Behavioral Rhythmicity, Age, Division of Labor and *period* Expression in the Honey Bee Brain

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Abstract Young adult honey bees work inside the beehive "nursing" brood around the clock with no circadian rhythms; older bees forage for nectar and pollen outside with strong circadian rhythms. Previous research has shown that the development of an endogenous rhythm of activity is also seen in the laboratory in a constant environment. Newly emerging bees maintained in isolation are typically arrhythmic during the first few days of adult life and develop strong circadian rhythms by about a few days of age. In addition, average daily levels of *period* (*per*) mRNA in the brain are higher in foragers or forager-age bees (> 21 days of age) relative to young nest bees (~ 7 days of age). The authors used social manipulations to uncouple behavioral rhythmicity, age, and task to determine the relationship between these factors and *per*. There was no obligate link between average daily levels of *per* brain mRNA and either behavioral rhythmicity or age. There also were no differences in *per* brain mRNA levels between nurse bees and foragers in social environments that promote precocious or reversed behavioral development. Nurses and other hive-age bees can have high or low levels of *per* mRNA levels in the brain, depending on the social environment, while foragers and foraging-age bees always have high levels. These findings suggest a link between honey bee foraging behavior and *per* up-regulation. Results also suggest task-related differences in the amplitude of *per* mRNA oscillation in the brain, with foragers having larger diurnal fluctuation in *per* than nurses, regardless of age. Taken together, these results suggest that social factors may exert potent influences on the regulation of clock genes.

Key words social behavior, circadian rhythms, biological clock, *period* gene, division of labor, honey bee, social environment

Honey bees show postembryonic development of circadian rhythms (Stussi and Harmelin, 1966; Spangler, 1972; Stussi, 1972; Toma et al., 2000). This appears to be related to the ontogeny of their social behavior and, therefore, to contribute to the colony organization of their colony. Bees typically care for ("nurse") the brood and perform other tasks in the hive for the first 2 to 3 weeks of adulthood and then shift to foraging for nectar and pollen outside the hive for the remainder of their 4- to 7-week adult life

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(reviewed by Winston, 1987; Robinson, 1992). Bee larvae require constant care, and nurse bees work arrhythmically around the clock to provide it. Foragers, in contrast, have a highly developed internal circadian clock that is used for sun compass navigation, dance communication, and timing visits to flowers for maximum nectar and pollen availability (von Frisch, 1967; Crailsheim et al., 1996; Moore et al., 1998).

To study the underlying relationships between division of labor and the development of activity rhythms, Toma et al. (2000) cloned a bee homolog of the "clock" gene *period* (*per*). *per* was selected because it interacts directly, or indirectly, with all other known components of the circadian clock (for recent review, see Dunlap, 1999; Wager-Smith and Kay, 2000; Williams and Sehgal, 2001). Furthermore, analyses of mutant flies have revealed pleiotropic effects of the *per* locus on diverse aspects of neural and behavioral function (Wheeler et al., 1991; Hall, 1998; Andretic et al., 1999; Andretic and Hirsh, 2000; Williams and Sehgal, 2001). Analysis of brain mRNA levels in bees showed that *per* oscillates with peak levels during the night, as in other insects (Reppert et al., 1994; Hall et al., 1995; Hardin et al., 1996). In addition, bee foragers had a 1.5- to 3-fold increase in average daily levels relative to younger bees.

Postembryonic development of circadian rhythms also occurs in vertebrates (Turek and Zee, 1999), but not in *Drosophila* (Sehgal et al., 1992). The association between age-related division of labor, ontogeny of rhythms, and the apparent up-regulation of a clock gene makes the bee an attractive model to study how social and developmental factors regulate components of a biological clock, two poorly understood themes in chronobiology. We explored three hypotheses relating to the possible connections between behavioral rhythms, age-related division of labor, and developmental regulation of *per* expression in the bee brain. The "rhythm hypothesis" predicts that a higher daily average level of *per* is associated with the expression of behavioral rhythmicity. The "age hypothesis" predicts that nurse-age bees (~7 days old) always have lower *per* mRNA levels relative to forager-age bees (> 21 days old). Toma et al. (2000) showed that elevated *per* mRNA levels cannot be attributed exclusively to aging, because bees induced to forage precociously had levels similar to normal-age foragers. However, there are additional social manipulations that uncouple behavioral development from chronobiological aging in bees that we used to provide a more rigorous test of this hypothesis. The "task hypothesis" predicts an association between the average daily level of *per* and the bee's current occupation. We tested whether low levels of brain *per* are associated with nursing while higher levels are associated with foraging.

MATERIALS AND METHODS

We performed three experiments that uncoupled behavioral rhythmicity, age, and task to determine which factor, if any, is most closely associated with variation in average daily levels of *per* mRNA in the brain. In Experiment 1, we uncoupled age and behavioral rhythmicity by individually isolating bees in cages in the laboratory and exploiting the fact that there is variation in the age at which locomotor behavior becomes rhythmic (Toma et al., 2000). This enabled us to address the rhythm hypothesis by measuring *per* levels in same-age young bees that showed either rhythmic or arrhythmic locomotor behavior. In Experiment 2, we uncoupled age and task by establishing single-cohort colonies initially composed of all young bees. This manipulation enabled us to address both the age and task hypotheses by measuring *per* levels in normal-age nurses and precocious foragers that were the same age, and over-age nurses and normal-age foragers that were the same age. In Experiment 3, we uncoupled rhythmicity, age, and task by inducing foragers to revert to nursing in reversion colonies initially composed only of foragers. This enabled us to measure *per* levels in foragers and foragers that reverted to nursing (reverted nurses) that were the same age. Reverted nurses care for brood around the clock with no diurnal rhythms (Bloch and Robinson, 2001), as do normal-age nurses (Moore et al., 1998). Experiment 3 thus addressed the rhythm, age, and task hypotheses. Experiments 2 and 3, which exploit the natural plasticity of honey bee division of labor (Robinson, 1992, 1998), were performed in the field with free-flying colonies.

