Monitoring Circadian Rhythms of Individual Honey Bees in a Social Environment Reveals Social Influences on Postembryonic Ontogeny of Activity Rhythms

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Abstract Social factors constitute an important component of the environment of many animals and have a profound influence on their physiology and behavior. Studies of social influences on circadian rhythms have been hampered by a methodological trade-off: automatic data acquisition systems obtain high-quality data but are effective only for individually isolated animals and therefore compromise by requiring a context that may not be sociobiologically relevant. Human observers can monitor animal activity in complex social environments but are limited in the resolution and quality of data that can be gathered. The authors developed and validated a method for prolonged, automatic, high-quality monitoring of focal honey bees in a relatively complex social environment and with minimal illumination. The method can be adapted for studies on other animals. The authors show that the system provides a reliable estimation of the actual path of a focal bee, only rarely misses its location for > 1 min, and removes most nonspecific signals from the background. Using this system, the authors provide the first evidence of social influence on the ontogeny of activity rhythms. Young bees that were housed with old foragers show ~24-h rhythms in locomotor activity at a younger age and with stronger rhythms than bees housed with a similar number of young bees. By contrast, the maturation of the hypopharyngeal glands was slower in bees housed with foragers, similar to findings in previous studies. The morphology and function of the hypopharyngeal glands vary along with age-based division of labor. Therefore, these findings indicate that social inhibition of task-related maturation was effective in the experimental setup. This study suggests that although the ontogeny of circadian rhythms is typically correlated with the age-based division of labor, their social regulation is different.

Key words social, circadian rhythms, *Apis mellifera*, ontogeny, pattern-recognition, division of labor

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Worker honey bees, like infants of human and other primates (Mirmiran et al., 2003; Rivkees, 2003), show postembryonic development of overt circadian rhythms. Little is known, however, about the environmental regulation of the ontogeny of overt circadian rhythms and other developmental changes in the circadian clock (e.g., Page, 1990). We tested the hypothesis that the development of overt circadian rhythms in bees is influenced by the social environment. We were motivated to pursue this line of research because almost every aspect of honey bee biology is influenced by the social environment, and there is evidence for social influences on the phase and strength of circadian rhythms in bees (Southwick and Moritz, 1987; Moritz and Sakofski, 1991; Moritz and Kryger, 1994; Frisch and Koeniger, 1994; Moore et al., 1998; Bloch and Robinson, 2001).

Newly emerged bees that are isolated individually in a constant environment typically have no circadian rhythms during their first days of adult life but later manifest significant circadian rhythms (reviewed in Moore, 2001). By contrast to old bees that typically have strong rhythms in an LD illumination regime, many young bees either have no diurnal rhythms, or they lose rhythmicity when released to constant conditions (Bloch et al., 2001; Bloch and Meshi, 2007). Young workers also show an ontogeny of circadian rhythms in colonies in which bees freely forage for food outside the hive. In these more natural conditions, the ontogeny of circadian rhythms meshes with their age-related division of labor. Young bees typically care for brood around-the-clock with no circadian rhythms during their first 1 to 2 weeks of adult life and later (typically > 3 weeks of age) switch to foraging activities with strong circadian rhythms and a sleep-like behavior during the night (Kaiser and Steiner-Kaiser, 1983; Moore et al., 1998; Sauer et al., 2003). Worker bees also show age-related variation in the expression of the clock gene *period*, providing a molecular correlate to the ontogeny of overt circadian rhythms (Toma et al., 2000; Bloch et al., 2001, 2004; Shimizu et al., 2001).

To study social influences on the ontogeny of circadian rhythmicity, we developed a method of obtaining high-quality activity data on freely interacting bees, over extended periods, and under socially relevant contexts. This was imperative because currently used methods are hampered by a trade-off. Human observers can record the activity of individually identified bees in a small observation hive (with transparent walls) while maintaining a reasonably natural and ecologically relevant context (e.g., Crailsheim et al., 1996; Moore et al., 1998; Bloch and Robinson, 2001). Bees forage for food, colonies are self-sustained, the division of labor appears normal, and there are no indications of abnormal behavior (von Frisch 1967; Robinson, 1992; Seeley, 1995). The main drawback is that data quality is relatively low because human observers cannot observe bees constantly, and their performance is variable and may be influenced by experience, fatigue, and emotional state. A second approach is to monitor circadian rhythms with automatic data acquisition systems (e.g., Spangler, 1972; Frisch and Koeniger, 1994; Bloch et al., 2002a; Yerushalmi et al., 2006). These systems monitor activity objectively and continuously around-the-clock throughout long periods and with high sampling resolution. However, these systems are designed to monitor individuals that are isolated and therefore are limited in their capacity to assess social influences on circadian rhythms. There is evidence suggesting that the social environment may indeed influence circadian rhythms in honey bees; nurse-age (6-10 days of age) bees in a colony are active around-the-clock with no circadian rhythms but manifest strong circadian rhythms in locomotor activity shortly after transfer to individual cages in the laboratory (Shemesh et al., 2007). To overcome this data quality/natural context trade-off, we adapted a commercially available, pattern recognition–based video tracking system to automatically monitor honey bees in a relatively complex social environment. After adjusting and validating the system, we used it to test the influence of old foragers on the development of circadian rhythms in young bees.

We focused on foragers because their inhibition of task-related behavioral development is well documented in honey bees, and the ontogeny of circadian rhythms is associated with the division of labor (Moore et al., 1998; Toma et al., 2000). Young bees that are housed exclusively with other young bees develop faster and start to forage precociously compared to bees that are housed with foragers or in typical field colonies. The transition from in-hive tasks to foraging is not merely a behavioral phenomenon; it is associated with profound changes in bee physiology, endocrinology, neuroanatomy, and brain gene expression (Winston, 1987; Robinson and Huang, 1998; Bloch et al., 2002b; Whitfield et al., 2003). For example, in nurses, the hypopharyngeal glands, which are located in the bee's head, produce proteinaceous secretions ("jelly") that are fed to larvae. Along with the switch to foraging activities, these glands decrease in volume, stop producing larval food, and produce enzymes that are important for processing floral nectar into honey instead (Winston 1987; Kubo et al., 1996; Naiem et al., 1999; Deseyn and Billen, 2005). This switch in gland function is accelerated in groups composed solely of young bees (Robinson, 1992; Robinson and Huang, 1998). We therefore used the hypopharyngeal gland morphology as an index for division of labor-related behavioral development. We hypothesized that if social inhibition occurs in bees in the small groups used in our experiments (see below), then bees housed with foragers should develop more slowly and have larger glands at 10 days of age.

