to identify which of the sleep stages differed between callows and foragers, we ran complementary t-tests and found that in colony H3 the bout duration of the first sleep stage was longer in callows than in foragers, whereas in colony S25 in the second sleep stage the bout duration was shorter in callows (t-test, P<0.05; Fig. 2D).

We found no consistent differences in the percentage of time that foragers and callows spent sleeping (supplementary material Fig. S2). In the experiment with bees from colony H3, callows slept more than foragers (t-test, P<0.05), whereas in colony S25 callows slept less (P<0.05). It is not clear whether this variation across trials reflects genetic differences between colonies, or stems from variability in experimental procedures (lab *vs* hive environment before monitoring sleep; see Materials and methods).

In an analysis of all motionless bees (including all behavioral states besides active), we found that bees slept in 80% of all bouts in which they did not move for ⩾5 min. This suggests that lack of movement for >5 min can serve as an indirect measure of sleep in studies of locomotor activity.

Response threshold

The response threshold varied with arousal states for both forager and callow bees (Kruskal–Wallis tests, P<0.0001, followed by one-tailed multiple comparisons, P⩽0.05 for both foragers and callows;

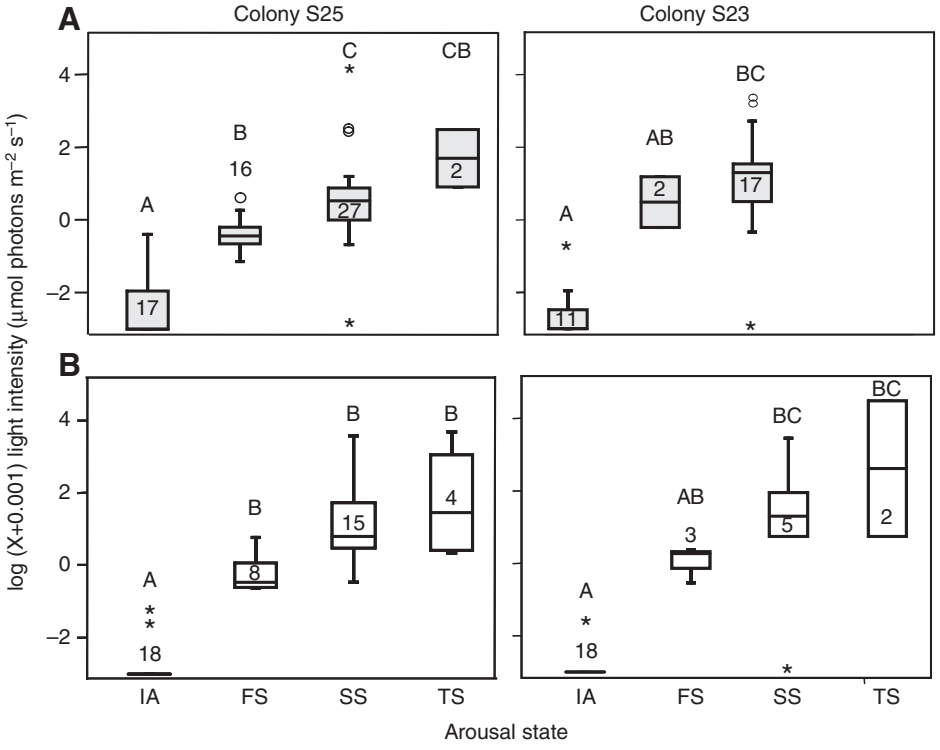

Fig. 3. Response threshold of bees in different arousal states. (A) Foragers. (B) Callows. Right panels: colony H23; left panels: colony S25. The response threshold to a light pulse differed significantly across arousal states, but not between callows and foragers. The central horizontal line in each box indicates the median, the box borders indicate the 75th and 25th percentile, and the error bars outline the range. Circles indicate outliers; values in a range spanning between 1.5 and 3 box lengths. Asterisks indicate extreme values: values in a range spanning more than three box lengths. Sample size (number of bees) is shown within or above each box. Different capital letters indicate arousal states that are statistically different (Kruskal–Wallis tests followed by multiple comparisons, P<0.0001 for both foragers and callows). FS, first sleep stage; SS, second sleep stage; TS, third sleep stage. Response threshold, at TS, was not determined for foragers in colony S23.

