Changes in *period* mRNA levels in the brain and division of labor in honey bee colonies

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Previous research showed that age-related division of labor in honey bees is associated with changes in activity rhythms; young adult bees perform hive tasks with no daily rhythms, whereas older bees forage with strong daily rhythms. We report that this division of labor is also associated with differences in both circadian rhythms and mRNA levels of *period*, a gene well known for its role in circadian rhythms. The level of *period* mRNA in the brain oscillated in bees of all ages, but was significantly higher at all times in foragers. Elevated *period* mRNA levels cannot be attributed exclusively to aging, because bees induced to forage precociously because of a change in social environment had levels similar to normal age foragers. These results extend the regulation of a "clock gene" to a social context and suggest that there are connections at the molecular level between division of labor and chronobiology in social insects.

Age-related division of labor, the most common form of division of labor among workers in insect societies (1), is based on a pattern of behavioral development by individual workers. Workers typically spend the first part of their adult life working in the nest and the final part of their adult life foraging for food outside the nest (2). This division of labor is also flexible, as workers are able to respond to changes in colony needs by showing plasticity in the rate of behavioral development. In honey bees, where this plasticity has been studied most extensively, the pace of behavioral development is known to be influenced by environmental, social, and genetic factors (3). That division of labor is a highly regulated process is evident by the fact that behavioral development in honey bees involves changes in circulating hormone levels (4), brain chemistry (5, 6), and brain structure (7).

Changes in neural function that underlie long-term changes in memory are subserved by transcription-dependent mechanisms (8). This also might be the case for the long-term changes in neural function that underlie age-related division of labor in social insects. In animal species in general, there is as yet little evidence for changes in gene expression in the brain that are associated with behavioral plasticity in naturalistic contexts (9–12). There is only a single report of developmental regulation of gene expression in the bee brain: age-related increases in brain mRNA levels of royal jelly protein-3 (13), which is very similar to a protein produced by the hypopharyngeal glands and fed to the brood. The function of this protein in the brain is not known.

Studying "candidate genes" is one way to gain insight into the extent to which transcription-dependent mechanisms are involved in division of labor. To use this approach, it is necessary to choose a gene whose function in another organism may plausibly be related to division of labor. Our choice was guided by recent findings of intriguing changes in activity rhythms associated with division of labor. Forager honey bees have a highly developed internal circadian clock that is used for sun compass navigation, dance communication, and for timing visits to flowers for maximum nectar and pollen availability (14). However, tasks such as brood care are performed around the clock, arrhythmically (15, 16). Bees showed activity rhythms in the hive before they began to forage, and genotypes that resulted in younger ages at onset of foraging also showed an earlier onset of activity rhythms (16). Based on these findings, we hypothesized that division of labor in honey bee colonies also involves behavioral and molecular changes in circadian processes.

To determine whether the changes in diurnal activity rhythms observed under natural conditions (15, 16) reflect changes in endogenous circadian rhythms, we studied the locomotor activity of newly emerged adult bees under controlled laboratory conditions. We also tested the hypothesis that division of labor in honey bees is associated with changes in a molecular component of the circadian clock. We chose *period* (*per*) because in *Drosophila melanogaster* it shows pleiotropy for temporal phenomena. Mutations of *per* affect circadian locomotor rhythms (17), mating behavior (18), and development time from egg to adult (19), processes with dramatically different timescales. Honey bee behavioral development—from hive tasks to foraging—is not a rhythmic process, but it has a strong temporal component. Here, we report on changes in *per* mRNA levels in the brain associated with division of labor.

Materials and Methods

Bees. Bee colonies were maintained in the field according to standard commercial techniques at the University of Illinois Bee Research Facility (Urbana, IL). The colonies in this region are derived from a mixture of European races of *Apis mellifera*. Bees used in all experiments were from colonies headed by queens that were each instrumentally inseminated with semen from a single (different) drone. Different colonies were used in each experiment; in some experiments, a colony was used as a "source" to make smaller experimental colonies. Sample sizes for bees and colonies are given for each experiment.

