

Developmentally determined attenuation in circadian rhythms links chronobiology to social organization in bees

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Summary

We investigated labor-related plasticity in the circadian clock of the bumblebee *Bombus terrestris*. Bumblebee workers vary enormously in body size, and we found that size, division of labor, and diurnal rhythms in activity are correlated in *B. terrestris* colonies. Large workers typically perform foraging activities with strong diurnal rhythms and low activity at night, whereas small bees typically care for (nurse) brood around the clock with weak or no diurnal rhythms. Under constant laboratory conditions, circadian rhythms in locomotor activity were weaker, less stable, and developed at a later age in small (nurse-size) bees compared to their larger (forager-size) sisters. Under a light:dark illumination regime, many small bees, particularly at a young age, were active during the dark

phase, fewer small bees developed rhythms, and they did so later compared to large bees. Taken together these findings reveal naturally occurring attenuation or suppression in the circadian clock of small bees that is determined during pre-adult development. This deficiency in clock function, however, does not result in pathology but rather appears to be functionally significant, because it is associated with around-the-clock brood care activity and therefore apparently improves divisions of labor and colony efficiency. This in turn suggests that variation in social biology influences traits of the circadian clock.

Key words: bumblebee, *Bombus terrestris*, circadian rhythm, division of labor, size polymorphism.

Introduction

Circadian rhythms are ubiquitous in animals and are assumed to be functionally significant, because they enable the temporal organization of endogenous processes and the anticipation of predictable day–night fluctuations (reviewed in Dunlap et al., 2004). In some species however, individuals manifest prolonged periods with no circadian rhythms. For example, in the naked mole rat, the only known eusocial mammal, most individuals have robust circadian rhythms when isolated individually, but are arrhythmic when studied in the social context of the colony (Davis-Walton and Sherman, 1994; Riccio and Goldman, 2000). Arrhythmicity cannot be attributed solely to their fossorial lifestyle because solitary subterranean mole rats exhibit strong circadian rhythms in locomotor activity (e.g. Lovegrove and Papenfus, 1995). In honey bees, activity with no circadian rhythms characterizes young workers that typically stay in the dark and thermoregulated hive. By contrast foragers, which are typically older, have a pattern similar to those of other diurnal species, with strong circadian rhythms typified by outside activity during the day and sleep-like behavior at night (Kaiser and Steiner-Kaiser, 1983; Moore et al., 1998). Because both honey bees and naked mole rats are highly social (eusocial) species,

it is tempting to hypothesize that the incidence of individuals with no circadian rhythms is associated with eusociality. This hypothesis is particularly attractive for honey bees, whose activity with no circadian rhythms is linked to brood care (nursing) behavior and appears to be functionally significant because it enables around-the-clock care for the developing larvae. Consistent with this premise is the finding that old foragers who reverted to nursing brood switched back to around-the-clock brood care activity similar to that of young nurses in typical colonies (Bloch and Robinson, 2001). The hypothesis that social evolution shapes the circadian system has significant evolutionary, sociobiological and chronobiological implications because it predicts molecular and physiological differences in the clock system of species with different levels of social organization. Studies on honey bees have indeed shown that age-based division of labor is correlated with age-related development of circadian rhythms, but it is not clear to what extent the circadian behavior of nurse-age bees in the laboratory corresponds to that of nurses in the social context of the hive. Around-the-clock brood care may not be governed by the internal pacemaker: it could also result from an uncoupling of the pacemaker from locomotor activity, or from nursing activity overriding (‘masking’) the clock

output. Indeed, many nurses that are removed from the colony and monitored individually manifest circadian rhythms in locomotor activity even at a young age (Bloch et al., 2006).

To test the hypothesis that division of labor is associated with endogenous variation in the circadian clock, we investigated the circadian system in the bumblebee *Bombus terrestris*, in which division of labor is based largely on polymorphism in worker body size (Alford, 1975; Michener, 1974). Thus, many task-related differences are determined during pre-adult development and are expected to be more permanent than in honey bees and naked mole rats. Bumblebees are taxonomically related to honey bees but differ in the specifics of their social organization; their societies are annual, simpler and much smaller than for honey bees (a few dozen to a few hundred compared with some tens of thousands, respectively). Division of labor in bumblebees relates to size, with foragers significantly larger than nest bees. Age plays a minor role, if any (age polyethism is weak or absent) (Alford, 1975; Michener, 1974).

We first analyzed activity rhythms for *B. terrestris* foragers and nurses in free-flying colonies in the field. We then sought to determine whether task-related differences in activity rhythms are endogenous by analyzing locomotor activity for small (nurse-size) and large (forager-size) bees in both constant and oscillating laboratory environments. Our findings indicate that small bees emerge from the pupae with an attenuated circadian clock, and tend to have a nocturnal pattern of activity during their first days of adulthood in a light:dark (LD) illumination regime, whereas larger bees typically manifest strong circadian rhythms early in life and tend to have a diurnal activity pattern in a LD illumination regime. This variation in clock function corresponds closely to the division of labor in the colony: small bees specialize in around-the-clock brood care activity whereas large bees tend to perform foraging activity, starting as early as 2 days of age.