Below we describe the validation of our method for monitoring focal bees in groups of about 30 individuals. Using this method, we found that bees housed with foragers showed earlier and stronger ~24-h rhythms compared to those housed with only young bees.

MATERIALS AND METHODS

Bees

Bee colonies were maintained according to standard beekeeping techniques at the bee research facility on the Givat-Ram Campus of the Hebrew University of Jerusalem, Jerusalem, Israel. The bees were derived from a mixture of European races of *Apis mellifera* typical to Israel. We used bees from colonies in which the queen was instrumentally inseminated with semen from a single drone (carried out at the Bee Breeding Station of the Israeli Ministry of Agriculture at Tzrifin). Because drones are haploid, the coefficient of relatedness among offspring of single drone inseminated queens is 0.75 (Winston, 1987). In our experiments, we used 1-day-old (0- to 24-h) workers and foragers of unknown age. To obtain 1-day-old bees, we removed all adult bees from a honeycomb frame containing pupae (sealed in cells), transferred it in a lightproof container to an incubator (30 ± 1 °C, 45% ± 5% RH), and collected bees that emerged during the next < 24 h. To obtain foragers, we temporarily blocked the hive entrance with a piece of 8-mesh wire hardware cloth and collected bees that returned to the hive with pollen loads on their hind legs. We minimized exposing bees to external factors that could influence the function of biological clocks (e.g., light, variation in temperatures).

Measuring Hypopharyngeal Gland Size

Bees collected for hypopharyngeal gland analysis were stored at –20 °C until gland dissection. The hypopharyngeal glands are paired structures, each composed of hundreds of small vesicle-like bodies called acini that are attached to the central duct (Winston, 1987; Deseyn and Billen, 2005). We dissected hypopharyngeal glands from bee heads in bee saline (Huang et al., 1991) and disconnected a group of 12 to 49 acini that we photographed under a microscope (×40 magnification). We then analyzed the digital image with Image-Pro Plus image analysis software (MediaCybernetics Ltd.) that calculates several indices for size (e.g., maximal diameter, mean diameter, area). Acini size is correlated with exocrine activity and is commonly used as an index of hypopharyngeal gland activity (e.g., Crailsheim and Stolberg, 1989; Huang and Robinson, 1996; Naiem et al., 1999; Deseyn and Billen, 2005).

Tracking Focal Bees in a Social Environment

We tracked focal bees automatically with the EthoVision Color-Pro 3.0 (Noldus Information Technology, Wegeningen, The Netherlands) pattern recognition system, which detects color patterns within defined arenas. Each pixel is evaluated by the HSI (hue, saturation, intensity) color-coding scheme. Once the EthoVision system detects the defined color patterns, it extracts their locations; stores the information as a series of X, Y coordinates; and uses these data to calculate the distance covered by each bee in the interval between 2 successive samples. The distance is converted from pixels into centimeters based on prior size calibration. The video images are deleted immediately after extracting the spatial information, which enables continuous storage of information over prolonged periods.

We marked the focal bees with blue, green, and red paint that did not overlap with HSI values and were distinct from background colors. We painted both the dorsal and ventral sides of the abdomen following preliminary experiments that showed that tracking efficiency is low when only the thorax is painted (Fig. 1A). To paint bees, we chilled them at 4 °C (~1 min) and harnessed them in plastic collars that prevented contact between the painted abdomen and other body parts (e.g., the wings). We were careful that the paint did not reach openings of the tracheal system, which are located on the lateral sides, or Nasanov glands, which open at the distal part of the abdomen. We used DecoArt (Americana) dyes that dry fast even when substantial amounts are applied. In a preliminary experiment, we found no difference in behavior or survival over 10 days between painted and control bees (78% ± 13% and 72% ± 17%, respectively, $n = 60$ for each treatment group; t test, $p = 0.76$).

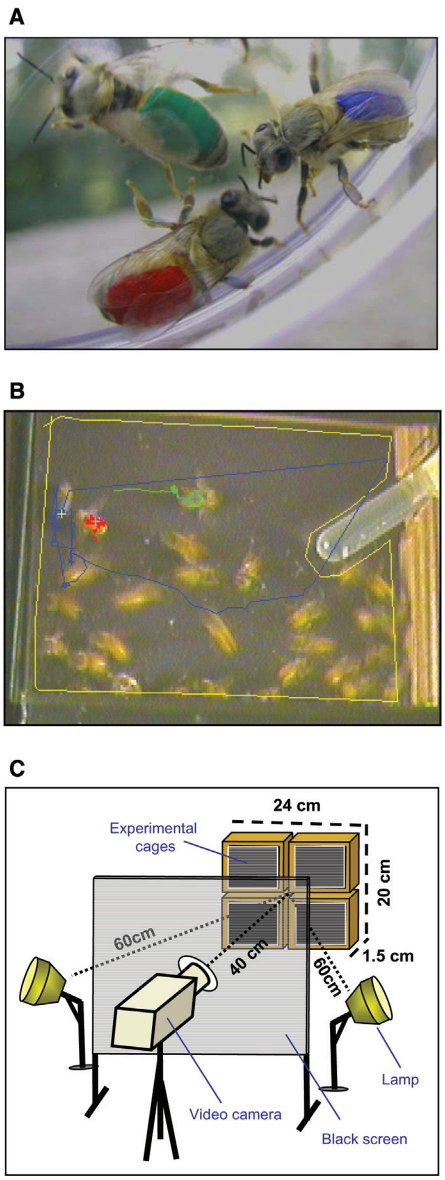

Figure 1. Automatic tracking of focal bees in a social context with the EthoVision Color Pro pattern recognition–based video tracking system. (A) Three focal bees painted on their abdomens with red, green, or blue marks. (B) Experimental arena for simultaneous tracking of 3 color-marked bees. Each cage was defined as an experimental arena into which we introduced 3 marked and 30 unmarked bees. The yellow line delineates the boundaries of the tracking arena in which the EthoVision system searched for the predefined color patterns. Note that the feeder was excluded from the arena. The + sign and a color patch indicate that the system is locked onto a paint-marked bee. Each colored line connects the latest 100 locations for a focal bee painted with a corresponding color. Note that the system ignores unmarked bees. (C) Experimental setting for the parallel tracking of 4 arenas. We housed all 4 cages in an environmental chamber illuminated with 2 lamps directed toward the walls. We placed a black screen between the camera and the cages to prevent light reflections on the glass cover of the cages. The camera was connected to a computer with the EthoVision system that was placed outside the experimental chamber. This setup enabled us to continuously monitor the bees with minimal disruption.