Measuring the Ontogeny of Circadian Rhythms for Locomotor Activity. Care was taken to minimize exposure of bees to extrinsic factors that could entrain activity rhythms. We collected frames of pupae from colonies in the field and placed them in a light-proof container in an incubator (33°C, 95% relative humidity). Under brief exposure to dim red light (which bees cannot see; ref. 14), we transferred 0- to 24-h-old adult bees to individual clear Plexiglas cages with sugar syrup, pollen substitute, and water ad libitum and maintained in constant darkness (DD) at 26°C in an environmental chamber. Locomotor activity was monitored automatically with an infrared sensor (20) and recorded by the Datacol 3 acquisition system (Mini-Mitter, Sunriver, OR). We determined whether patterns of activity had circadian rhythms in the first experiment by eye with double-plotted data (20) and in experiments 2 and 3 first by eye and then with χ^2 periodogram

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Abbreviations: *per*, *period*; *EF-1α*, *elongation factor 1α*; DD, constant darkness; LD, light/dark.

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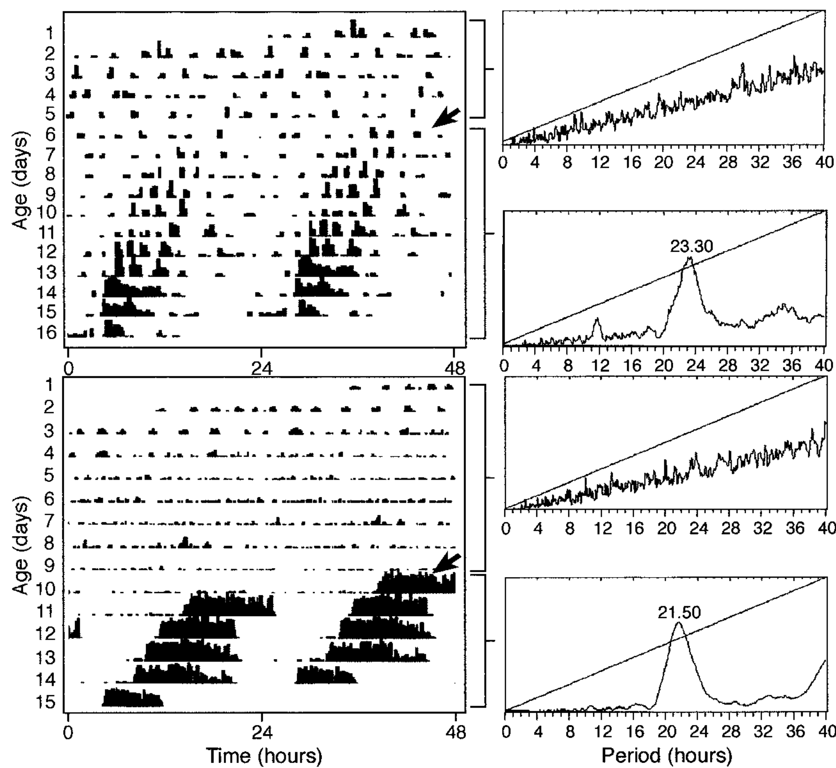

Fig. 1. Ontogeny of circadian rhythm in honey bee locomotor behavior. Representative actograms (*Left*) and periodogram analyses (*Right*) for two unrelated bees. Brackets indicate the arrhythmic and rhythmic portions of each actogram, as determined by eye, with a periodogram for each portion. Arrows on (double-plotted) actograms indicate the onset of circadian rhythm. A significant ($P = 0.01$) circadian rhythm is indicated by a peak above the diagonal line on the periodogram.

analysis (Tau program; Mini-Mitter) (21). The criterion for the onset of rhythmicity was set at $P < 0.01$; we used this criterion because periodic rhythms significant with a criterion of $P < 0.05$ were detected even in the absence of a single clearly defined peak of activity. This is probably because of the large sample sizes for each bee, which are a reflection of the high level of bee activity in the cages, and data being collected every 10 min. Using $P < 0.01$ as the criterion resulted in only those activity records with a single clearly defined peak of activity showing statistical significance. Experiment 1: $n = 55$ bees from 13 different colonies ($\approx$4 bees per colony); experiment 2: $n = 33$ bees from colony R1; and experiment 3: $n = 23$ bees from colony R20.

Laboratory Colonies. We measured brain mRNA *per* levels from bees collected from "single-cohort" colonies maintained in an environmental chamber (31°C day and 28°C night, 65% relative humidity). Each single-cohort colony was composed initially of 450–650 1-day-old bees. Colonies were entrained to light/dark (LD) 12:12. Fluorescent lights were positioned to ensure even lighting, minimizing the possibility that bees could avoid entrainment by hiding in dark corners of the hive (22). A queen pheromone strip (Bee Boost; Phero Tech, BC, Canada) was used instead of a live queen because a live queen can influence the rhythms of worker bees (23).