Materials and methods

Bees

Bombus terrestris L. colonies were purchased from Polyam Pollination Services, Kibbutz Yad Mordechai, Israel. Colonies were obtained a few days after the emergence of the first worker and contained a queen, 1–10 young workers, and brood at various stages of development. We reared the colonies in wooden nest-boxes (30×23×20 cm) with a Plexiglas™ cover. We performed all treatments and inspections under dim red light invisible to bees (Peitsch et al., 1992).

Division of labor and circadian rhythms in colonies foraging in the field

Newly emerged bees were measured and marked individually with colored numbered tags (Graze, Weinstadt, Germany). We replicated this experiment with three colonies. In colonies 2 and 3, we used the forewing length (measured immediately after emergence) as an index of body size. In colony 2, we also measured the length of the forewing marginal

cell (at the end of the experiment); these two size indices were highly correlated ($N=61$, $R^2=0.90$, $P<0.001$, $y=3.76x+0.07$). We did not measure size for bees from colony 1.

To enable detailed observations, we housed focal colonies in a specially designed observation hive (made of transparent Plexiglas™, 32×24×13 cm) that was placed inside an environmental chamber (29±2°C). We placed the observation hive on shock absorbers to minimize vibrations, to which bumblebees are very sensitive. During the first few (9–15) days the colony was fed *ad libitum* with commercial syrup (Polyam Pollination Services) and fresh pollen (collected by honey bees). The hive was connected to the outside by a clear plastic tube (~1 m length, 2 cm diameter) and food provision was gradually stopped when a focal colony contained 10–30 workers (7–13 days from the emergence of the first worker). We began the first observation session when a focal colony contained at least 28 workers. By that time, all colonies were self-supporting and collecting all the nectar and pollen they required.

For each colony we performed two 4-day sessions of observations separated by a 3–4 day break (Session A and Session B, respectively). This observation protocol allowed us to monitor the development of behavior of individually identified bees. We carried out observations every 3 h. Each observation included six scans of 10 min each (Bloch and Robinson, 2001). During the first 3 min we recorded all active bees and then recorded all behaviors for each tagged bee. Observers ($N=4$) were trained for several days before the experiment began to minimize inter-observer variability. Each observer was assigned to different shifts on each day to minimize the time×observer interaction. We recorded the following clearly identified behaviors: flying out of the hive, returning to the hive (with or without pollen), handling wax, inspecting a larvae cell, inspecting an egg cell, feeding larvae (a bee inserting its head into an open larval cell for >2 s; this behavior was followed by a quick contraction of the abdomen). The first two activities characterize foraging, and the remaining four characterize nursing (Brian, 1952; Free, 1955; O'Donnell et al., 2000). We defined a foraging trip as a bee departing from the nest and returning after ≥5 min. An additional category included all active bees. This category covered all behaviors of an active bee that did not fit the categories listed above (e.g. fanning, eating pollen, drinking sugar syrup, incubating brood, removing debris, walking). A bee that was inactive and suddenly toddled a few steps after being shoved by another bee was not considered to be active if it became quiescent again after four steps or less. An additional observer recorded foraging activity. In order to study the age of first foraging, in colony 3 we used a video recording system to automatically monitor foraging activity (CQMax; ADS, Inc., Gardena, CA, USA). This system employs motion detection technology to automatically record movement of bees moving between the hive and the outside. The monitoring system recorded activity for 24 h a day over the first 20 days following the connection to the outside (including the period spanning the two sessions of observations). For the video analysis of age at first foraging, we limited ourselves to pollen foraging. This was done to ensure that we did not record bees

performing orientation flights. To confirm that our analysis captured most if not all bees that foraged, we compared the video analyses and human observations during sessions A and B in Colony 3. We found that out of a sample of 47 bees that were observed foraging during the two sessions with human observers, 43 returned at least once with pollen and the other 4 were seen departing but were never seen to return.

We arbitrarily classified all bees within each 4-day observation session as a 'nurse', an 'intermediate', or a 'forager'. Nurses were defined as bees that performed <5 foraging events (a scan during which the bee was foraging), foragers were bees that performed >20 foraging events, and were recorded in at least twice as many foraging as nursing scans. These arbitrary definitions created two clearly distinct classes of bees. The first class covered bees that never or almost never foraged (nurses), and the second class included bees that specialized in foraging activities (foragers). Bees that did not fit into either of these two classes were defined as 'intermediates'. We only assigned category labels to bees that were observed performing any of these activities in ≥ 80 scans (total number of scans=192, 4 days \times 8 observations \times 6 scans). Circadian rhythms in activity (scans in which the bee was active) were assessed with ClockLab software (Actimetrics Co., Wilmette, IL, USA).