Bees

To reduce genetic variability, bees used in all experiments were from "source" colonies that were each headed by a queen instrumentally inseminated with semen from a single (different) drone. All bees in each trial of an experiment thus had an average coefficient of relatedness of 0.75 due to haplodiploidy (Page and Laidlaw, 1988). Each source colony was maintained in

the field at the University of Illinois Bee Research Facility, Urbana, according to standard commercial techniques and had a population of about 40,000 bees, typical for field colonies. The colonies were derived from a mixture of European races of *Apis mellifera*, typical for this region. Two source colonies (R30 and R36) were used in both Experiments 2 and 3.

Care was taken to minimize the exposure of bees to extrinsic factors that can influence the functioning of circadian clocks. To obtain 0- to 24-h-old adult (1-day-old) bees, we removed honeycomb frames containing pupae (sealed in cells) from colonies in the field and immediately transferred them to a light-proof container, which was then placed in a dark incubator (33 °C, 95% relative humidity). All other manipulations, observations, and collections were performed under dim red light, which bees cannot see (von Frisch, 1967). Bees for mRNA analysis were collected directly into liquid N_2 and stored at –80 °C until brain dissection. Average daily levels of *per* were calculated from bees collected at three or four different time points during the day. Because *per* oscillates with a circadian rhythm, multiple time points were necessary to ensure that differences in phase or amplitude were not interpreted as differences in average daily levels.

Experiment 1: *per* Brain mRNA Levels in Behaviorally Rhythmic and Arrhythmic Bees

Locomotor activity was monitored as in Toma et al. (2000). We transferred 1-day-old bees and foragers from the same source colonies to individual clear Plexiglas cages with sugar syrup (50%, w/v) and pollen supplement (a ball 4-6 mm in diameter). To synchronize behavioral rhythms, bees were maintained at 26 °C in an environmental chamber for 3 days under light-dark (LD). Because we collected foragers from the field, we entrained the young bees with an LD regime similar to the natural photoperiod conditions experienced by foragers at the time. In the first trial, which was performed in June, the LD regime was set to 15:9 (colony R1), and in the second trial, which was performed in September, 12:12 (colony R7). After 3 days of LD, the bees were kept in constant darkness (DD). Locomotor activity was monitored under LD during Days 1 to 3 and then under DD during Days 4 to 7. Bees were collected every 6 h starting on the 4th day of DD, that is, when the young bees were 7 days old (locomotor activity was monitored for foragers only in the first trial). The foragers were used as positive controls; when maintained under similar conditions, they have well-developed locomotor rhythms (Splanger, 1972; Kaiser and Steiner-Kaiser, 1983; Moore and Rankin, 1985) and high levels of brain *per* mRNA (Toma et al., 2000).

Locomotor activity was monitored automatically with an infrared sensor and the DataCol 3 acquisition system (Mini-Mitter Co., Sunriver, OR, USA). Locomotor events were analyzed in 10-min bins. χ^2 periodogram analysis (Tau program, Mini-Mitter; Sokolove and Bushel, 1978) was used to determine whether patterns of activity had circadian rhythms. The criterion for rhythmicity was set to $p < 0.01$ (as in Toma et al., 2000) because it resulted in only those activity records with a single clearly defined peak of free-running period showing statistical significance.

Two trials of this experiment were performed, and they differed in several respects. In each trial, bees were from a different unrelated source colony (colonies R1 and R7, $n = 93$ young bees and 93 foragers in each trial). We also used two different methods to quantify *per* mRNA, Northern blot analysis in Trial 1 and real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in Trial 2. This allowed us to compare results from the relatively new technique of real-time quantitative PCR with a more traditional technique. Finally, as stated above, we used different LD regimes so that each trial could be conducted under the conditions appropriate for that time of year.

Experiment 2: *per* Brain mRNA Levels in Bees from Single-Cohort Colonies

We used standard methods to make a single-cohort colony (e.g., Robinson et al., 1989). We marked ca. 1200 one-day-old bees (obtained from a source colony as described above) with a paint spot on their thorax over 2 to 3 days and placed them in a small beehive with a queen (unrelated to the workers), one honeycomb frame of honey and pollen, and one empty honeycomb frame (for the queen to lay eggs). Each single-cohort colony was placed in an incubator for the first 4 days and then taken outside to a small building. Single-cohort colonies were placed in a building rather than set outside in the open, to enable us to regulate colony ambient temperature and to minimize exposure to light when the hives were opened for inspections and bee collections. The building was maintained at about 28 °C in constant darkness; a dim red

light was used for hive inspections and bee collections. To allow for free flight, the hive entrance was connected to an opening in the wall with a Plexiglas tube (4 cm diameter, 40 cm long). We opened the hive entrance 1 day after moving the colony to the small building.

Nurse bees and foragers were identified according to standard criteria (e.g., Moore et al., 1998). A nurse bee was an individual with its head in a honeycomb cell containing an egg or larva. A forager was a bee returning to its hive with a distended abdomen (swollen with a load of nectar, or, rarely, water; Robinson and Page, 1989) or with loads of pollen in its pollen baskets, conspicuously located on the hind legs. Observations of foraging were facilitated by temporarily obstructing the hive entrance with a piece of 8 mesh wire hardware cloth. Observations of foraging were conducted for 4 to 5 h/day at times when there were no orientation flights occurring in front of the hive (from 1000 to 1700 h). These flights, usually distinguishable from foraging flights, are taken by young bees before they become foragers (Winston, 1987; Capaldi et al., 2000).

Approximately 5% to 10% of the members of a single-cohort colony develop into precocious foragers about 2 weeks earlier than usual because older bees are not present to inhibit the behavioral development of young bees (Huang and Robinson, 1992). We collected young bees, both normal-age nurses and precocious foragers, when they were 8 to 10 days old (n = 15-25 per group, except young foragers in R30 for which we collected 2-15 per group). We then removed the frame of pupae and replaced it with an empty frame to prevent the emergence of new adult bees (that typically work as nurse bees). Old bees, both over-age nurses and normal-age foragers, were collected when they were 23 to 25 days old (n = 10-25 per group). Bees from each behavioral group were collected every 6 h around the clock (0500, 1100, 1700, and 2300 h).