Brain mRNA *per* levels were measured from three age groups of bees. First, we collected young bees (4–6 days old) from three single-cohort colonies (one colony each from source colonies 19, 45, and 101) maintained for 4 days under LD (12:12) and for 2 days under DD. During DD, bees were exposed to dim red light produced by fluorescent bulbs specially coated (Chemical Products, North Warren, PA) to only emit 660- to 670-nm wavelengths. This light was used to allow for collections to be made during dark phases. Bees were collected on day 4 of LD and the 2 days of DD. We collected the next sample of young bees (7–9 days old) from three different single-cohort colonies (again, one

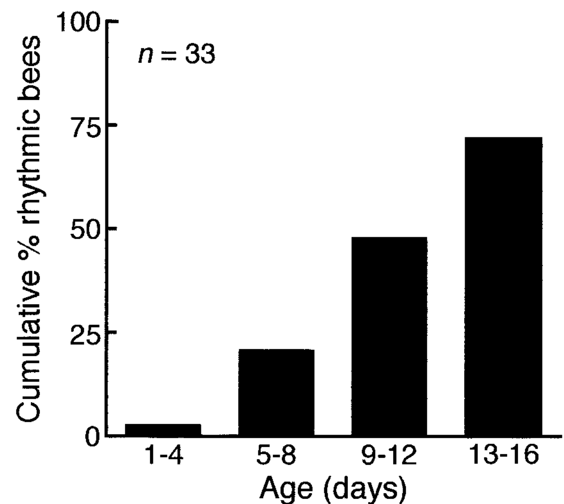

Fig. 2. Age-related changes in percentage of bees showing circadian rhythms for locomotor behavior. For each bee, periodogram analysis was performed on data from successive 4-day intervals to determine when there was significant circadian rhythmicity ($P < 0.01$). Cumulative proportions of rhythmic bees are depicted on graph; G tests were performed on actual frequencies to determine whether the distribution of rhythmic bees changed with age (results in text). Data are from the second trial of this experiment, in which 25% of the bees never showed circadian rhythms during the observation period. Results of the other two trials are not shown (descriptive statistics in text).