Circadian rhythms in locomotor activity for small and large bees in the laboratory

We monitored locomotor activity from the moment of emergence from the pupa. We collected small and large cocoons from young colonies (1–3 weeks after the emergence of the first worker) and placed each in a separate monitoring cage (8.5 cm diameter). Cocoons were fixed to the cage bottom with bumblebee wax such that its upper part faced upward, similar to their position in a colony. We provisioned each monitoring cage with a pea-size ball of pollen (collected by honey bees) mixed with sugar syrup, and a tube with sugar syrup; empty monitoring cages served as a control for spontaneous background noise. Environmental conditions were $29\pm 1^\circ\text{C}$, relative humidity=30–34% and constant dim red light (DD) in the first experiment, and $28\pm 2^\circ\text{C}$, relative humidity=25–55%, 12 h light (430–650 candela steradian m^{-2}): 12 h dim red light (LD) illumination regime, in the second experiment. Adult bees emerged from 42–70% (in four trials) of the cocoons in the first experiment (in DD), and 65–90% (in two trials) in the second experiment (in LD). Locomotor activity was monitored with the ClockLab data acquisition system using 1–2 light-sensitive black and white Panasonic WV-BP334 (Laguna, Philippines), 0.08 lux CCD cameras (each camera recorded activity from 24 cages), and a high-quality monochrome image acquisition board (IMAQ 1409, National Instruments Co., Austin, TX, USA). Data were collected continuously at a frequency of 2.5–3 Hz.

We measured the length of the forewing marginal cell at the end of each trial. Bees above and below the median of our bumblebee population (estimate based on a sample of 254 bees from three free-flying colonies, median marginal cell length=2.75 mm; median wing length=10.41 mm) were

classified as 'large' or 'small', respectively. These size classifications fit with the division of labor (χ^2 analysis, d.f.=2, $P<0.001$). Most bees defined as 'large' were also classified as foragers or intermediates, with only about 10% classified as nurses: the majority of bees defined as 'small' were also classified as nurses, with only about 10% falling into the forager category. Nevertheless, it should be pointed out that size distribution in *B. terrestris* is continuous rather than discrete, and the relationship between size and division of labor is probabilistic and not deterministic. Circadian rhythms and other properties of locomotor activity were analyzed using the ClockLab software (see below). Differences in the age at onset of circadian rhythms in locomotor activity between small and large bees were subjected to non-parametric Kaplan–Meier survival analyses with the Breslow statistic (SPSS) (for details, see Bloch et al., 2002). We pooled data from the four trials of the DD experiment and from the two trials of the LD experiment following analyses indicating no significant differences on any of the parameters tested (two-way ANOVA with trial and body size as factors, $P>0.05$).

Analysis of circadian rhythms

Circadian rhythms were determined using a χ^2 periodogram analysis (Sokolove and Bushel, 1978; ClockLab software). The criterion for rhythmicity was set at $P<0.01$ (Toma et al., 2000; Bloch et al., 2001; Bloch et al., 2002). The free-running period (FRP= τ , the average period for a cycle of activity under constant environmental conditions) was determined for the longest stable period sequence (ranging from 3 to 15 days). The FRP of some individuals ($N=21$, see Results) changed during the DD experiment. For these individuals, we also determined the new FRP, termed 'second FRP'. Accordingly, the initial FRP was termed 'first FRP'. The onset of rhythmicity for each bee was the first of at least 2 consecutive days in which a periodogram analysis for 3 successive days produced a statistically significant rhythm with a period of 19–29 h. We used the 'power' as an index for the strength of rhythm. The 'power' was defined as the height of the periodogram peak above the $P=0.01$ significance threshold. The periodogram peak is high when the rhythm is strong, for example, when a consolidated period of activity is limited to one part of the day, and a period with no activity to another part. The periodogram peak is low when periods of activity are spread throughout the day (for more details see Klarsfeld et al., 2003). The power value was determined for a sequence of 5 consecutive days starting with the first day of statistically significant circadian rhythms (we assigned a power=0 to five bees that did not develop circadian rhythms; one bee was excluded from analysis because she had a maximal sequence of <5 days of significant rhythms). The criteria for anticipation of light transition during the LD experiment were: (1) the bee had significant diurnal rhythms; (2) the bee was active during ≥ 3 successive bins, with an activity level $>1/3$ of the maximum for that day, before the light was turned on (for day-active bees), or off (for night-active bees); (3) the bee met criteria 1 and 2 for at least 2 consecutive days.

For the field experiment, circadian parameters were determined for each session of 4 successive days. Locomotor activity in the laboratory was analyzed for 5 min (in DD) or 10 min (in LD) bins and activity in field colonies with 30 min bins. Data from bees during the last 2–3 days before they died were excluded from the analyses.