We provisioned each monitoring cage (internal space = 12 × 10 × 1.5 cm) with a sugar-syrup feeder (5-ml tube, 50% w/w, replenished every 2 days) and 2 honeycomb wax cells filled with pollen. To decrease background colors and reduce noise detection, we covered the inner wall of the cage with nonreflective black heavyweight Bristol paper. The cage cover was made of transparent glass through which we video tracked the activity of focal bees. The space between the cage bottom and cover (1.5 cm) was similar to the typical gap between honeycombs in a colony (known as "bee space"; Winston, 1987). We positioned the cages vertically, again similar to the comb stance in natural colonies. Within the EthoVision image of each cage, we outlined an arena in a way that excluded elements such as the sugar-syrup feeder and pollen cells that reflect color falling within our target HSI definitions (Fig. 1B). We simultaneously tracked 4 cages (arenas), placed next to each other in an environmental chamber (29 ± 1 °C, 35%-50% RH). The total area, including the 4 experimental cages, was 768 × 576 pixels. The chamber was constantly illuminated by 2 dim yellow lamps (1.55 μmol photons/m^2/sec), directed toward the walls such that light intensity on the cages was uniform and there was no direct reflection from the glass cover. To further minimize light reflection, we placed a black screen in front of the cages, with an aperture for the camera lens (Fig. 1C).

To further reduce noise detection, we applied a filter that limited detection to objects with a surface area of > 15 pixels; the surface area of the paint spots on the bees, as detected by the EthoVision system, was 170 ± 1.2 pixels. We set the frame capture rate at 5 frames/sec. To enable efficient color detection under such dim illumination, we used a light-sensitive video camera (Ikegami, Tsushinki Co. Ltd., CCD Icd-870P, 0.08 lux at F1.4). We placed the camera in front of the cages inside an environmental chamber and connected it with an S-video cable to a computer with the EthoVision software placed outside the chamber.

Postacquisition Data Processing

The data acquired by the EthoVision system were exported as Excel files and processed with a specifically developed algorithm we term *EthoClean*. Each data file contains a time series of X, Y coordinates from each color pattern (focal bee) and missing values for the times when no object was detected. The EthoClean algorithm was designed to interpolate over missing data points in which the tracking system lost the focal bee and to remove nonspecific background noise reflected from objects other than the painted bees. The EthoClean algorithm is based on the assumption that whenever the EthoVision system detects an object over a series of consecutive samples, it is likely to be a color-marked bee. Other color objects can come either from a marked bee or from other objects with similar HSI characteristics. The EthoClean distinguishes between these possibilities by computing a "reasonable" distance (X_n) that a bee could have traveled since the last series of successive pattern detections. Only color objects that are within the "reasonable" distance are added to the bee's path. To calculate X_n, the EthoClean uses the following equation:

$$X_n = \frac{1 - r^n}{1 - r} \cdot D_{\max}.$$

The parameters are as follows: $D_{\max}$ = the maximal distance a bee can travel in an interval between successive video frames, n = the number of consecutive samples with missing values, and r is an arbitrary constant. To find parameters that fit the typical behavior of bees, we videorecorded marked bees and compared their actual tracks with data acquired by the EthoVision system. Using this approach, we found that a series of ≥ 5 successive samples of the same color object was always produced by a marked bee. The maximal distance that a bee traveled in 0.2 sec ($D_{\max}$, the interval between successive video frames) was 1.8 cm. We verified $D_{\max}$ by measuring the maximal distance made by foragers that were disrupted and moved rapidly (n = 12). To set the value of r, we used a trial-and-error approach and found that a value of $r = 0.09$ produced the most reliable distinction between background noise and genuine bee signals. The EthoClean algorithm produces a text file with the total distance per 10 min for each color-painted bee. The format of this output file was set to be imported by the ClockLab software with which we performed the circadian analyses (see below).

Experiment 1: Determining Tracking Efficiency over Time

We simultaneously tracked 2 cages with 3 young focal bees and 30 unmarked ("background") young bees, as well as 2 cages with 3 marked young bees and 30 unmarked foragers. We set the EthoVision to capture 5 frames per second and tracked the bees over 10 successive days. We repeated this experiment 5 times (each with 4 cages) with bees from source colony S22. To minimize the Position × Treatment interaction, we randomly switched the positions of the cages in which unmarked bees were young or foragers in each repetition. For each day, we determined the number of video frames (samples) in which marked bees were detected.

Experiment 2: Determining the Reliability of Data Obtained with the EthoVision System and Processed with the EthoClean Algorithm

We simultaneously recorded the activity of focal bees with the EthoVision system and with a video camera. To estimate the actual track for each focal bee, we manually analyzed the video records with Image-Pro Plus image-processing software and determined the bee's precise location (X, Y coordinates) in each video frame. We repeated this experiment twice. In the first experiment, we tracked 5 marked callow bees from 2 cages (tracking arenas). Their activity was recorded in 5 sessions of 5 min each. In the second experiment, we tracked 3 marked foragers in 1 arena for which we recorded 10 sessions of 5 min each. In both experiments, we placed the focal bees with a background of 30 unmarked bees. We used linear regression to compare the paths calculated with the Image-Pro frame-by-frame analysis against the paths estimated based on the EthoVision data before and after processing with the EthoClean algorithm.

To assess the suitability of the data acquired by the EthoVision system for circadian analyses, we exported EthoVision data, before and after processing with the EthoClean algorithm, to the ClockLab circadian analysis software. Circadian rhythms were determined using chi-square periodogram analyses. The criterion for rhythmicity was set at $p < 0.01$ as in previous studies (e.g., Toma et al., 2000; Bloch et al., 2001, 2002a). For each bee, we determined the following parameters:

(1) Free-running period (FRP)
(2) Overall level of activity, which is the average distance a bee moved (cm/10 min) during the entire 10 days of the experiment
(3) Power, an index of the strength of the rhythm. Power was defined as the height of the periodogram peak above the p = 0.01 significance threshold (Klarsfeld et al., 2003). We defined the "initial power" as the power during the first 4-day interval in which the bee manifested a circadian rhythm and "maximal power" as the 4-day interval with the highest power value.
(4) Age at onset of circadian rhythmicity. To obtain this value, we carried out periodogram analyses over a sliding 4-day window (days 1-4, days 2-5, etc.). The onset of circadian rhythmicity was defined as the first day in an interval with a significant period of 20 to 28 hours. We considered a bee as rhythmic only if circadian rhythms were significant in 2 successive 4-day intervals.

We used survival analysis (Kaplan-Meier with the Breslow statistic; Bloch et al., 2002a) to compare the age at onset of circadian rhythmicity. This method was appropriate because not all bees developed circadian rhythms by the end of the trial. We used multivariate analysis of variance (ANOVA) with trial, cage within trial, and social environment as fixed factors in comparisons of power, activity, and FRP, for which values were normally distributed.