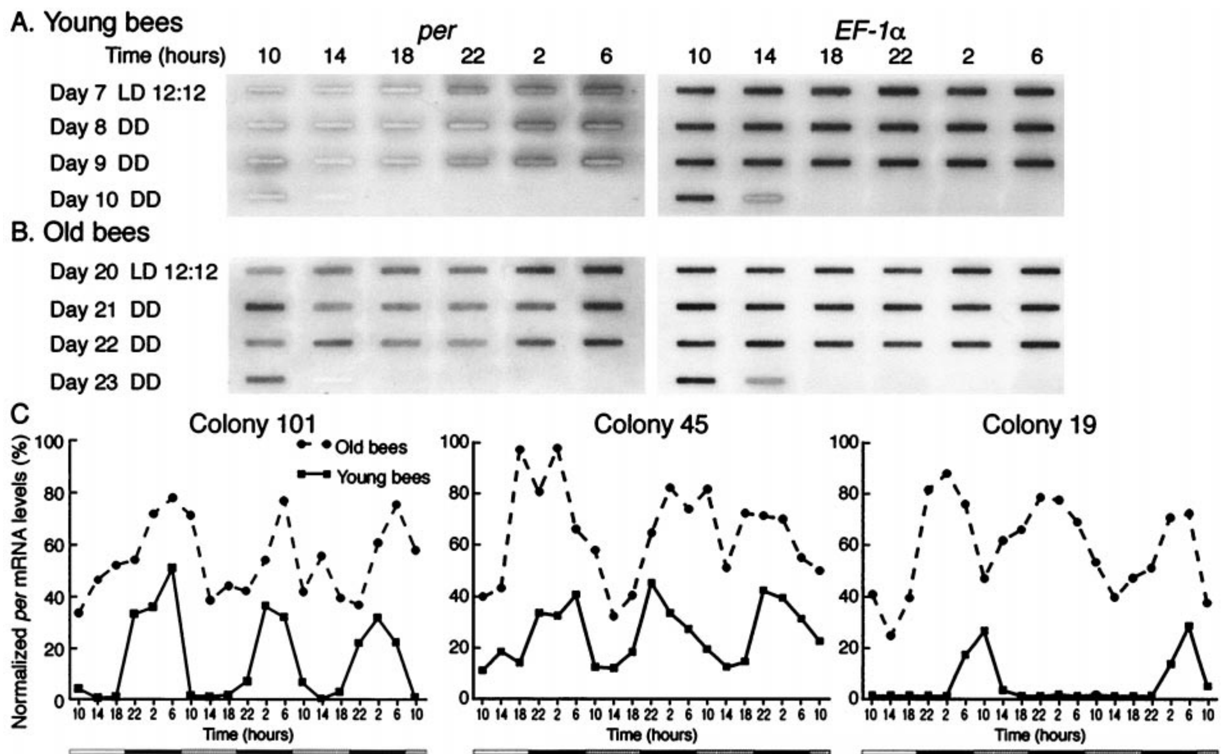

Fig. 3. Differences in *per* mRNA levels in the brains of young and old bees. Representative RNA slot blots (*A* and *B*) from one of the three laboratory colonies studied and densitometric analyses (*C*) from all three colonies (National Institutes of Health IMAGE version 1.6.1 software) for 7- to 9- and 20- to 22-day-old bees. Blots were quantified by scanning only a rectangular region within each slot (darkened background areas around some slots are artifacts and were not used for quantification). Images were scanned to produce the figure by using ADOBE PHOTOSHOP. Results normalized to *EF-1*α mRNA values for each point. Bar below *x* axis: day (open bar) and night (filled bar) for LD and subjective day (hatched bar) and night (filled bar) for DD. Levels of *EF-1*α mRNA in the brain (data not shown) did not vary with age (ANOVA; $P > 0.40$, $F = 0.774$, $n = 14$). Slots in the Day 10/Day 23 14-hour positions contain bovine rRNA as a negative control (same quantities as the bee RNA).

colony each from source colonies 19, 45, and 101) maintained for 7 days under LD and for 2 days under DD. Bees were collected on day 7 of LD and the 2 days of DD. Finally, we collected old bees (20–22 days old) from two single-cohort colonies (from source colonies 45 and 101) maintained for 20 days under LD and for 2 days under DD. Bees were collected on day 20 of LD and the 2 days of DD. Old bees derived from source colony 19 were taken directly from the source colony itself in the field; the bees were entrained (12:12 LD) in the environmental chamber for 5 days and then were collected during day 6 of LD and 2 days of DD. For each age group, we collected bees every 4 h during the three collection days ($n = 20$ time points, 20 bees per time point). Bees were entrained under LD for a minimum of 4 days (S. M. Reppert, personal communication) because mRNA analyses required the use of material pooled from multiple bees for each data point. This protocol prevented us from analyzing bees younger than 4 days of age. We collected two groups of young bees to maximize chances of detecting differences relative to old bees.

Field Colonies. To determine whether results obtained from laboratory colonies accurately reflect results that occur under more natural conditions, we also measured brain *per* mRNA levels from bees collected from one free-flying colony. These bees were from one of the same source colonies used above (source colony 45). We marked 1-day-old bees ($n = 500$) with a spot of paint on the thorax and introduced them into an unrelated colony in a standard hive maintained indoors at 27°C with an opening to the outside. The colony was exposed to natural sunlight at the hive entrance (LD cycle ≈12:12) and allowed free flight; it was kept indoors to enable us to efficiently remove bees during nocturnal collections. We collected marked bees from honeycombs containing brood in the hive at 7 days of age (under red light) and as returning foragers 17–24 days of age at the hive entrance every 3 h during 1 day. Some marked foragers were also marked with an additional paint dot as they entered the hive and then were sampled from combs during the nocturnal collections.

To determine whether differences in brain *per* mRNA levels detected in laboratory and field colonies are associated solely with differences in age, we also measured brain mRNA levels from bees taken from two single-cohort colonies in the field. These single-cohort colonies each consisted of *ca.* 2,000 1-day-old bees, a queen, and three frames of comb, two partially filled with honey and pollen, and one empty for the queen to lay eggs (24). Each single-cohort colony was made from a different source colony than in previously described experiments. About 5–10% of the members of a single-cohort colony develop into precocious foragers about 2 wk earlier than usual because older bees are not present to inhibit their behavioral development (24, 25). We collected precocious and normal-age foragers every 6 h for 1 day when they were 10 and 22 days old, respectively. (The laboratory colonies described above also were single-cohort colonies, but we did not expect that sampling them at early ages would be compromised by the presence of precociously developing bees; they had very small populations and few bees in a single-cohort colony are precocious.)