Results

Division of labor and circadian rhythms in colonies foraging in the field

There was a clear division of labor between *B. terrestris* workers. Some workers specialized in nursing activities, whereas others specialized in foraging activities. In each of the three colonies we observed bees that cared for brood but did not forage during the observation sessions. By contrast, all bees that performed foraging activities also performed nursing activities. Division of labor was strongly correlated with body size. Large bees were more likely to perform foraging activities (linear regression analysis; sessions A and B in colonies 2 and 3, $R^2=0.25-0.51$, $N=20-50$, $P<0.001$; Fig. 1A). Bees that were classified as nurses were smaller than those classified as foragers or intermediates (see Materials and methods for classifications; one-way ANOVA, $P<0.001$, followed by Bonferroni multiple comparison, $P<0.05$, in both sessions in colonies 2 and 3). In a sample of 64 workers we found that 92% of the bees that were classified as nurses in session A were also classified as nurses in session B (Fig. 1B). By contrast, only half of the bees classified as foragers in session A fit this classification in session B; the rest were classified as intermediates. Intermediates constituted the most flexible group, with only 25% of those classified as intermediates in session A fitting this definition in session B, where 50% were classified as foragers and 25% as nurses (Fig. 1B). Intermediates were younger than both foragers and nurses (one-way ANOVA, $P<0.001$; Bonferroni, $P<0.005$ for both sessions in all three colonies, with the exception of

session B in colony 2). In Colony 3, in which we used an automatic motion-detection based video recording system, we found that large bees performed their first pollen foraging earlier than small individuals (Fig. 1C). This analysis revealed that some bumblebees performed successful foraging trips as early as 2 days of age (Fig. 1C).

Division of labor was associated with variation in diurnal rhythms. The proportion of bees with activity rhythms in the 19–29 h range was significantly higher for foragers as compared to nurses in session B in all three colonies (Fisher's Exact Test; $P<0.05$, two-tailed, Fig. 2A–C), and in session A (in which colonies contained fewer bees) in colony 2 ($P<0.001$). A similar trend was obtained in colonies 1 and 3 ($P=0.58$, $P=0.095$, respectively), and the proportion was significantly higher in a pooled analysis of all three colonies (Fisher's combined P -

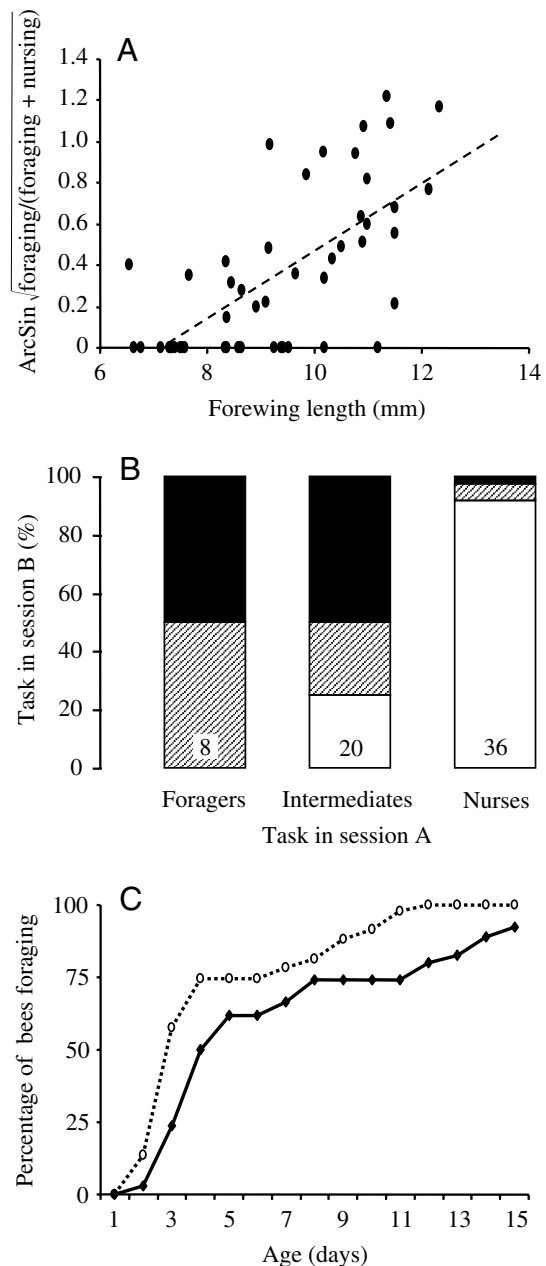
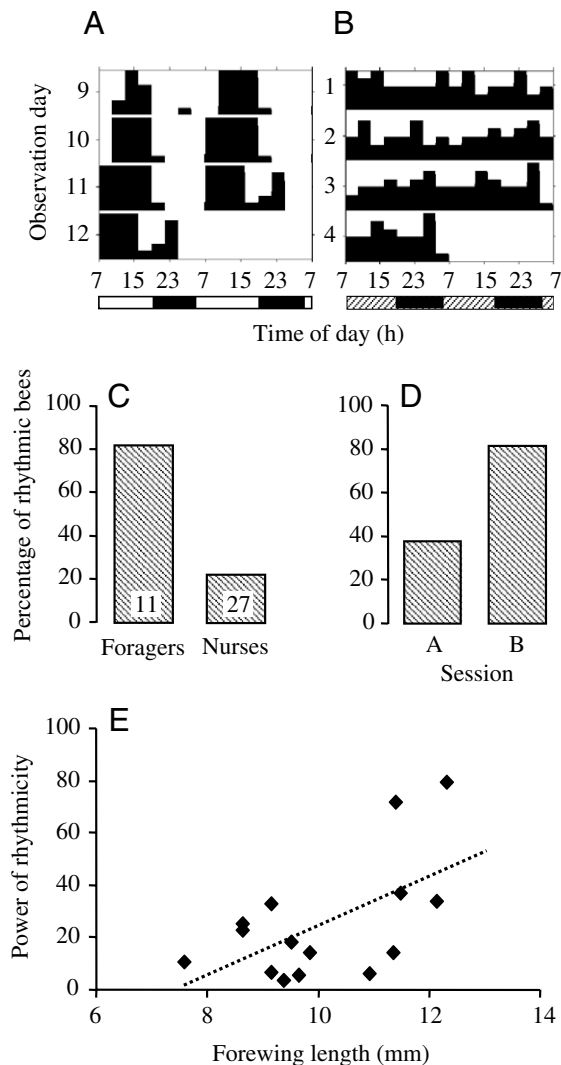