Measurements of *per* mRNA were made from bees from two single-cohort colonies; in one single-cohort colony, there were bees from two different source colonies, for a total of three replicate analyses (each with bees from an unrelated source colony, R14, R30, and R36). mRNA was quantified with real-time quantitative RT-PCR.

Experiment 3: *per* Brain mRNA Levels in Bees from Reversion Colonies

We placed ca. 2000 to 2500 identified foragers in a glass-walled observation hive containing a queen, one honeycomb frame with young brood (eggs and 1- to 3-day-old larvae), and one frame with honey and pollen. Foragers were obtained by vacuum as they returned to their hive (entrance temporarily obstructed as above). The foragers were vacuumed into small cages, chilled on ice, and transferred into the observation hive. Previous studies have shown that making a colony solely of foragers results in 10% to 20% of them showing behavioral reversion, from foraging to nursing (Page et al., 1992; Robinson et al., 1992; Huang and Robinson, 1996).

For each reversion colony, the foragers, queen, and brood were taken from the same source colony, so they were all related to each other. Each observation hive was housed in a room at the Bee Research Facility maintained at 25 ± 3 °C under dim red light. To allow for free flight, the hive entrance was connected to an opening in the wall as described above. The hive entrance was opened 1 day after colony establishment; observations began on Day 3 to allow the bees time to acclimate to the new hive. Foragers and reverted nurses were collected following 3 days of observations (6-7 days after colony establishment). Observations of nursing behavior were facilitated by marking each bee with a numbered colored tag on the thorax and a paint symbol (corresponding to the last digit in the tag) on its abdomen. The abdomen marking, visible when the bee is inside a cell contacting a larva, alerts the observer to the presence of a focal bee that can be identified after it emerges from the cell.

We performed three trials of this experiment, each with bees from a different source colony (R30, R36, and R39). Nurse bees and foragers were identified and then marked with individually unique colored number tags (Colony R36: n = 99 and 86 reverted nurses and foragers, respectively; Colony R30: n = 180 and 91, respectively). Bees in these two trials were of unknown age; since all reverted nurses were once foragers, we assume that their age distributions were not different than those of the foragers from their colonies. In one trial (with Colony R39), *per* levels were measured in foragers and reverted nurses of known age. They were obtained by marking (with a paint spot on the thorax) about 3100 one-day-old bees in source colony R39 over a 7-day period and collecting about 500 of them as returning foragers when they were 21 to 28

days old (typical ages for foragers; see Winston, 1987). Identified foragers and reverted nurses were collected at three time points, 0400, 1300, and 2200 h. *per* mRNA was quantified with real-time quantitative RT-PCR.

The reversion colonies from which we sampled for *per* analysis in this study showed a strong reorganization of their division of labor (Bloch and Robinson, 2001). Most bees that reverted to nursing stopped foraging. Instead, they attended brood around the clock, with no diurnal rhythms, just as normal-age nurses do in more typical colonies (Moore et al., 1998). The nursing behavior of reverted nurses was effective; adults of normal appearance emerged from the brood they reared.

Brain Dissection

Brains were dissected on a frozen dissecting dish in dry ice and remained frozen during the entire dissection procedure. The compound eyes, ocelli, hypopharyngeal glands, and any other glandular tissues were removed during dissection. Because *per* mRNA levels are expressed on a per-brain basis, brains in which pieces of tissue were lost were discarded and only intact brains were analyzed. Each brain was stored individually at –80 °C until *per* mRNA quantification.

Northern Blot Analysis

Northern blot analysis were performed as in Toma et al. (2000). Total brain RNA was isolated using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA (5 µg), from 4 to 10 brains per time point, was separated on 1% agarose/0.6 M formaldehyde gels. We measured levels of *per* relative to *elongation factor 1 alpha* (*EF-1*α). mRNA levels for *EF-1*α do not vary diurnally, in bees of different ages, or between nurses and foragers (Toma et al., 2000). Filters were first probed with *per* and then stripped and reprobed with *EF-1*α. We used a 1083-bp *per* riboprobe from a PCR fragment, which spanned most of the C2 region, and a 600-bp honey bee *EF-1*α riboprobe (Toma et al., 2000). Both probes were digoxygenin (DIG)-11-UTP labeled and were used with DIG Easy Hyb buffer (Boehringer Mannheim, Indianapolis, IN, USA, manufacturer's protocol); *per* blots were probed at 62 °C with 35 ng/ml probe.

Real-Time Quantitative RT-PCR

In a real-time PCR, DNA amounts are quantified by measuring fluorescence emission (from a probe specific to the focal gene) during the linear phase of the amplification step of a PCR reaction, which is the most reliable time for measurement (Heid et al., 1996; Gibson et al., 1996; Winer et al., 1999). The specificity and sensitivity of this method enabled us to measure *per* mRNA levels from individual bee brains. Measurements were performed on individual brains for all analyses except those in Experiment 1 (performed on RNA pooled from 4-7 brains). Total brain RNA was isolated using the RNeasy total RNA isolation kit followed by treatment with DNase (1 U RQ1 DNase, Promega, Madison, WI, USA, or on-column Rnase-Free DNase, Qiagen, according to manufacturer's instructions). Total RNA (200 ng) from a single brain was reverse-transcribed in 50 µl 1X RT buffer, 5.5 mM MgCl, 500 µM deoxy NTPs mixture, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, and 1.25 U/µl MultiScribe Reverse Transcriptase (TaqMan Reverse Transcription Reagent kit, PE Applied Biosystems, Haywood, CA, "ABI"). Reverse transcription was carried out at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min, and then incubated at 4 °C.