Experiment 3: The Influences of Foragers on the Development of Circadian Rhythms in Young Bees

The experimental setup was similar to that in experiment 1. We conducted 6 trials (10 days each) with bees from colonies S15 and S22, as well as 5 trials with colony S17, between March and November 2005. We used the ClockLab software to compare the FRP, initial power, maximal power, and level of activity for focal bees from the 2 social environments (as explained in experiment 2). In these analyses, we used a 3-way ANOVA with trial, cage (arena), and social environment as fixed factors. This model enabled us to detect variation due to social environment while controlling for the effects of trial and arena. We carried out 2 additional analyses to compare the ontogeny of circadian rhythms. First, we compared the percentage of bees with circadian rhythms at 2 time frames: days 2 to 5 and days 6 to 9 (with Fisher's exact test). Only bees with a significant periodogram during this interval were considered rhythmic. Second, we performed a Kaplan-Meier survival analysis with the Braslow statistic, with which we compared the proportion of rhythmic bees as a function of age (see above). In the survival analyses, a bee that showed a significant circadian rhythm was considered rhythmic from that time on (even if the periodogram value fell below $p < 0.01$ on subsequent days). To confirm that the presence of foragers affected the physiology of young bees, we measured the size of the hypopharyngeal gland for bees in the 2 social environments (see above).

RESULTS

Experiment 1: Determining Tracking Efficiency over Time

The EthoVison system detected the marked bees in 129,375.1 ± 3785 samples/day (mean ± SE), which corresponds to 29.9% ± 0.9% out of a total of 432,000 frames sampled/day (n = 33 marked bees). Tracking efficiency varied over time; detection levels were significantly lower on the first and last days of the experiment (around 18% on days 1 and 10, compared to highest levels of 37.6% ± 2.6% on day 4; $p < 0.01$, 2-way ANOVA with day and trial as fixed factors, Bonferroni post hoc test; data not shown). The reduced detection efficacy on day 1 probably stems from low activity and the typical clustering behavior of newly emerged bees; the reduced detection on day 10 probably reflects accumulation of dust and dirt on the internal part of the glass cover and attrition of the color marks. A mean of ~129,000 samples/day implies that marked bees were detected on average 90 times/min. This, as well as even lower values than those obtained for days 1 and 10, should provide a good estimation of variation in locomotor activity during the day if samples are distributed rather evenly and sequences of missing values are short. We tested these assumptions and found that out of 20,443 ± 2286 cases of missing values per day, more than 58% of the sequences lasted for < 0.01 min, and about 98% to 99% lasted for < 1 min (n = 12 focal bees per day and social environment; Fig. 2). We further found that the EthoVision detected an object in 88.41 ± 6.99 samples/min in 87 randomly chosen hours of data from various bees, days, and trials for bees tracked in experiment 1. For this analysis, we only used samples that were part of a series and therefore likely to be emitted from a painted bee (see Materials and Methods). Using the noise recognition protocol of the EthoClean algorithm, we found that 7076 ± 1329 samples/day were likely to come from subjects other than the marked bees and were therefore considered "noise" (see Materials and Methods). We found that these putative background noise objects increased with time (from 0.85% ± 0.06% at day 1 to 2.5% ± 0.27% samples/day at day 10), probably due to the accumulation of dust and dirt on the glass cover.

These results indicate that the acquired data, including during days 1 and 10 in which tracking efficiency was lower, are appropriate for analyses of circadian rhythms because resolution is high, pattern detections are distributed evenly over time, and cases in which bees are not detected for more than 1 min are fairly rare.

Experiment 2: Determining the Reliability of Data Obtained with the EthoVision System and the EthoClean Algorithm

Figure 3A shows a representative path obtained from a video analysis (left panel; estimated from a

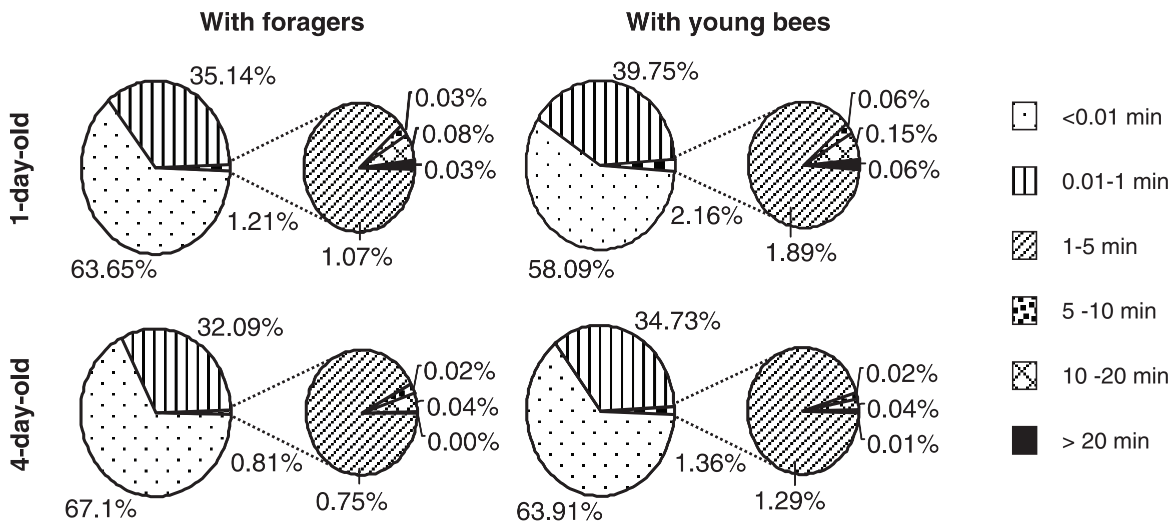

Figure 2. Distribution of sequences of EthoVision samples with missing values. Each large pie chart shows the percentage of cases of sequences of missing values as a function of their length. The small pie charts detail sequences lasting > 5 min. Upper panels, data for 1-day-old bees; lower panels, data for 4-day-old bees. Right panel, marked bees were housed with foragers; left panel, marked bees were housed with callow bees. Each pie chart shows the average percentage for *n* = 6 bees.

frame-by-frame analysis of video records, see Materials and Methods), compared with the path for the same bee generated with data acquired by the EthoVision system, before (middle panel, EV) and after (right panel, EC) processing with the EthoClean algorithm. The path estimated by the EthoVision system generally exhibited good fit with the actual trajectory of marked bees. The representative paths in Figure 3A also show that the EthoClean algorithm improves the estimation of the bee trajectory by interpolating the sequences of missing values and deleting background noise. Bee activity as estimated by the EthoVision was significantly correlated with the actual activity of a focal bee with an R^2 of approximately 0.6 (Fig. 3B). The correlation was stronger after processing the data with the EthoClean algorithm and accounted for approximately 80% of the variance. The improved estimation was also manifested by an increase in the slope of the regression line (a = 0.27 and 0.45 before and a = 0.57 and 0.51 after processing with the EthoClean algorithm). These results indicate that the estimate of activity based on data acquired by the EthoVision system and processed by the EthoClean algorithm corresponds well with the actual activity of the bee.