Fig. 1. Size and division of labor in *B. terrestris*. (A) The relationship between body size (forewing length) and the propensity to forage. Each filled circle represents a single bee observed in Session A of Colony 2. The broken line depicts the regression of foraging propensity on wing length ($y=0.16x-1.18$, $R^2=0.46$). Similar results were obtained in Session B for this colony and in both sessions for Colony 3 (size was not recorded in Colony 1). (B) Age-related plasticity in worker task. Individually marked workers were observed for two sessions of 4 successive days each. The two sessions (A and B) were separated by a period of 3–4 days. The figure depicts the percentage of foragers (filled bars), intermediate (hatched bars), and nurses (open bars) in session B as a function of their task in session A. Data were pooled from the three colonies. Numbers within bars indicate sample size. (C) Body size and age at first pollen foraging. Foraging trips (a bee returning to the hive with pollen) of individually marked bees were recorded using an automatic video system equipped with motion detection technology. Large bees (marginal cell ≥ 2.75 mm; dotted line, $N=59$) began to forage at a younger age than small bees (marginal cell <2.75 mm; continuous line, $N=34$; Kaplan–Meier Survival Analysis with Breslow statistics, $P=0.007$).

values, $P < 0.005$). An increase in overall activity in the hive typically started approximately 1 h before sunrise, consistent with the premise that the endogenous clock influences diurnal activity rhythms. To determine whether bees develop circadian rhythms with age, we compared the proportion of rhythmic bees in sessions A and B in a group of bees that were observed for both sessions (only included are bees < 3 days of age on the first day of session A). We found no changes for nurses (Fisher's Exact Test, $P > 0.37$, two-tailed in all three colonies, data not shown) but in a group that included all bees classified as foragers and intermediates in session A (both were typically large, see above), there was an increase in the proportion of bees with a circadian rhythm in session B (Fig. 2D, $N = 13$, $P < 0.05$). Worker size was positively correlated with power in both sessions of colonies 2 and 3 (in which we measured worker size, Fig. 2E, Linear regression analysis, $R^2 = 0.22\text{--}0.34$, $P < 0.05$).

Circadian rhythms in locomotor activity for small and large bees in the laboratory

In the first experiment we analyzed locomotor activity in DD for all bees that emerged from the pupa and lived for 6 days



or more [$N = 42$, age when they died or at the end of experiment = 17 ± 4.7 days (mean \pm s.e.m.); see representative actograms in Fig. 3A,B]. Five small bees did not develop circadian rhythms until the end of the experiment (≥ 16 days) or the day they died (≥ 10 days). We calculated the power of the circadian rhythm for the period after the bee developed significant circadian rhythms. The power was positively correlated with forewing marginal cell length (Fig. 3C). This correlation was significant even when we excluded bees that never showed circadian rhythms ($R^2 = 0.17$, $N = 36$, $F_{1,35} = 7.35$, $P = 0.01$). Large forager-size bees had stronger circadian rhythms compared to small nurse-size bees (Fig. 3D). Large bees also developed circadian rhythms at a younger age compared to small bees (Fig. 3A,B,E; large bees: $N = 17$, mean age = 2 days, 95% confidence interval = 2,3; small bees: $N = 25$, mean age = 6 days, 95% confidence intervals = 3,8). There were no significant differences between small and large bees in either the first (Mann-Whitney test, $N = 37$, $P > 0.5$) or second ($P > 0.1$) FRP. However, a larger proportion of small workers had a second FRP > 24 h compared to large bees (Fisher's Exact Test, $P < 0.05$, two-tailed).

In the second experiment we analyzed circadian rhythms in LD for bees that lived for 10 days or more ($N = 20$, $N = 27$, in the first and second trials, respectively). The majority (91%) of the large bees but only up to 67% of the small bees developed diurnal rhythms during their first 12 days after emergence from the pupa. It is notable that many bees, particularly during the first days after emergence, were active during the dark phase. This nocturnal activity pattern was more common for small bees. In fact, all small bees with statistically significant diurnal