We measured levels of *per* relative to *EF-1*α. Quantitative RT-PCR was performed with real-time TaqMan technology (Heid et al., 1996; Gibson et al., 1996; Winer et al., 1999) using an ABI Prism 5700 sequence detector. Primer Express software program (ABI) was used to help design the specific primers and probe. F: 5'-CACTATGTACGGCAGCGATGAA-3' (anneals between residues 1689-1710); R: 5'-ACCACTGCTAAGGTTTTCTGCACTA-3' (1839-1815); TaqMan probe: 5'FAM (6-carboxy fluorescein)-CAGCGGTCAGGAAATGCATGTCACC-3' TAMRA (6-carboxy-tetramethylrhodamine) (1760-1784). To quantify *EF-1*α, a 82 bp fragment was amplified. F: 5'-GCAGTTGATCGTTGGAGTGAAC-3' (153-174); R: 5'CCTCTTTCTTGATCTCCTCGAAAC- 3' (235-212); TaqMan probe: 5'FAM-AGATGGACATGACCGATCCCCCG-3'TAMRA (176-198). PCR primers and TaqMan probes were obtained from ABI.

Each sample was analyzed in triplicate for both *per* and *EF-1*α (i.e., 6 PCR reactions per sample), loaded on the same reaction plate (96 wells). Amplification reactions (25 µl) contained 1X TaqMan Universal PCR Master Mix, 1 µM of each primer, 0.6 µM TaqMan probe, and 10 ng cDNA (control samples had no

reverse transcriptase). Amplification thermal profile: 50 °C for 2 min, 95 °C for 10 min (95 °C for 15 sec, 60 °C for 1 min) × 40 cycles. Outliers (standard deviation among triplicates > 1.0) were excluded from analyses.

To quantify *per* mRNA, we recorded the number of PCR cycles (Ct) required for each reaction's fluorescence to cross a threshold value of intensity, set to pass through the linear portion of the amplification curve. For each sample, the difference in Ct was used to calculate the amount of *per* relative to *EF-1*α (ΔCt). Samples in each run were normalized relative to a control sample ($2^{-\Delta\Delta Ct}$, according to ABI User Bulletin #2; see also Winer et al., 1999). Statistical analyses (see Results) were performed on ΔΔCt values, which were normally distributed. Measurements with the real-time RT-PCR method indicated that levels of *EF-1*α did not vary with age, task, or time of day (data not shown).

RESULTS

Experiment 1: *per* Brain mRNA Levels in Behaviorally Rhythmic and Arrhythmic Bees

We tested the rhythm hypothesis by comparing *per* mRNA levels in rhythmic and arrhythmic young bees and by comparing young rhythmic bees with rhythmic foragers. Behavioral analyses of rhythmic and arrhythmic bees are presented in Figure 1A and Table 1. All foragers had strong and significant circadian rhythms of locomotor activity (periodogram analysis, χ^2, $p < 0.01$) and high levels of *per* mRNA in the brain (Figs. 1 B-D), as in previous studies (Spangler, 1972; Moore and Rankin, 1985; Toma et al., 2000).

Most young bees (70.1% and 76.7% in Trials 1 and 2, respectively) were rhythmic under LD conditions (Days 1-3), but only 47.4% and 38.4% (Trials 1 and 2, respectively) expressed circadian rhythms under DD in Days 4 to 6 (Table 1, Fig. 1A). Bees that were rhythmic in LD were more likely to exhibit a rhythm in DD in Trial 2 (Fisher's 2×2 test, $\chi^2 = 8.87$, $p = 0.003$); a similar trend was seen in Trial 1, but the differences were not statistically significant ($\chi^2 = 1.0$, $p = 0.3$). The free-running periods (*tau*) of these rhythms varied between 16.5 and 29.8 h, as in Toma et al. (2000). We monitored the locomotor activity of foragers only in Trial 1; in this trial, they had significantly less overall activity relative to both groups of young bees, rhythmic and arrhythmic (Table 1). Foragers also had a significantly shorter *tau* than did young rhythmic bees (Table 1).

Analyses of *per* mRNA with Northern blots in Trial 1 provide comparable results to those obtained with real-time quantitative PCR in Trial 2. This occurred even though we used different source colonies, different methods for *per* mRNA quantification, and different LD regimes. Foragers had significantly higher average daily levels of brain *per* mRNA compared with both groups of young bees (Fig. 1., ANOVA, $p < 0.05$). Moreover, in both trials there was no significant difference in average daily levels of brain *per* mRNA between young rhythmic and arrhythmic bees ($p > 0.05$). Because RNA was analyzed in bees collected after 3 days in DD, the bees were probably not well synchronized when collected. This makes it difficult to compare patterns of *per* oscillation among the different groups. Apparent differences between peak levels in the two trials may have been due to differences between the two source colonies used (Table 1, Toma et al., 2000) and differences in light regimes (Qiu and Hardin, 1996; Majercak et al., 1999; Nuesslein-Hildesheim et al., 2000).

Experiment 2: *per* Brain mRNA Levels in Bees from Single-Cohort Colonies

In this experiment, we tested the task and age hypotheses by uncoupling age and task in colonies, each established with a single cohort of young bees. In these single-cohort field colonies, there were no consistent age- or task-related differences in average daily levels of *per* brain mRNA (Fig. 2; two-way ANOVA, $p > 0.05$ for both age and task, in all three trials). This is in contrast to the differences between foragers and young bees in Experiment 1 when bees were maintained in individual cages in the laboratory. Previous results indicate that precocious foragers in single-cohort colonies have *per* levels that are similar to normal-age foragers and other older bees (Toma et al., 2000, Fig. 4). We therefore assume that both nurses and foragers in single-cohort colonies showed a premature up-regulation of *per*.

Our results suggest task-related differences in the diurnal oscillation pattern of *per* mRNA in the brain (Fig. 2A). Levels were highest during the night and lowest during the day, as in Toma et al. (2000) and other insects (reviewed by Reppert et al., 1994; Hall, 1998; Young, 1998; Dunlap, 1999). Foragers (both young = 8-10 days of age, and old = 23-25 days of age)

Table 1. Analysis of locomotor activity for bees from Experiment 1 (mean ± SE [*n*]).