We further tested the influence of processing the data with the EthoClean algorithm on the analysis of circadian rhythms. The ClockLab software calculated higher activity values for data processed with the EthoClean algorithm (only EthoVision [EV] = 13.69 ± 0.95 [n = 42], with EthoClean [EC] = 24.3 ± 1.58 [n = 46]; t test, $p < 0.01$), which was expected because the EthoClean algorithm interpolates over missing values. The EthoClean processing, however, did not affect the estimated values for the FRP (EV = 23.94 ± 0.29, EC = 24.23 ± 0.26; t test, $p = 0.45$), initial power (EV = 22.91 ± 3.15, EC = 16.37 ± 2.21; $p = 0.09$), maximal power (EV = 76.91 ± 10.78, EC = 75.49 ± 10.52; $p = 0.92$), and the age at onset of circadian rhythmicity (Kaplan-Meier survival analysis with Breslow statistic, $p = 0.21$, $n = 52$, data not shown).

Experiment 3: The Influences of Foragers on the Development of Circadian Rhythms in Young Bees

Focal bees that were housed with foragers had larger acini in their hypopharyngeal glands (Table 1). These results are consistent with earlier studies and are thought to represent the inhibition of maturation in young bees that interact with foragers (Huang and Robinson, 1992, 1996). The 2 treatment groups did not differ in their FRP (3-way ANOVA, with trial, cage, and social environment as factors; the influence of the social environment, $p = 0.06$, $p = 0.55$, and $p = 0.86$ for colonies S15, S17, and S22, respectively; Fig. 4A). Bees that were housed with foragers had stronger rhythms, as is evident from their higher maximal power ($p < 0.01$, $p = 0.03$, and $p < 0.01$, in colonies S15, S17, and S22, respectively; Fig. 4C). We noted a similar trend for initial power, but the differences were not statistically significant ($p = 0.20$,