Fig. 3). Awake, immobile–active bees responded to very low light intensities (<0.05 µmol l photons m^{-2} s^{-1}), whereas bees in the third sleep stage typically responded only to intense light (>1000 µmol l photons m^{-2} s^{-1}). The responses of bees in the first and second sleep stages were between these two extremes (Fig. 3). There was no significant difference in the response threshold of foragers and callows in the same arousal state (Mann–Whitney tests, $P>0.085$ for all behavioral states).

The dynamics of sleep behavior

Foragers were typically active throughout the subjective day, and limited their sleep to the subjective night (Fig. 4A). The temporal pattern of activity was more variable in callow bees. Callows from colony S25 were typically active around-the-clock, with periods of sleep behavior distributed throughout the day (N=6). A similar pattern of activity was also observed in 45% (N=9) of the callows from the two other source colonies (Fig. 4B). By contrast, in 55% (N=11) of the callows from these two colonies, sleep behavior tended to be more common during the subjective night, reminiscent of the pattern in foragers (Fig. 4C). All sleep bouts, in both foragers and callows, were interrupted by brief episodes of awakening (transitions from sleep stages to immobile–active or grooming; Fig. 4A–C). We could not determine clear sleep cycles as those commonly reported for mammals. The average sleep bout duration was shorter in foragers (two-way ANOVA, age effect: P=0.04; colony effect: P<0.001; 'age × colony' effect: P=0.04; Fig. 4D). Consistent with this trend, the average number of bouts per day was higher in foragers than in callows (two-way ANOVA, age effect: P=0.016; colony effect: P=0.4; 'age × colony' effect: P=0.002; Fig. 4E).

We further characterized the likelihood of transitions between behavioral states, using first-order Markov chain analysis (see Materials and methods). Both foragers and callows typically passed from the active state to either the grooming or immobile–active state (Fig. 5A,B). The transition to sleep was gradual, typically through the first sleep stage, less frequently through SS and hardly ever directly to the third sleep stage. When bees returned from sleep to wakefulness, they almost always did so by passing through the immobile–active or grooming state, rather than by passing directly to the active state (Fig. 5A,B). However, we found that the transition matrices of foragers and callows differed significantly (LOOCV analysis, P=0.002; see Materials and methods). When examining the overall differences between the transition patterns of foragers and callows (Fig. 5C), we found that the largest differences were in the transitions from the second and third sleep stages to the other behavioral states. The likelihood of transitions from the second and third sleep stages, but not from activity (A, IA and G) and the first sleep stage, to the other states differed between foragers and callows (P=0.009 for SS; P=0.006 for TS, LOOCV analysis with a Bonferroni correction; Fig. 5C). Callow bees reverted from the second and third sleep stages to the first stage more commonly, whereas foragers typically exited sleep from these stages and entered either the immobile–active or grooming states (Fig. 5A–C). Callows and foragers did not differ in the number of brief awakenings (with the exception of colony S25; Fig. 6A), however, callows passed more often between the sleep stages than foragers (two-way ANOVA, age effect: P=0.001; colony effect: P=0.81, interaction: P=0.89; Fig. 6B, see also Fig. 5).