	Trial 1			
	Percentage Rhythmic in DL	*Percentage Rhythmic in DD*	*Period (*tau*) (h)*	*Activity (events/10 min)*
A. All Bees	70.1 (78)	47.4 (78)	23.4 ± 0.6 (37)	26.9 ± 2.2 (78)
All young	ND	100 (73)	22.2 ± 0.2 (73)	14.4 ± 1.0 (73)
All foragers		$p < 0.01$	$p < 0.01$	$p < 0.001$
B. Bees Used for per Analysis	33.3 (18)	0 (18)	—	29.6 ± 4.7 (18) a
Young arrhythmic	84.2 (19)	100 (19)	23.1 ± 0.6 (19)	24.1 ± 3.9 (19) a
Young rhythmic	ND	100 (47)	22.1 ± 0.1 (47)	14.9 ± 1.4 (47) b
Foragers			$p < 0.05$	$p < 0.05$
	Trial 2			
	Percentage Rhythmic in DL	*Percentage Rhythmic in DD*	*Period (*tau*) (h)*	*Activity (events/10 min)*
A. All Bees				
All young	76.7 (86)	38.4 (86)	22.5 ± 0.3 (33)	19.0 ± 1.6 (86)
B. Bees Used for per Analysis	60.0 (25)	0 (25)	—	15.4 ± 2.9 (25)
Young arrhythmic	94.1 (17)	100 (17)	22.2 ± 0.3 (17)	26.2 ± 3.5 (17)
Young rhythmic				$p < 0.05$

NOTE: The last row in each table shows the results of statistical analyses (unpaired *t*-tests, besides Trial 1B activity, ANOVA with Fisher PLSD post hoc test groups with different letters were significantly different). Activity was measured as the number of times a bee crossed the infrared sensor ("event") during each 10-min period (see Materials and Methods). Locomotor activity was not monitored for foragers in Trial 2. ND = not determined.

showed significant diurnal differences in *per* mRNA levels in 6 out of 6 cases (ANOVA, $p < 0.05$). In contrast, there were no significant diurnal differences ($p > 0.05$) for nurse bees in 5 out of 6 cases (normal-age nurses in Colonies R14, R30, and R36 and over-age nurses in Colonies R30 and R36). In addition, peak levels appeared to be higher for foragers compared with nurses, irrespective of age (Fig. 2A).

Experiment 3: *per* Brain mRNA Levels in Bees from Reversion Colonies

This experiment addressed the rhythm, age, and task hypotheses. We compared foragers with foragers that reverted to nursing behavior (reverted nurses). Reverted nurses cared for brood with no diurnal rhythms and were of similar age to foragers (Bloch and Robinson, 2001). Average daily levels of brain *per* mRNA for reverted nurses (Fig. 3) were not significantly different from foragers in 2 out of 3 colonies (two-way ANOVA for time and task effects; task effect, $p > 0.05$). They were significantly lower ($p < 0.05$) in one colony (R30). An experiment-wide analysis revealed no effect of reversion on *per* levels (three-way ANOVA, task effect, $p = 0.098$; Table 2).

There was some evidence to support the idea that there are task-related differences in *per* oscillation. There were significant differences in *per* levels for foragers collected at different times of day in all three colonies and for nurses in 2 out of 3 colonies (R30 and R36) (Fig. 3A; ANOVA, $p < 0.05$). A more detailed analysis revealed that the difference between the highest and lowest daily values was significantly greater for foragers than for reverted nurses ($p < 0.05$, Wilcoxon Signed Rank test; data from all three colonies pooled).

DISCUSSION

This study provides new insights into the ontogeny of behavioral rhythms and the regulation of *per* in honey bees. At the behavioral level, we describe three different behavioral responses to controlled chronobiological conditions, which may reflect three stages of circadian system functioning in young adult bees (Experiment 1). In the first stage, bees are arrhythmic; they show no rhythmicity in either constant darkness or light-dark conditions. In the second stage, bees can respond to a light cue and thus synchronize their activity with the environment, but they cannot sustain endogenous rhythms (in the absence of light). In the third stage, bees can both respond to light and sustain endogenous rhythms. Forager bees correspond to this most advanced stage (e.g., Fig. 1A;