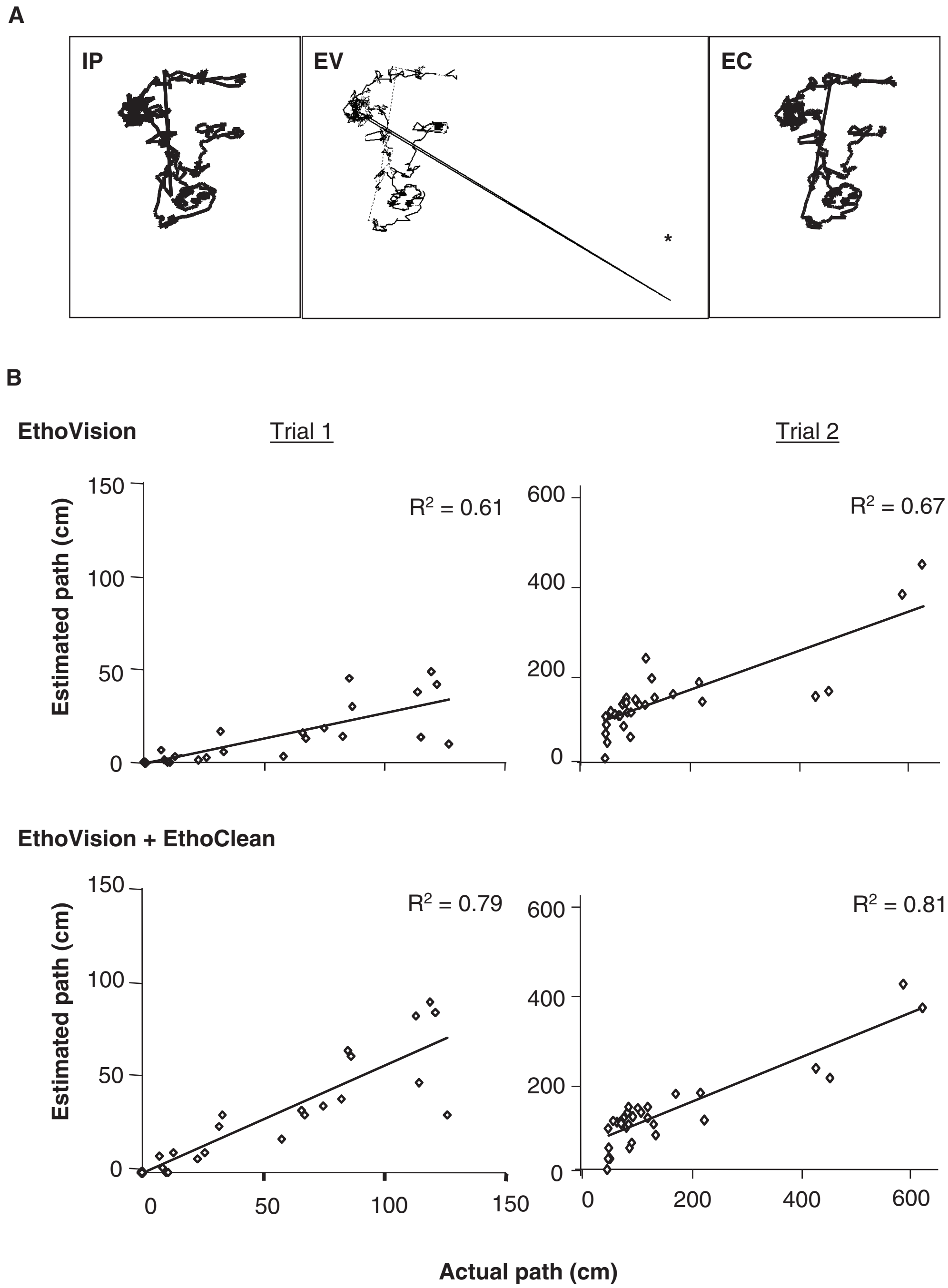

Figure 3. Validation of the data acquired by the EthoVision system and processed with the EthoVision and EthoClean algorithms. (A) Comparison of the actual path made by a bee, as estimated by a frame-by-frame video analysis with Image-Pro Plus image-processing software (left panel, IP), with estimates based on data acquired and processed by the EthoVision system (middle panel, EV) or further processed by the EthoClean algorithm (right panel, EC). The panels depict the path of a bee marked in red during a 5-min tracking session. Thick lines show the estimated paths. Dotted lines in the middle panel are interpolations made by the EthoVision algorithm that were not saved to the data file. The asterisk shows a noise signal that the EthoVision algorithm added to the bee path. (B) The relationships between actual and estimated trajectories. Upper panels, regression plot for estimated path based on EV data. Left panel: trial 1, marked bees were 1 day old ($n = 25$). Regression line parameters, $y = 0.27x - 0.75$, $R^2 = 0.61$. Right panel: trial 2, marked bees were foragers ($n = 30$). Regression line parameters, $y = 0.45x + 63.7$, $R^2 = 0.67$. Lower panels: the same data as in the upper panels but after processing with the EthoClean algorithm. Left panel: trial 1, regression line parameters, $y = 0.57x - 0.62$, $R^2 = 0.79$. Right panel: trial 2, regression line parameters, $0.51x + 50.3$, $R^2 = 0.81$. There was an increase in both the slope and R^2 in data processed with the EthoClean algorithm.

Table 1. Influence of Foragers on the Development of Hypopharyngeal Glands in Young Bees

	"Background" Bees		*Results of ANOVA, p-Value*		
Colony	*Foragers*	*Young Bees*	*Background*	*Trial*	*Trial × Background*
S15	7816.5 ± 467.5 (31)	6100.8 ± 246.9 (34)	< 0.001	< 0.001	0.015
S17	5908.0 ± 329.8 (20)	5525.7 ± 167.8 (19)	0.07	< 0.001	0.045
S22	12,361.2 ± 575.7 (19)	6733.6 ± 349.3 (23)	< 0.001	0.014	0.02

Values are mean ± SE acinus diameter (square microns); sample size in parentheses. Acinus size was calculated from a sample of 12 to 49 acini/bee. We repeated this experiment with bees from colonies S15, S17, and S22. The 3 columns to the left summarize the results of a 2-way analysis of variance (ANOVA) with treatment ("background" bees) and trial within experiment as factors. The treatment effect was also statistically significant in a pooled analysis that included bees from all 3 colonies ($p < 0.001$, 2-way ANOVA with colony, and treatment as fixed factors).

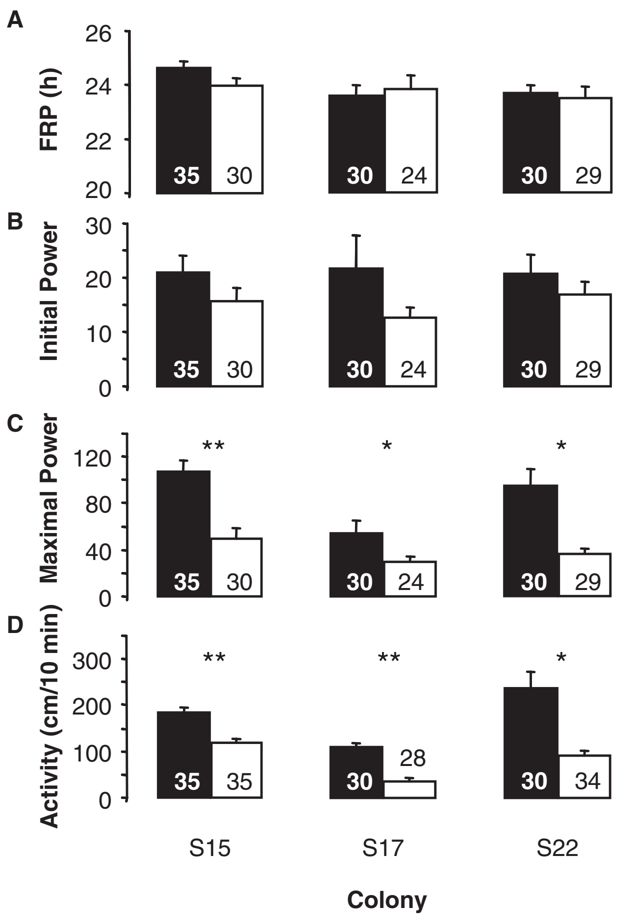

Figure 4. The influence of foragers on locomotor activity in young bees. (A) The free-running period (FRP), (B) initial power, (C) maximal power, and (D) overall activity. Filled bars, bees housed with foragers; open bars, bees housed with young bees. Small bars show standard error; sample sizes inside bars. *p < 0.05 and **p < 0.01 refer to the effect of the social environment in a 3-way analysis of variance (ANOVA). The analyses of the FRP, initial power, and maximal power only included bees that developed circadian rhythms.

$p = 0.24$, and $p = 0.21$, respectively; Fig. 4B). Young bees that were housed with foragers were also more active in all 3 colonies ($p < 0.001$; Fig. 4D).

Figure 5A,B shows the ontogeny of circadian rhythms of representative focal bees that were housed with only young bees or with foragers, respectively. The proportion of rhythmic bees during days 2 to 5 was significantly higher in bees housed with foragers in colonies S15 and S22 ($p < 0.01$, Fisher's exact test); a similar trend was seen in colony S17, but the differences were not statistically significant ($p = 0.4$, Fig. 5C). As expected, the proportion of bees with circadian rhythms increased with age and was higher for bees 6 to 9 days of age (average of 64.7% ± 8.3% compared to 32.5% ± 7.4% during days 2-5; Fig. 5D). As in the first time frame, the proportion was higher in bees that developed in the presence of foragers ($p < 0.01$, $p = 0.02$, and $p = 0.2$, in colonies S15, S17, and S22, respectively; Fig. 5D). In a complementary survival analysis, we found that focal bees that were housed with foragers showed circadian rhythms in locomotor activity at an earlier age ($p < 0.01$ and $p = 0.01$, in colonies S15 and S22, respectively). At the end of the experiment, the proportion of rhythmic bees appeared higher in bees housed with foragers in all 3 colonies (Fig. 6). This experiment shows that in the presence of foragers, the maturation of the hypopharyngeal glands is inhibited, but the ontogeny of activity rhythms accelerated in young honey bee workers.

DISCUSSION

We developed and validated a method for studying locomotor activity rhythms for individually identified focal bees in a relatively complex social environment. This method allows for prolonged high-quality tracking of bees and sets the stage for studies on social influences on circadian rhythms, locomotor behavior, and spatial distribution. Our method and validation procedure provide a framework for devising similar systems for studying social influences on locomotor behavior in other species.