Individually Reared Bees. To determine whether differences in brain *per* mRNA levels detected in laboratory and field colonies

Table 1. Results of ANOVA for mRNA levels in the brains of young vs. old bees

Laboratory colonies			Field colony		
Effect	*F* value	*P*	Source	*F* value	*P*
Colony	103.23	0.0001	Age	29.53	0.0006
Age	407.8	0.0001	Time	4.20	0.0309
LD/DD	2.31	0.1661			
Time	26.97	0.0001			

Data in Figs. 3 and 4. $n = 19$ and 9 time points per colony, for laboratory and field colonies, respectively.

completely depend on the social environment, we reared bees individually in the laboratory, as described above. One-day-old bees (from a different source colony than in previously described experiments) were kept in the laboratory at 26°C for 6 days [3 days of LD 15:9 (similar to natural conditions at that time of year) and 3 days of DD]. Foragers were collected in the field from the same colony and kept in the laboratory for 3 days (DD). Bees were collected every 6 h the following day.

Molecular Cloning of a Honey Bee Ortholog of *Period*. Total RNA was isolated from bee brains (RNeasy total RNA isolation kit; Qiagen, Valencia, CA) followed by treatment with DNase (1 unit RQ1 Dnase; Promega). mRNA was isolated with the Poly(A) Quik kit (Stratagene). First strand synthesis was done according to GIBCO/BRL protocols for Superscript II using 200 ng of poly(A) RNA, 150 ng of random hexamers, and 200 units of Superscript reverse transcriptase. PCR was with degenerate primers based on amino acid sequences CVISMH and YNQLNYN (26) and conditions reported therein (except final primer concentration 400 nM and one set of 35 cycles). A 1,083-bp PCR fragment was sequenced, and a BLAST search of GenBank revealed that it has significant amino acid similarity to *per* from other insects: 58% for *Periplaneta americana*, 55% for *Drosophila melanogaster*, and 48% for *Hyalophora cecropia* and *Manduca sexta*. The full-length sequence for cDNA (*AmPer*) was obtained by probing a UniZAP vector honey bee brain cDNA library (made for us by Stratagene) with the PCR fragment. *AmPer* shows several regions of similarity to *per* in all other species, including the presence of the PAS domain, which mediates interactions between PER and other clock proteins (27). The GenBank accession number for the full cDNA and amino acid sequence is *AmPer* AF159569.

Quantification of mRNA. *Slot blot analysis.* Bees were collected directly into liquid N_2, and brains were dissected on dry ice. Total brain RNA was isolated (as above), and 7.5 μg and 1.5 μg, respectively, were used for blots with *per* and *elongation factor 1 alpha (EF-1α)* as a control (28). We used a 1,083-bp *per* riboprobe from the PCR fragment, which spanned most of the c2 region (29), and a 600-bp honey bee *EF-1α* riboprobe (from a clone given to us by P. R. Ebert, University of Queensland). Both were digoxygenin (DIG)-11-UTP labeled and were used with DIG Easy Hyb buffer (Boehringer Mannheim, manufacturer's protocols); *per* blots were probed at 62°C with 35 ng/ml probe.

Northern blot analysis. To verify the results of slot blot analyses, we also measured levels of brain *per* mRNA with Northern blot analysis. Total RNA (5 μg), extracted from 4–10 brains per time point, was separated on 1% agarose/0.6 M formaldehyde gels. Filters were first probed with *per* and then stripped and reprobed with *EF-1α*. Procedures and probes were as described above.

Statistical analyses. More than 2,000 brains were dissected and pooled (4–10 per time point) for mRNA quantification. For laboratory colonies, we used split-plot ANOVA with colony and age as whole plots, LD/DD as split levels and time as reported within; for the field colony, two-way ANOVA (time and age); for field single-cohort colonies and individually reared bees, paired *t* tests; (SAS Institute, Cary, NC). For behavioral analyses, G tests were used to determine whether the proportion of bees with circadian locomotor rhythms increased with age.