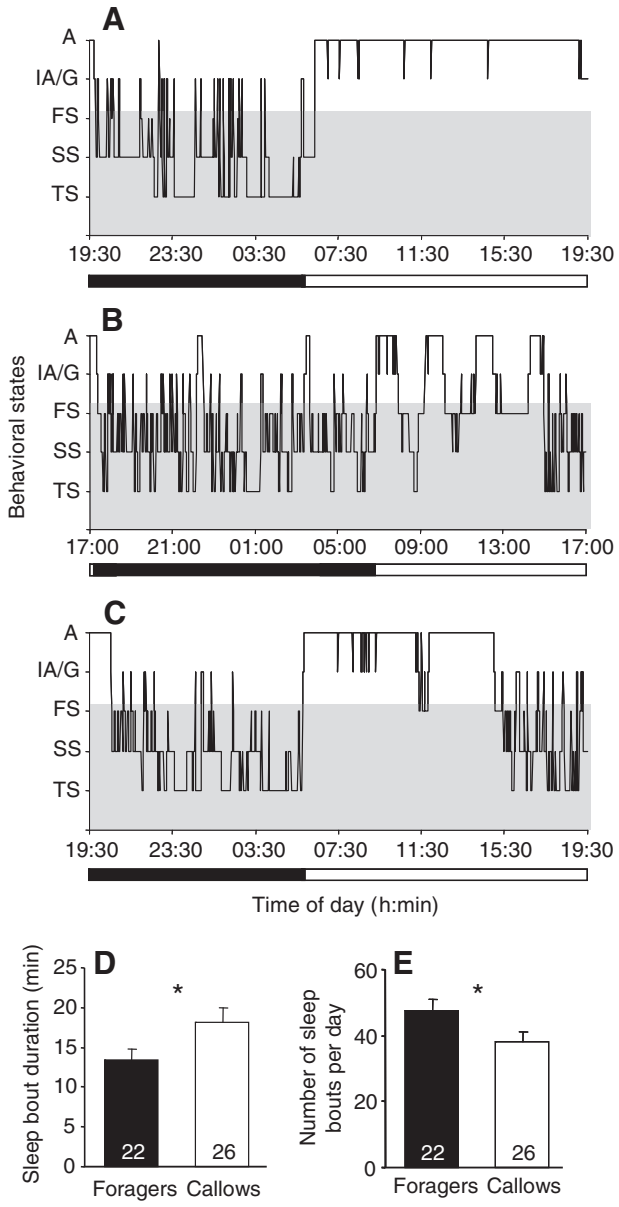

Fig. 4. Transitions between behavioral states throughout the day. (A) An example of a forager. (B) An example of a callow with apparent around-the-clock activity. (C) An example of a callow with apparent circadian rhythm in activity. The three bees are from colony H3. Note that the two callow bees manifested all three sleep stages. A, active; IA/G, immobile–active or grooming; FS, first sleep stage; SS, second sleep stage; TS, third sleep stage. For details on behavioral states see Fig. 1 and Table 1. Gray background indicates sleep stages; white background indicates awake states. The horizontal bars at the bottom of the plots depict the subjective time: black bars, subjective night; hatched bars, subjective day. (D) Sleep bout duration (mean ± s.e.m.). Bout duration differed between foragers and callows (two-way ANOVA, age effect, P=0.04) (E) Number of sleep bouts per day (mean ± s.e.m.). The number of sleep bouts differed between foragers and callows (two-way ANOVA, P=0.016). Numbers within boxes indicate the sample size (pooled from the three colonies). Filled bars, foragers; open bars, callows. Asterisks indicate a statistically significant difference between foragers and callows.

DISCUSSION

We characterized three distinct sleep stages in honeybees that differ in body and antennae posture, bout duration, antenna movements, and response threshold. We further provided the first analysis of sleep in young bees, which we found to include the same sleep stages observed in foragers, even in individuals that were active around-