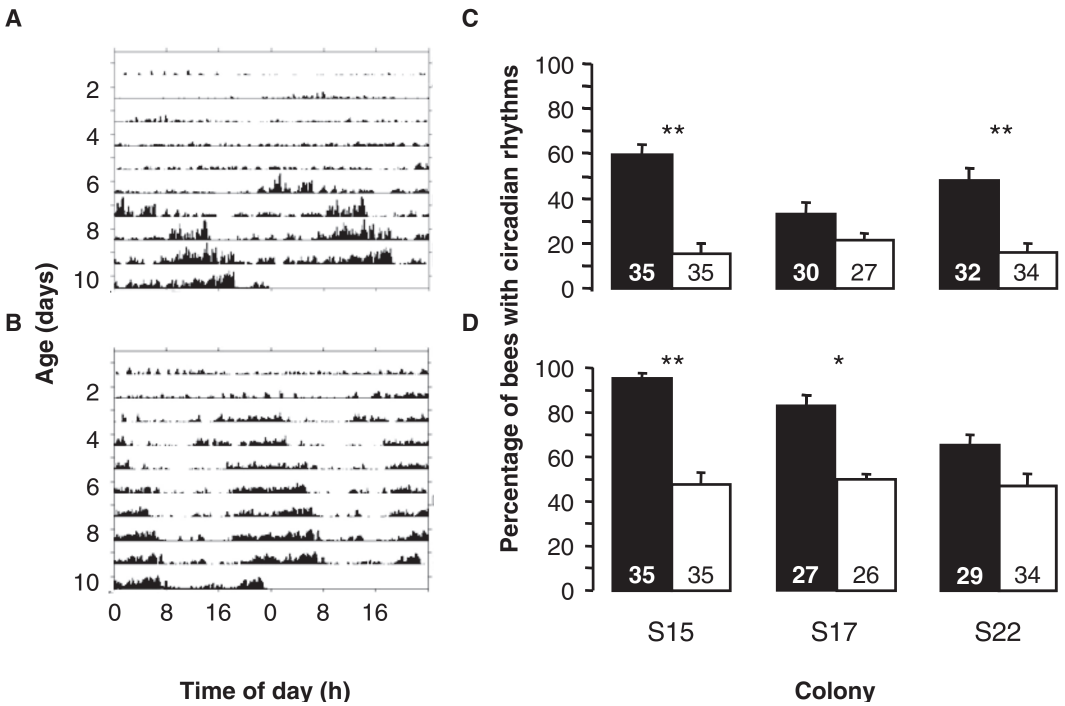

Figure 5. The influence of foragers on circadian rhythmicity in young bees. (A) Representative double-plot actogram for a callow bee that was housed only with young bees. The actogram was generated with the ClockLab software for data acquired with the EthoVision system and processed with the EthoClean algorithm. The numbers on the *y*-axes show the age of the focal bee. The height of the bars within each day corresponds to the distance covered by the bee in 10-min bins. (B) Representative actogram for a callow bee that was housed with foragers. The actograms in A and B are plotted with the same distance scale and are for bees of similar age and genotype that were tracked in parallel (colony S15; trail 11). Note the lower initial activity and later onset of circadian rhythmicity for bees housed with only young bees. (C) The proportion (average ± SE) of focal bees with circadian rhythms in locomotor activity when 2 to 5 days of age. (D) Same as C but when 6 to 9 days of age. Filled bars, bees housed with foragers; open bars, bees housed with young bees. Bees from the 2 treatments were compared with Fisher's exact test. Other details as in Figure 4.

Using this method, we show that young bees housed with foragers develop earlier and stronger ~24-h rhythms compared to bees housed with only young bees, providing the first support for the hypothesis that the social environment influences the development of the circadian system.

Experiment 1 shows that the EthoVision Color-Pro system efficiently tracked focal bees that are housed with a background of 30 unmarked bees. The system obtains data of high quality and with a resolution that is almost always higher than 1 sample per minute, which is very high for analyzing rhythms of about 24 h (i.e., circadian). There were relatively few cases in which the system recorded the locations of objects other than focal bees ("background noise"). The reliability of the system was demonstrated in experiment 2, which shows that there is good fit between the data acquired by the EthoVision system and the actual activity of focal bees, as estimated from frame-by-frame video analyses. The assessment of bee activity was further improved by processing these data with the EthoClean algorithm.

The actograms generated with data acquired by the EthoVision system in the current study for bees in small groups are reminiscent of those obtained for individually isolated bees using conventional data acquisition systems. In both cases, bees typically do not show circadian rhythms during their first days after eclosion but later display clear circadian rhythms with a FRP of about 24 h (Spangler, 1972; Toma et al., 2000; Bloch et al., 2002a; Bloch and Meshi, 2007). An ontogeny of ~24-h rhythms was also documented for the behavior (Moore et al., 1998) and metabolic rates of bees in colonies (Hepburn et al., 1984). Together, these studies suggest that the ontogeny of circadian rhythms is a basic feature of the honey bee circadian system that is manifested in various environments. The pace of rhythm development, however, is modulated by social factors as discussed below.

Our experimental design allowed us to focus on the influence of a single main social factor—foragers—on behavioral development in young bees. The situation in natural colonies is more complex, with multiple social factors (e.g., the queen, various brood stages,

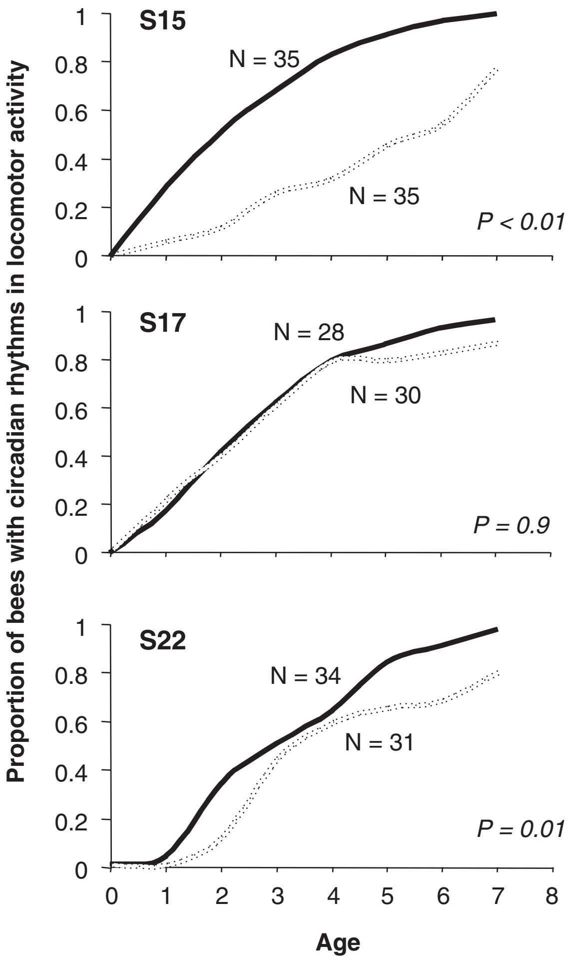

Figure 6. The influence of foragers on the age at onset of circadian rhythms in young bees. Black line, focal bees housed with foragers; double dotted line, focal bees housed with callow bees. A bee that showed significant circadian rhythms in a 4-day interval was considered rhythmic thereafter. Differences between the groups were tested with survival analysis (Kaplan-Meier, Breslaw statistic, *p* values on the bottom of plots). Numbers above and below curves show the sample size for bees housed with foragers or young bees, respectively.