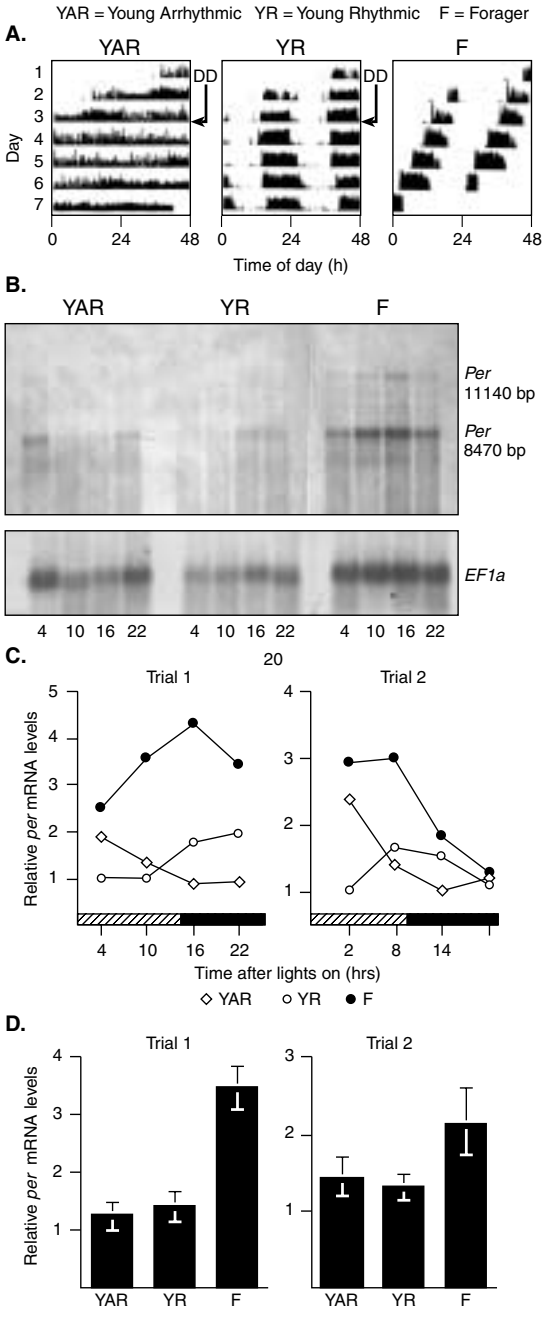

Figure 1. Circadian rhythms in locomotor activity and *per* brain mRNA levels in honey bees. Bees were entrained for 3 days and then transferred to constant darkness (DD). (A) Representative actograms of young arrhythmic (YAR), young rhythmic (YR), and forager (F) bees. (B) Northern blot analysis for Trial 1 (Trial 2 performed with real-time reverse transcription (RT) quantitative polymerase chain reaction). Two transcripts were detected; we do not know whether they are alternative transcripts of alleles, splice variants, or whether the larger transcript is a pre-mRNA. Values for both transcripts were summed for the analysis in Panel C. Portions of this figure appeared in Toma et al. (2000). (C) Quantification of *per* mRNA levels over time. Each data point represents the value of a single, pooled mRNA sample (4-7 brains). (D) Average daily *per* mRNA levels. Same data as in Panel C but averaged over each day to facilitate group comparisons. Results of statistical analyses appear in text.

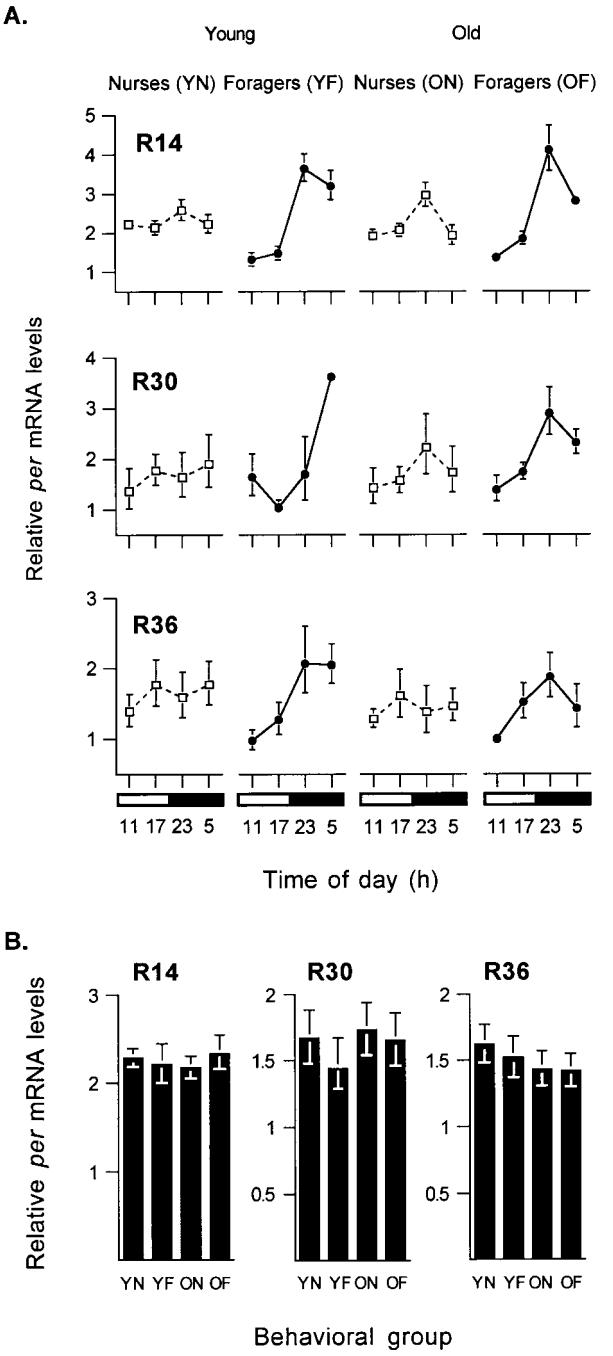

Figure 2. Age, task, and *per* brain mRNA levels (mean ± SE) in bees from single-cohort colonies. Young nurses and foragers are 8 to 10 days of age, old nurses and foragers are 23 to 25 days of age. (A) Quantification of *per* mRNA levels over time (n = 7-8 brains/behavioral group/time point analyzed individually from R14; 8 from R36; and 5-6 from R30, except n = 3 young foragers for CT 11, 2 for CT 23, and 1 for CT 5). The x-axis depicts the time of day. The bars in the bottom of each plot depict the ambient photoperiod: filled bars = dark (sunset to sunrise). Note that the bees' exposure to light did not necessarily match daylight time (e.g., bees can leave the dark hive some time after sunrise). (B) Average daily *per* mRNA levels. Same data as in Panel A. Results of statistical analyses appear in text. Levels of mRNA were measured with real-time quantitative reverse transcriptase polymerase chain reaction.

Table 2. Results of three-way analysis of variance for brain levels of *per* mRNA for bees from reversion colonies (Experiment 3).

	DF	*Sum of Squares*	*Mean Square*	F *Value*	p *Value*
Time	2	32.657	16.329	20.268	<0.0001
Task	1	2.234	2.234	2.773	0.0980
Time × Task	2	5.107	2.554	3.170	0.0448
Colony	2	9.488	4.744	5.889	0.0034
Time × Colony	4	7.158	1.789	2.221	0.0693
Task × Colony	2	5.580	2.790	3.463	0.0338
Time × Task × Colony	4	4.779	1.195	1.483	0.2100
Residual	152	122.457	0.806		

NOTE: Data in Figure 3.

Spangler, 1972; Moore and Rankin, 1985), and abundant experimental evidence indicates that a highly functional circadian system is required for foraging (von Frisch, 1967). The reasons why adult workers do not have a fully functional circadian system upon emergence are not known, but these results suggest that this delayed development is one of the factors that limit honey bees from foraging in the first few days of adulthood. This maturational factor may be important in structuring age-related division of labor, a key feature in the organization of insect societies.

At the molecular level, our findings suggest a link between honey bee foraging behavior and *per* upregulation in the brain; foragers are the only behavioral group that consistently had high average daily levels. Our results show that the previously reported (Toma et al., 2000) association between variation in average daily levels of *per* expression and age-related division of labor in honey bees cannot be completely explained by variation in behavioral rhythmicity, task, or age. In addition, we found that *per* mRNA levels were different in young bees housed in different social environments. Taken together, these results suggest that social factors influence *per* expression in the bee brain.