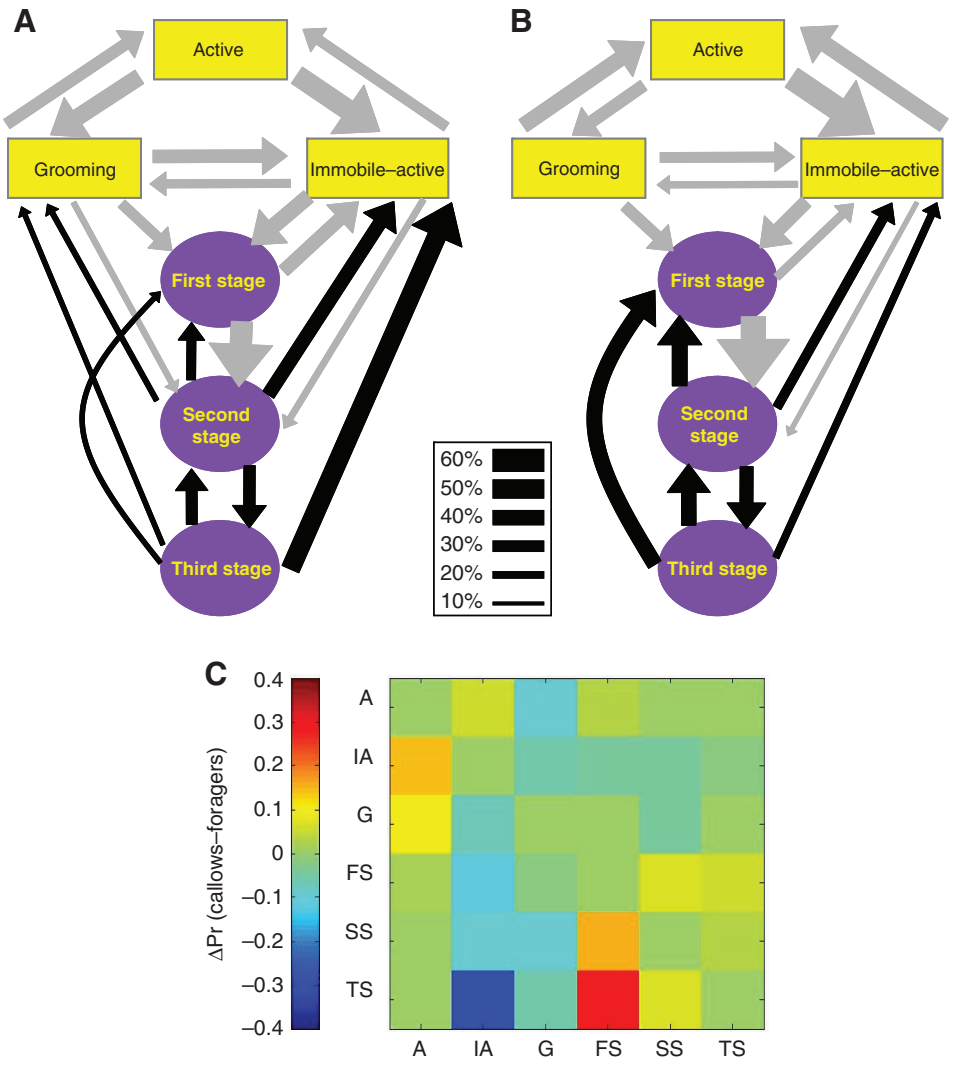

Fig. 5. The dynamics of transitions between behavioral states. Schematic representation of average transition matrix for foragers (A), and callows (B). The width of arrows is proportional to the average probabilities of transitions from each behavioral state to all other states. The patterns are analyzed from a first-order Markov chain (see Materials and methods), by using 3127 and 3928 behavioral transitions, from 16 foragers and 17 callows, respectively (pooled from colonies H3, H12 and S25). The overall matrices of callows and foragers differ from each other (LOOCV analysis, P=0.002; supplementary material Fig. S1). Transitions that are statistically significantly different between callows and foragers are highlighted in black. For clarity, we show only transitions with average probability >0.1. See Fig. 1 and Table 1 for more details on behavioral states. (C) The difference between average transition probability of foragers and callows. ΔPr=the probability matrix of foragers subtracted from that of callows. Blue colors represent transitions that are more frequent in foragers; red colors represent transitions that are more frequent in callows (see scale).

the-clock. However, the sleep architecture of young bees differed from that of foragers, which may suggest variation in the underlying sleep neuronal network.

Our detailed characterization of sleep behavior confirms and extends earlier studies that focused on sleep in forager bees (Kaiser and Steiner-Kaiser, 1983; Kaiser, 1988; Schmolz, 2002; Sauer et al., 2003; Sauer et al., 2004). An important aspect of the current work is that the bees were free to move in their cages, and were not tethered, as in most previous studies on sleep in bees. Our experimental procedure allowed bees to choose their resting place, and change their body posture freely. Although the experimental setup of the current study differs from previous ones, we also found that sleep in honeybees is a dynamic process, and that deep sleep is associated with an increased response threshold, relaxation of the antennae and body, and reduced antennal movements.