Fig. 2. Division of labor and activity rhythms in free flying *B. terrestris* colonies. (A) Representative actogram for a forager. The numbers on the y-axis depict the day of observation. The height of the bars within each day corresponds to the number of scans in which the bee was active (see Materials and methods for details). Data for each day are double plotted to facilitate visual detection of rhythmicity. This individual has significant diurnal rhythms in activity. The bar at the bottom of the plot depicts the illumination regime. Open bar = day (sunrise to sunset); filled bar = night (sunset to sunrise). (B) Representative actogram for a nurse. Details of plot as in A. Hatched part of the illumination regime bar depicts the time of day for a bee that stays inside the constantly dark hive. This individual has no diurnal rhythms in activity. (C) Representative summary of the percentage of rhythmic nurses and foragers (Colony 2, session B). A higher proportion of foragers have significant circadian rhythms (Fisher's Exact Test, $P < 0.001$; similar results were obtained for two additional colonies in session B and for colony 2 in session A). (D) Development of diurnal rhythms in young foragers and intermediates in free-flying colonies (pooled data from all three colonies). The proportion of bees with circadian rhythms was higher in session B (Fisher's Exact Test, $N = 13$, $P < 0.05$). There was no similar development of rhythm in nurses. (E) Representative summary for the relationship between body size and the power of circadian rhythms (Colony 2, session A, $R^2 = 0.34$, $N = 15$, $P < 0.05$). Similar results were obtained for an additional colony. Note that in this experiment foragers experienced day–night oscillations whereas nurses typically stayed inside the relatively constant environment of the hive.

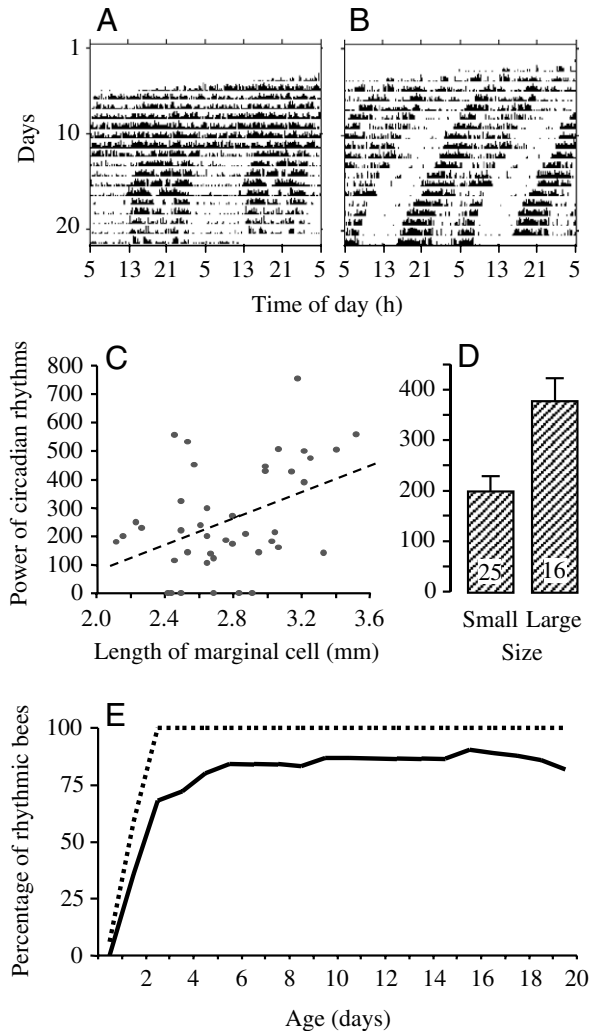


Fig. 3. Body size and circadian rhythms in locomotor activity for *B. terrestris* workers in a constant laboratory environment. Individual workers were monitored from the day they emerged from the pupa. (A) Representative actogram for an individual small bee. The height of the small bars within each day corresponds to the locomotor activity in a 5 min bin. Other details of the actogram are as in Fig. 2A. This individual emerged from the pupa at day 4, and had significant circadian rhythms at 6 days of age (power=273, determined after 6 days of age). There was an alternation in FRP at 13 days of age. (B) Representative actogram for an individual large bee. Details of actogram as in A. This individual is from the same trial as the one in A, emerged from the pupa at day 3, and had significant circadian rhythms for the first time at 3 days of age (power=505). (C) The relationship between body size and the strength of circadian rhythms. The broken line represents the regression for the power of circadian rhythms on the length of the forewing marginal cell (an index for body size; regression analysis; $N=41$, $R^2=0.2$, $P<0.005$; $y=264x-447$). (D) The power of circadian rhythms in small and large bees. Numbers within bars indicate sample size. The differences are statistically significant (unpaired t -test, $P=0.0015$). (E) Age at onset of circadian rhythms for large (broken line, $N=17$) and small (continuous line, $N=25$) workers. The differences between small and large bees were statistically significant (Kaplan–Meier Survival Analysis, Breslow statistic, $P<0.025$).

rhythms during the first 3 days after emergence had a nocturnal activity pattern (Fig. 4A,B). There appeared to be a trend for large bees to anticipate the light transition (light on or light off, for bees with diurnal and nocturnal activity patterns, respectively) at a younger age than small bees but this was not statistically significant (Survival Analysis, $P>0.05$, Fig. 4C).