Results

All individual bees reared in the laboratory were very active, but none showed any discernible circadian rhythm of locomotor activity for the first few days (Fig. 1). The mean (±SE) age at onset of circadian rhythm was 7.6 ± 0.6 in the first experiment ($n = 55$ bees from 13 different colonies, ≈4 bees per colony), 8.3 ± 0.4 in experiment 2 ($n = 33$ bees from colony R1), and 7.0 ± 0.6 days in experiment 3 ($n = 23$ bees from colony R20). The proportion of rhythmic bees increased significantly with age in experiments 1, 2, and 3 ($P < 0.01$, G tests) (Fig. 2).

Per mRNA cycled in bee brains in an endogenous circadian manner. *Per* mRNA levels oscillated in both young and old bees (Fig. 3) in laboratory colonies under conditions of DD (Table 1, significant "time" effect). Exposure to light did not cause significant variation in absolute levels (Table 1, LD/DD), again indicating endogenous regulation. These results are consistent with analyses of *per* in other species (27).

There was a significant effect of age on *per* mRNA levels in the brain of bees from in laboratory colonies (Table 1). Young bees

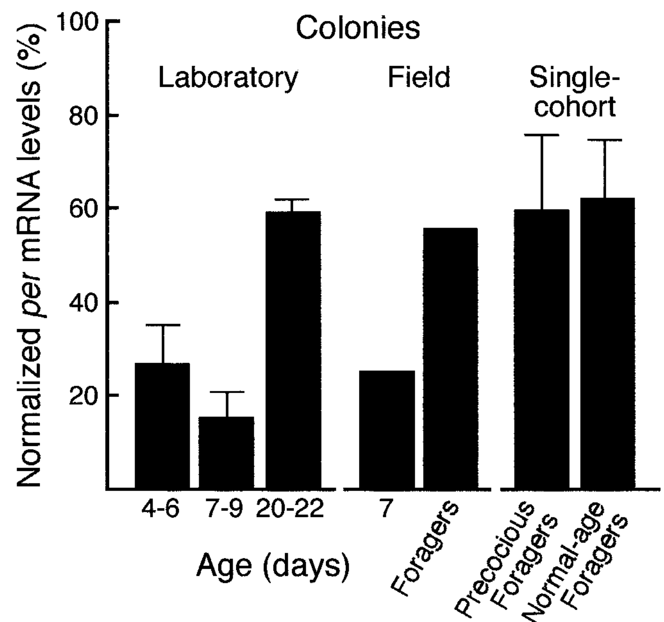

Fig. 4. Differences in *per* mRNA levels in the brains of young and old bees, 7-day-old hive bees and 17- to 24-day-old foragers and precocious and normal age foragers. Means (±SE) of colony means given for laboratory ($n = 3$) and single-cohort ($n = 2$) colonies; $n = 1$ field colony. Representative slot blots in Fig. 3; results of statistical analyses for laboratory and field colonies in Table 1 and for single-cohort colonies in the text.

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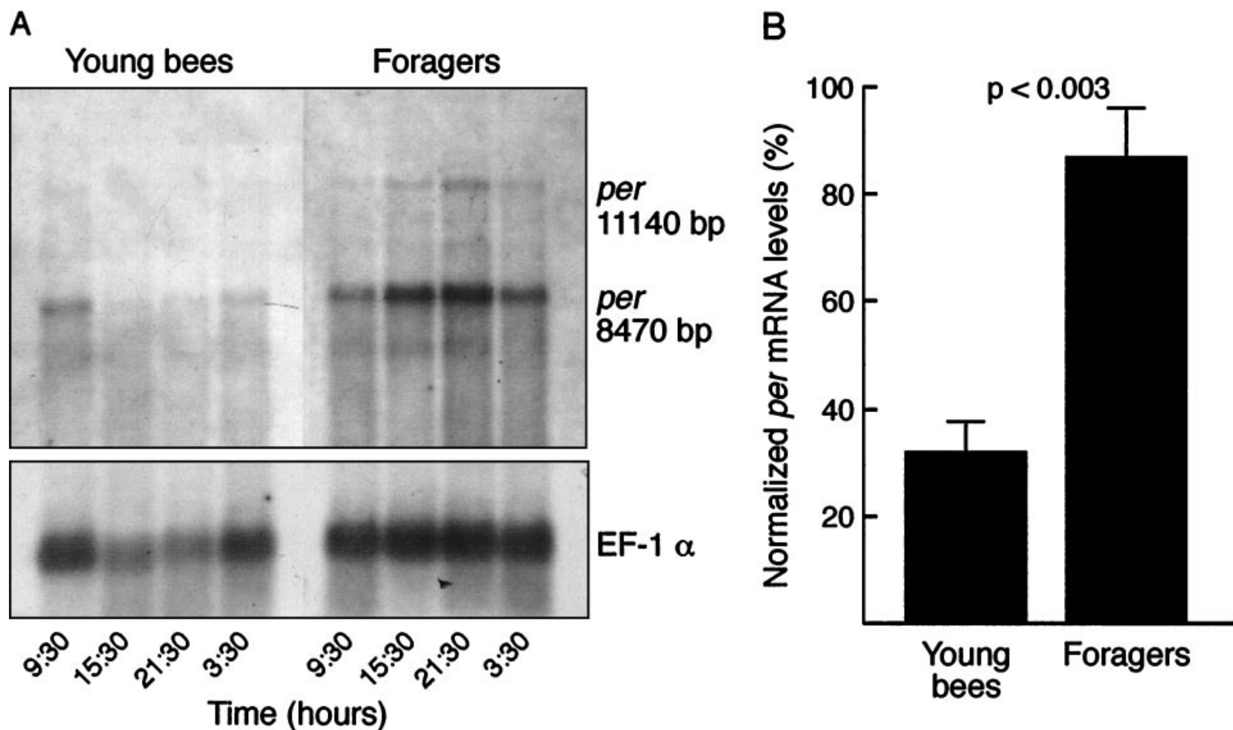