The description of three sleep stages in bees is reminiscent of the classification of sleep into distinct stages in mammals. For example, human sleep is divided into five stages: NREM (non rapid eye movement) stages 1–4 and REM (rapid eye movement) sleep. These sleep stages are categorized mainly by their electroencephalographic (EEG) pattern, but they also differ in other behavioral and physiological parameters such as response threshold, muscle tonus and activity level (e.g. Grahnstedt and Ursin, 1980; Thoman and Glazier, 1987; Wilde-Frenz and Schulz, 1983; Keenan et al., 1993). NREM1 and NREM2 are characterized by a relatively low arousal threshold and high muscle tonus and body movements, and are therefore considered ‘light sleep’; NREM3 and NREM4 have higher arousal thresholds and reduced muscle tonus and body movements, and are considered ‘deep sleep’. REM sleep is accompanied by a near-to-complete loss of muscle tonus (Keenan et al., 1993). As in mammals, sleep depth in honeybees varies with stage. The first sleep stage seems to be the lightest one, and appears as a transitory stage between wakefulness and deep sleep. Bees in the first sleep stage exhibit the most frequent antennae movements, are most sensitive to light stimuli, and have the shortest bout duration. Nevertheless, the behavior and response threshold of bees in the first sleep stage still differ significantly from those of bees that are inactive but awake. The third sleep stage of honeybees appears to be the deepest. Bees in the third sleep stage show the lowest number of antennae movements, have the highest response threshold, the most reduced muscle tonus, and the longest bout duration. Deep sleep in bees is also associated with an increase in ventilatory cycle duration (Sauer et al., 2003), and reduced body temperature (Kaiser, 1988). In both bees and mammals, the transitions from arousal to deep sleep and from deep sleep to awake states are typically gradual [for mammals, see Feinberg and Ucbida

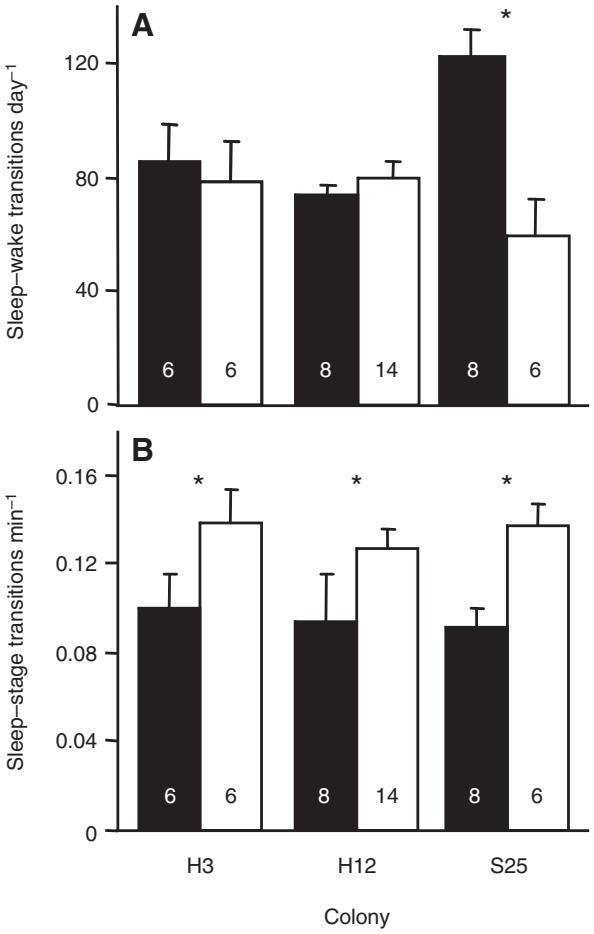

Fig. 6. Transitions between arousal states. (A) Number of sleep–wake transitions during the day. (B) Number of transitions between sleep stages during a single sleep bout. Numbers within boxes indicate the sample size. Filled bars, foragers; open bars, callows. Asterisks indicate a statistically significant difference between foragers and callows.