Discussion

Large bumblebees forage with strong circadian rhythms, like most solitary diurnal species, but small bees emerge from the pupae with an attenuated clock and typically specialize in around-the-clock nursing activity. Our laboratory experiments provide the first evidence that this remarkable behavioral plasticity is associated with intrinsic differences between forager- and nurse-destined bees. The relationship between body size, circadian rhythmicity, and the propensity to forage are probabilistic and not deterministic. Larger bees are more likely to forage and to have strong circadian rhythms early in life, but there is an overlap in the size of nurses and foragers. Likewise, both the propensity to forage and the strength of circadian rhythms are correlated with size within the group of bees classified as foragers. The size-related variation in clock function is apparently determined during pre-adult development. An important aspect of our findings is that this variation in circadian rhythms is naturally occurring and not the outcome of treatments such as constant light or artificially induced mutations.

This study confirms and extends earlier studies that indicate that division of labor in bumblebees is based primarily on size (Alford, 1975; Michener, 1974). We found that in contrast to honey bees, *B. terrestris* nurses were not younger than foragers and were not likely to switch to foraging activities as they aged (during the period we studied, Fig. 1B). Our findings of differences in the circadian clock of newly emerged bees are congruent with recent evidence for size-related variation in the organization and function of the visual and chemosensory systems in *B. terrestris* (Brockmann et al., 2003; Spaethe and Chittka, 2003) and in learning performance in *B. impatiens* (Worden et al., 2005). The emerging picture is that large and small bees differ not only in size and allometry but also in function. Taken together, these studies suggest the existence of physical, size-related, subcastes in bumblebees, reminiscent of the caste polymorphism division of labor of many species of ants in which morphologically distinct subcastes (e.g. minor, media, soldier) differ in the pace at which they progress from nest to outside activities (Wilson, 1971). In both bumblebees and ants, properties of division of labor are established before the adult worker emerges from the pupa and are closely linked to processes determining body size. It is important to note that these differences in circadian clock function fit closely with the division of labor: strong and early circadian rhythms, as well as a diurnal activity pattern, make large bees more efficient foragers; low functioning of these same systems perhaps makes small bees inferior foragers, or even renders them incapable of foraging. This may account for earlier reports that small bees refrained from switching to foraging activities even under

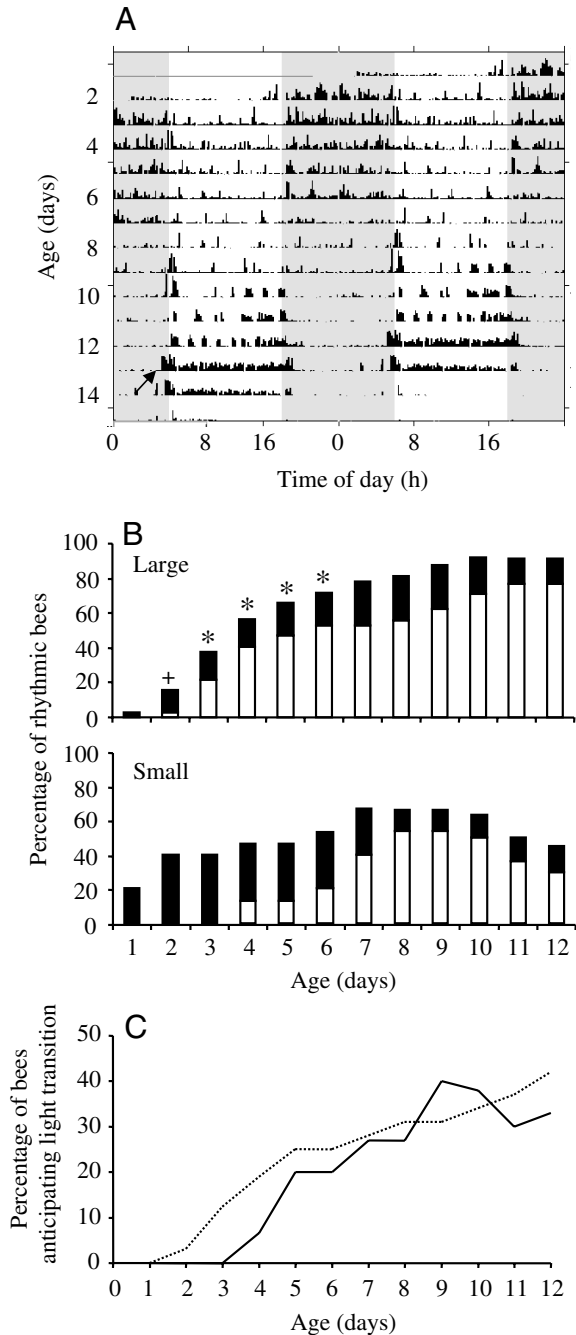


Fig. 4. Body size and circadian rhythms in locomotor activity for *B. terrestris* workers in an oscillating (LD) laboratory environment. (A) Actogram for a small bee. Details of the actogram as in Fig. 3A. The background of the plot delineates illumination regime: white background, light phase; gray background, dark phase. This individual emerged from the pupa at day 1, had a nocturnal activity pattern for the first 8 days, and then switched to a diurnal activity pattern. Anticipation of light on is evident at 12 days of age (black arrow). (B) Development of diurnal rhythms for large (top) and small (bottom) workers. Filled bars, significant rhythms with peak activity during the dark phase; open bars, peak activity during the light phase. Asterisks and plus signs indicate days in which there were significant differences in the proportion of small and large bees that were active during the light or dark phase, respectively (Fisher's 2×2 Exact Test, $P < 0.05$). (C) Anticipation of light transition in small (continuous line, $N=14$) and large (dotted line, $N=33$) workers. Included are bees with nocturnal activity that anticipated light-off and bees with diurnal activity that anticipated light-on (Survival Analysis, $P > 0.05$).