other workers) that may interact in various ways. We found that bees that were housed with foragers showed ~24-h rhythms in locomotor activity at an earlier age and that the rhythms were stronger (higher power) relative to those in bees housed with a similar number of young bees. These findings provide the first evidence for the social modulation of the ontogeny of ~24-h rhythms. These ~24-h rhythms were not diurnal because bees were monitored in a constant environment and their rhythms free-ran with a variable period. It is difficult, however, to determine whether the observed rhythms were entirely internal (circadian) because we cannot rule out social masking. Young bees that were housed with foragers could have been aroused or prevented from resting during the foragers' subjective day or influenced by fluctuations in ambient temperature due to heat produced by the foragers' activity (Hepburn et al., 1984; Southwick and Moritz, 1987; Moritz and Kryger, 1994). In groups composed solely of young bees, circadian rhythms could have been masked by their lower activity and tendency to form dense clusters that could interfere with color tracking. It is hoped that future improvements of the tracking method will allow us to simultaneously track sufficient numbers of focal and "background" bees to explicitly test whether they are synchronized, as predicted for social masking. Assessing the pattern of clock gene cycling in the 2 social environments should also prove useful for distinguishing between masking and genuine variation in clock development. Nevertheless, several lines of evidence lend credence to the proposition that the observed rhythms are internal. First, circadian rhythms in locomotor activity are typically correlated with brain clock gene expression in bees (Toma et al., 2000; Bloch et al., 2001, 2004; Shimizu et al., 2001; Shemesh et al., 2007). Second, there is evidence that the (social) environment influences the development of the circadian system. Callow bees that experienced 24 or 48 h in a colony before transfer to individual cages were more likely to show circadian rhythms at 3 to 6 days of age compared to bees isolated immediately after emergence (Eban, Shemesh, and Bloch, unpublished observations). Third, bees from different genotypes that were housed in the same host colony differed in the age at which they show ~24-h rhythms, indicating that activity rhythms are not a mere masking by the hive environment (Moore et al., 1998). Fourth, the onset of activity on day 8 (an index for phase, data not shown) among 3 focal bees housed in the same cage with only callows was typically synchronized, similar to the synchronization among bees housed with foragers. These findings are more consistent with social entrainment because masking is not expected in groups exclusively composed of callows.

Assuming that the variation in overt rhythms is indeed circadian (internal), the next question is to define which social signals are used by foragers to influence the development of the circadian system of young bees. Plasticity in circadian rhythms is associated with the division of labor and therefore could be influenced by social signals that regulate the nurse-to-forager transition (reviewed in Robinson, 1992; Robinson and Huang, 1998; Bloch et al., 2002b). Our findings, however, are not consistent with this hypothesis because young bees housed with foragers showed earlier and stronger overt rhythms. The worker regulation of age-based division of labor fits a negative feedback loop model: foragers inhibit the development of young bees into more foragers. Such inhibition is effective even in groups of bees without brood or a queen (e.g., Huang and Robinson, 1992). Thus, the manifestation of a strong circadian rhythm that is typical of foragers was expected to be delayed in focal bees housed with foragers. The analyses of hypopharyngeal gland morphology were similar to those recorded for bees in free-foraging colonies (Robinson, 1992; Huang and Robinson, 1996; Deseyn and Billen, 2005), suggesting that the forager inhibitory signal was effective in our small experimental groups.

The conflicting influences of foragers on the ontogeny of the hypopharyngeal glands (inhibition) and activity rhythms (acceleration) suggest that the ontogeny of overt rhythms is not regulated as most other changes associated with the nurse-to-forager developmental syndrome. This proposition is consistent with studies showing that treatments with doses of juvenile hormone or octopamine that advanced the age of first foraging did not influence the development or function of the circadian system (Bloch et al., 2002a; Bloch and Meshi, 2007). In addition, 6- to 8-day-old nurse bees showed strong circadian rhythms shortly after transfer to individual cages in a constant environment, suggesting that their clock system was functional as well when they were in the hive and attended the brood around-the-clock (Shemesh et al., 2007). Thus, it appears that most nurses in a colony are active around-the-clock not because they are young and their clock is not yet fully functional, but rather because the brood influences the temporal pattern of worker activity.

Foragers may influence the ontogeny of rhythms in young bees by social signals other than those influencing division of labor. Such signals, however, have not been identified in honey bees, and the functional significance of foragers accelerating the ontogeny of circadian rhythms in young bees is not clear. Another possibility is that foragers provide nourishment or influence their microenvironment (e.g., temperature) in a way that supports the development of the circadian system in young bees. There is evidence for improved development in young bees paired with a 6- to 18-day-old companion bee. This effect was explained by the companion bee feeding royal jelly to the young bee by means of trophallaxis (mouth-to-mouth feeding). Foragers did not have a similar positive influence on the physiology of young bees; their glands did not produce royal jelly, and they did not perform trophallaxis (Naiem et al., 1999). Nevertheless, under conditions of a severe shortage of young bees, foragers may revert to a "nurse state," and their hypopharyngeal glands can regenerate, even if there is no brood to feed (Huang and Robinson, 1996). A similar reversion perhaps occurred in our experiments in which 30 foragers were housed for 10 days with only 3 young bees, but not in the experiments of Naiem et al. (1999), who replaced the companion bee every 2 days.

Postembryonic development of overt circadian rhythms is not unique to honey bees and has best been explored in infants of human and other primates. However, even in these well-studied systems, little is known about how ambient conditions influence the ontogeny of overt circadian rhythmicity (Mirmiran et al., 2003; Rivkees, 2003). Our evidence for social influences on the postembryonic development and strength of rhythms in locomotor activity in bees should stimulate further research designed to test the internal (circadian) nature underlying this behavior. In a broader perspective, our study suggests that the social environment should be taken into account in studies on the ontogeny of circadian rhythms.

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