(Feinberg and Ucbida, 1993)]. However, although we did observe a general tendency of movement toward and away from deep sleep, we did not recognize clear sleep cycles as reported for humans. We noted that sleeping bees occasionally showed bursts of rapid small-amplitude antenna movements, which were associated with a specific body posture. This behavior, which was observed for all bees and may correspond to the bursts of antennal activity described in Kaiser (Kaiser, 1988) and Sauer et al. (Sauer et al., 2004), was not analyzed systematically in the current report. It should be noted that the classification into distinct sleep stages was useful for sleep characterization and quantification, and enabled us to rigorously compare young bees and foragers, but does not imply a step-like transition between consecutive sleep stages or their underlying neuronal mechanisms.

An additional similarity to mammalian sleep is the interruption of all three sleep stages by brief awakenings, in both young bees and foragers. In mammals similar sleep–wake transitions are observed across different species, and the distribution of their episode durations follows a common scale-invariant pattern, leading to the hypothesis that brief awakenings have some yet unknown essential function in the process of sleep regulation (Halasz et al., 2004; Lo et al., 2004; Diniz Behn et al., 2007).

Prior to our study, it was not clear whether young bees sleep at all, since they are typically active around-the-clock with no circadian rhythms (reviewed by Moore, 2001; Bloch, 2008). Our findings show that young bees, even those that are active around-the-clock, exhibit sleep behavior. Moreover, body and antenna postures, antenna movements and response thresholds are similar to those of foragers in the same sleep stage. Both young bees and foragers progressed gradually from light sleep (FS) to deeper sleep (TS), and passed from sleep to awake states a similar number of times. However, their sleep architecture appears different. Overall, foragers had more sleep bouts during the day that were on average shorter than in young bees. They also tended to progress mainly from light to deep sleep, and from there tended to pass directly to awake states, switching less often between sleep stages. Young bees tended to pass more frequently between the three sleep stages, and had longer bouts in the first sleep stage and shorter bouts in the second and third stages.

The differences in sleep dynamics between young bees and foragers may represent variability in the neuronal network underlying sleep behavior. In mammals, the transitions between wake and sleep, and between sleep stages, stem from complex interactions between sleep and wake-promoting centers (reviewed by Merica and Fortune, 2004; Saper et al., 2001; Fuller et al., 2006; Lu et al., 2006). The differences between callows and foragers could represent developmental changes in the organization or function of the sleep neuronal network, since callows are younger than foragers. In humans, there is evidence for changes (‘maturation’) of sleep during early infant development (Jenni et al., 2004; Mirmiran et al., 2003). Young bees and foragers also differ in the environment they experience, which may contribute as well to the observed variation in sleep architecture (Ribeiro et al., 1999; Miyamoto et al., 2003; Ganguly-Fitzgerald et al., 2006). In this regard, it is interesting to note that electrophysiological recordings suggest that sleep in honeybee foragers is associated with distinct rhythmic activity in their mushroom bodies (Schuppe, 1995). The mushroom bodies, which differ in their neuroanatomy between young bees and foragers (Withers et al., 1993; Withers et al., 1995; Farris et al., 2001), have recently been implicated as the main brain region regulating sleep in *Drosophila* (Joiner et al., 2006; Pitman et al., 2006).

To the best of our knowledge, this study is the first to show plasticity in sleep behavior in insects. Even though both young bees and foragers have a characteristic sleep state, there appear to be notable differences in their sleep architecture. Since the behavior of bees is strongly influenced by the social environment in the hive (Shemesh et al., 2007), an important question for future research is whether similar plasticity in sleep behavior also occurs in field colonies, in which young bees typically care for the brood around-the-clock (Moore et al., 1998).

LIST OF ABBREVIATIONS

A	active state
FS	first sleep stage
G	grooming state
IA	immobile–active state
LOOCV	leave-one-out cross-validation
SS	second sleep stage
TS	third sleep stage

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