differences could be attributed to variation in the environment experienced by nurses and foragers; nurses stay in the dark and relatively constant environment of a hive, whereas foragers experience strong fluctuations in many environmental variables including light and temperature that influence both activity and the circadian clock.

The evidence for task-related differences in the endogenous circadian clock emerged from our analyses of locomotor activity in *B. terrestris* in the laboratory. We took advantage of the correlation between body size and task in our studies with free-foraging colonies (and in previous studies with bumble bees) (reviewed by Alford, 1975; Michener, 1974) to compare forager- and nurse-destined bees in controlled laboratory conditions. We found three size-related differences in circadian rhythms in constant environment (DD). Small bees manifested (internal) circadian rhythms in locomotor activity later in life, had weaker rhythms, and were more likely to show a spontaneous increase in FRP to values >24 h. In addition, in the LD illumination regime, small bees tended to anticipate the light transition later in life (an additional index for later onset of clock functionality), manifested a diurnal pattern later in life, and overall, fewer small bees had a diurnal pattern of activity. All the small bees that were 3 days or younger, and many at an older age, showed a nocturnal activity pattern. Small bees also had fewer and smaller cells immunoreactive to Pigment Dispersing Factor (PDF), a neuropeptide that is required for normal circadian rhythms in *Drosophila melanogaster*, and whose distribution is commonly used to define anatomical and functional properties of insect clocks (A. Dov and G. Bloch, unpublished data).

These findings in the laboratory fit with our observations of free-flying colonies in the field. Large bees typically perform their first foraging flight earlier in life (as early as 2 days of age, Fig. 1C), tend to specialize in foraging activities, and have a strong diurnal pattern of activity that anticipates sunrise. They therefore need a strong and stable circadian clock that is functional early in adult life. Small bees, on the other hand, typically perform nursing activities inside the dark and thermoregulated hive. They do not forage outside, or begin to

conditions of a severe shortage in foragers (e.g. Brian, 1952; van Doorn, 1987).

Around-the-clock activity in turn, may improve brood care behavior and make division of labor more efficient (Bloch and Robinson, 2001). Indeed, similar around-the-clock brood care activity was found in honey bees, in which division of labor is based on age and nurses are typically young (Moore et al., 1998). In both species foragers typically have strong circadian rhythms with reduced activity at night. Finding similar task-related plasticity in diurnal rhythms in two species that differ in the organization of division of labor lends credence to the premise that it has an adaptive value, but is not sufficient to imply that the circadian clock is involved. For example, these

forage relatively late in life. Their nocturnal activity pattern is perhaps related to a negative phototaxis. In honey bees, phototaxis is negative in nurses and positive in foragers (Ben Shahar et al., 2003); in *Drosophila* larvae, pacemaker cells and clock genes influence both phototactic and circadian behavior (Mazzoni et al., 2005). Nevertheless, the eventual (late) development of circadian rhythms and diurnal activity pattern in many small bees are potentially significant because they may enable small bees to perform foraging activities in situations of shortage in foragers (e.g. under strong predation pressure).

The differences in circadian rhythms in DD and LD were probably established during metamorphosis because in our experiments bees of different sizes were exposed to similar conditions during the last stages of pupal development and as adults. Variation in circadian rhythms of newly emerged workers was recently reported for honey bees tested during different months of the same year and is also attributed to developmental influences on the clock (Bloch et al., 2006). The only factor known to affect the development of the insect clock is light (Saunders, 2002), but light cannot account for the observed plasticity in the clock of *B. terrestris* (and honey bees) (Bloch et al., 2006). Our experiments were performed in constant darkness and environmental conditions were similar for small and large bees. Possible modulators of this developmental plasticity in the clock include variation in the microclimate or social environment of individual brood or differential feeding of larvae destined to develop into small and large individuals.

Our results establish a link between chronobiology and sociobiology by showing similar task-related plasticity in diurnal rhythms in two species of bees that differ in the organization of their division of labor, and by showing for the first time that this variability has an endogenous nature. Foragers have strong circadian rhythms, similar to those ubiquitous in solitary species, whereas nurses show a unique developmentally determined deactivation or attenuation of the circadian clock. This naturally occurring activity with no circadian rhythms does not result in pathology such as that produced by mutations and artificially induced arrhythmicity. Rather, it appears to be functionally significant because it improves task specialization by facilitating around-the-clock care for the brood. This finding, in turn, suggests that variation in social life can influence properties of the circadian clock, and that an ancient and conserved system such as the circadian clock can be shaped by natural selection to allow for better task specialization and proper integration of individuals into a society.

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