There was no consistent association between average daily levels of *per* brain mRNA and behavioral rhythmicity in honey bees. The rhythm hypothesis predicted that bees with circadian rhythms of locomotor activity would have higher brain *per* mRNA levels than arrhythmic bees. In *Drosophila*, genetically transformed lines that express very low levels of *per* tend to be behaviorally arrhythmic (Baylies et al., 1987). But mutations in *per* that shorten or lengthen the free-running period for locomotor activity affect other aspects of *per* mRNA and protein oscillation, not average daily levels (reviewed in Hall, 1995, 1998; Young, 1998; Williams and Sehgal, 2001). In our experiments, young bees—both with and without circadian rhythms of locomotor activity in the laboratory—had low levels of *per* mRNA in the brain, while foragers were rhythmic and had high levels. In addition, foragers that reverted to brood care under field conditions showed arrhythmic behavior (Bloch and Robinson, 2001), but we showed here that they tended to have high levels of *per* mRNA in the brain (in two out of three colonies). Based on previous results, we assume that foragers (before reversion) had strong circadian rhythms of activity (e.g., Spangler, 1972; Moore and Rankin, 1985; Toma et al., 2000; see also Table 1 and Fig. 1A).

In one colony in Experiment 3, reverted nurses had lower average daily levels of *per* mRNA in the brain than did foragers. Differences between colonies, which may reflect differences in genotypic or environmental factors, are routinely detected in behavioral and physiological analyses of bees (e.g., Giray et al., 1999), which is why it is important to replicate experiments with bees from different colonies. We do not know the basis for the observed difference among the reversion colonies. Also, because this was a field experiment, we could not measure the locomotor behavior rhythms of foragers and reverted nurses under precisely controlled chronobiological conditions as in Experiment 1.

There was no consistent association between average daily levels of *per* brain mRNA and chronological age in honey bees. The age hypothesis predicted that old bees (> 21 days of age) would always have higher *per* than young bees (about 7 days of age). Toma et al. (2000) also showed that elevated *per* mRNA levels cannot be attributed exclusively to aging, because bees induced to forage precociously in single-cohort colonies had levels similar to normal-age foragers. We confirmed and extended these findings by showing that both nurses and foragers, young and old, had high levels of *per*. The effects of age on the expression of clock genes have not been studied in other systems, but many other effects of aging on components of circadian clocks have been documented (Turek and Zee, 1999).

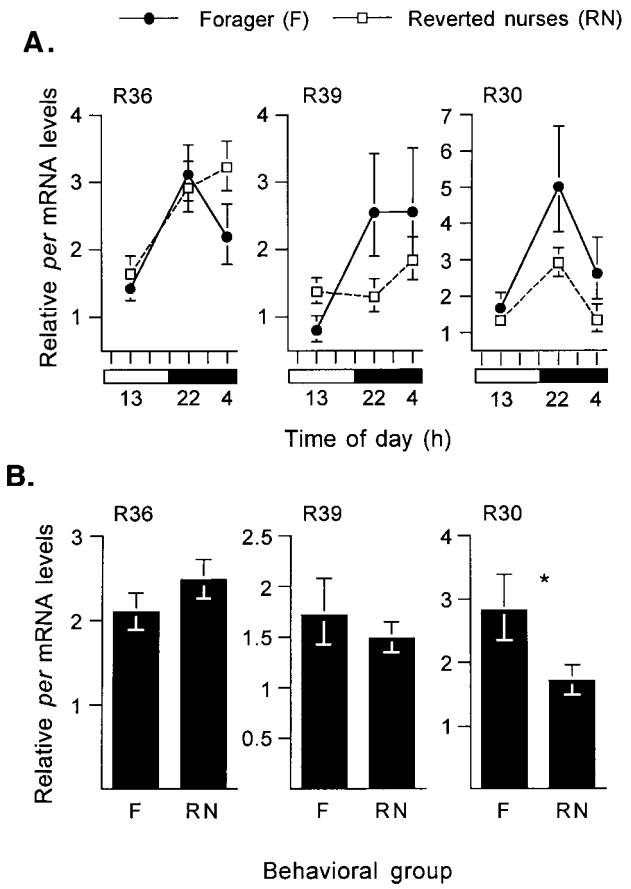

Figure 3. ***per*** **brain mRNA levels (mean ± SE) in bees from two groups from reversion colonies: foragers and foragers that reverted to nursing (reverted nurses). (A) Quantification of** ***per*** **mRNA levels over time (*n* = 6-9 brains/behavioral group/time point analyzed individually from R30; 7-12 from R36; and 9-11 from R39). Reverted nurses: open squares; foragers: filled circles. The *x*-axis depicts the time of day. The bars in the bottom of each plot depict the ambient photoperiod: filled bars = dark (sunset to sunrise). Note that the bees' exposure to light did not necessarily match daylight time (e.g., bees can leave the dark hive some time after sunrise). (B) Average daily** ***per*** **mRNA levels. Same data as in Panel A. Results of statistical analyses appear in text. Levels of mRNA were measured with real-time quantitative reverse transcriptase polymerase chain reaction.**

The task hypothesis predicted that foragers would have higher *per* than bees working in the hive. We speculated that *per* may influence the development of hive bees into foragers. If that were the case, we would expect levels of *per* mRNA to be low in nurses and high in foragers. Results from Experiments 2 and 3 indicate that there is no association between low levels of *per* brain mRNA and nursing behavior, which is not consistent with a prediction of the task hypothesis. Foragers, however, provided data consistent with this hypothesis. Although there were no differences between nurses and foragers in single-cohort colonies, we believe that both groups had high levels because in a previous study precocious foragers from single cohort colonies had the same high levels as normal-age foragers (Toma et al., 2000). This is also consistent with other studies in which normal-age foragers in single-cohort colonies were similar to foragers in typical field colonies with respect to several physiological and neuronal measures (e.g., Robinson et al., 1989; Withers et al., 1993; Schulz and Robinson, 1999). We speculate that there may be a link between the behavioral state of foraging and up-regulation of *per* expression in the brain. If this up-regulation involves an increase in the number of *per* expressing cells, it may result in more neural circuits coming under circadian regulation. Increased circadian regulation of neural circuitry might be needed to support some of the activities associated with foraging, including sun compass navigation, dance communication, and timing visits to flowers for maximum nectar and pollen availability. We also found that foragers had a shorter *tau* for locomotor activity in the laboratory than did young rhythmic bees. In other words, foragers had a shorter *tau* and higher levels of brain *per* mRNA, while young rhythmic bees had a longer *tau* and lower *per*. The association of *per* elevation and shorter *tau* was reported for flies, based on transgenic manipulations (Baylies et al., 1987) and crosses that produced lines with different dosages of the *per* gene (Smith and Konopka, 1982). Because free-running period data for foragers were obtained from only one trial, additional studies are necessary to determine the generality of this finding for bees.

What is different about life in a single-cohort colony compared with a more typical bee colony that may lead to a premature up-regulation of *per* expression in the brain? Possibilities include reduced social inhibition of behavioral maturation (Huang and Robinson, 1992) or other social (or environmental) factors associated with a smaller colony size. Perhaps the relatively small single-cohort colonies cannot regulate the hive environment as efficiently as larger field colonies. Another possibility is bees in a single-cohort colony take orientation flights at younger ages than in typical colonies (GE Robinson, unpublished observations), but preliminary results indicate that light and flight experience do not cause *per* up-regulation (Bloch, Rubinstein, and Robinson, unpublished).

Another instance of differences in *per* expression in different social contexts can be seen by comparing results of single-cohort colonies in the field and lab

studies with small single-cohort colonies or isolated bees. Young bees in single-cohort colonies in the field have similar levels to foragers (Fig. 2), but young bees housed in the laboratory individually (Fig. 1) or in small single-cohort colonies (Toma et al., 2000) had lower levels than foragers. The lab and field environments differ not only in climatic factors but also in many social parameters. For example, the field colonies had live queen and brood, while in the laboratory the bees had queen pheromone strip and no brood.

Together, our studies suggest that the regulation of *per* in the honey bee brain is influenced by environmental, social, and developmental factors. The details of this complex integration cannot be resolved at this stage. It is possible that developmental and social factors influence *per* expression in cells in different regions of the brain. Such changes cannot be effectively resolved in a whole brain analysis (as in the current study and in Toma et al., 2000). Precise manipulations of the social environment, uniquely possible with bees, combined with precise quantification of *per* levels in different parts of the brain (e.g., in situ hybridization or immunocytochemistry) should provide the means to uncover the social and developmental factors affecting *per* expression in honey bees.

Our experiments were designed to explore the regulation of average daily levels of *per* brain mRNA. The increase that occurs during honey bee behavioral development is relatively modest, but changes of similar magnitude can have important functional significance (Greenspan, 1997). The magnitude of the difference between high and low average daily levels is about the same as the differences that occur during diurnal oscillations of *per*, a phenomenon that is much better understood in chronobiology and is known to have functional significance.

Our experiments were not designed to rigorously test for differences in *per* oscillation. Nevertheless, based on a limited number of data points (3-4/day), our results hinted at a greater amplitude of *per* oscillation in foragers than in nurses, independent of age. The apparent task-related difference in amplitude reported here was not seen consistently in Toma et al. (2000), perhaps because most colonies in that study were synchronized by light and were not engaged in the task of brood care because the colonies did not have brood. If real, a *per* amplitude difference between nurses and foragers could be due to a number of factors. There are differences in activity patterns; foragers are active during the day and rest at night, while nurses are active around the clock (Kaiser and Steiner-Kaiser, 1983; Crailsheim et al., 1996; Moore et al., 1998; Bloch and Robinson, 2001). Changes in locomotor activity can cause changes in the pattern of *per* oscillation in hamsters (Maywood et al., 1999). If behavioral activity indeed influences *per* oscillation, then the presence of brood may be important because it affects nurse bee activity. Honey bee larvae need constant care and are attended around the clock by nurse bees. Alternatively, differences in amplitude may be associated with task-related changes in the molecular function of the internal clock. Changes that affect biochemical interactions among clock gene products can result in changes in the amplitude of *per* mRNA or protein (e.g., Schotland et al., 2000). It is also possible that the apparent differences in amplitude are because of better synchronization of *per* expression in foragers than in young nest bees. This might be because foragers spend more time outside of the dark and thermoregulated hive than do nurse bees and are therefore more exposed to strong zeitgebers (daily fluctuations in light and temperature). Lack of synchronization would lead to an apparent dampening of *per* amplitude (e.g., Hardin, 1994; Brandes et al., 1996; Cheng and Hardin, 1998). However, members of a bee colony can become synchronized to each other's rhythms via social interactions (Southwick and Moritz, 1987; Moritz and Sakofski, 1991; Frisch and Koeniger, 1994; Moritz and Kryger, 1994). Young bees display some synchronized behavior; they typically leave the hive at about the same time of day for brief orientation flights to learn the location of their hive relative to prominent landmarks in preparation for foraging (Winston, 1987; Capaldi et al., 2000). Additional studies are required to determine whether the apparent task-related differences in *per* amplitude are real and, if so, due to intrinsic or extrinsic factors.

Social regulation of a clock gene has not yet been shown in any other animal besides the honey bee. To better understand this phenomenon, it is necessary to extend this study to additional genes in the honey bee clock machinery. Clock gene expression is known to be influenced by light, temperature, and other clock genes (reviewed in Turek and Zee, 1999; Williams and Sehgal, 2001); recently, locomotor activity (Maywood et al., 1999) and seasonal time (Majercak et al., 1999; Nuesslein-Hildesheim et al., 2000) also have been implicated. Social regulation of circadian physiological and behavioral rhythms has been well documented (e.g., Kavaliers, 1980; Marimuthu et al., 1981; Mrosovsky, 1988; Duffield and Ebling, 1998; Rajaratnam and Redman, 1999), and some of these

rhythms are now known to be under the control of molecular mechanisms that involve *per*. Our results suggest that it will be fruitful to consider social factors when studying the regulation of clock genes in other species.

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