Fig. 5. Differences in mRNA levels in the brains of young laboratory-reared bees and foragers. Northern blot (*A*) and densitometric analyses (*B*) (normalization as in Fig. 3). Result of statistical analyses (paired *t* test) indicated on graph ($n = 4$ time points per group, 4–10 brains per time point). Two transcripts were detected, and both appear to cycle in circadian fashion. We do not know whether they are alternative transcripts of alleles, splice variants, or whether the larger transcript is a pre-mRNA; Southern analyses indicate one copy of *per* in the bee genome (D.P.T., Y. Ben-Shahar, A. Tam, and G.E.R., unpublished observations). Values for both transcripts were summed (analyses with major transcript alone gave the same results). The large size of both transcripts relative to the entire coding region is consistent with previous reports (26).

(4–6 days old and 7–9 days old) had *per* mRNA levels that were on average 45.8% and 26.3%, respectively, as high as old bees (Fig. 4). Differences in *per* mRNA levels between young and old bees were seen in all laboratory colonies despite the presence of significant intercolony variation in overall levels (Table 1) and phase and amplitude (Fig. 3).

There also were significant differences in *per* brain mRNA levels in bees collected from a field colony maintained under more natural conditions (Table 1). mRNA levels in 7-day-old bees were significantly lower than in foragers (Table 1), only 45.5% as high (Fig. 4). Northern blot analysis confirmed that foragers had significantly higher *per* levels than young bees (Fig. 5), even after social isolation for several days.

The increase in *per* mRNA levels in the brain was not strictly associated with aging. Precocious foragers from two single-cohort colonies maintained in the field had levels of *per* mRNA that were not significantly different from normal age foragers ($P > 0.81$; Fig. 4).

Discussion

Honey bees behave arrythmically during the first part of their adult life when they work at tasks that require around-the-clock activity. They then show strong rhythmic behavior before the onset of foraging and use their circadian clock for navigation and foraging-related activities (16). We showed that this change reflects an ontogeny of endogenous circadian rhythmicity. Postembryonic development of circadian behavioral rhythms has not been observed in other insects but does occur in reptiles, birds, and mammals (30). The absence of circadian behavioral rhythms in very young mammals is thought to be a result of a lack of entrainment rather than a lack of circadian function in the superchiasmatic nuclei, the location of the mammalian circadian clock (31). Eusocial naked mole rats that live their entire life as nest workers apparently have no circadian rhythm of activity, whereas dispersing males that leave the colony do (32). Behavioral results from bees and naked mole rats suggest that social evolution can influence the functioning of circadian rhythm systems.

We also demonstrated that changes in the expression of a clock gene are associated with age-related division of labor in a social insect. Although there is both development of rhythmic locomotor activity and change in the levels of brain *per* mRNA, we cannot conclude that these two processes are connected because they were not measured in the same bees. Based on the data presented in Fig. 2, it is likely that many, but not all, of the young bees used for mRNA analyses were arrhythmic; it was not possible to measure *per* levels in younger bees because of the constraints of the experimental design.

With the recent cloning of other genes that interact with *per*, the mechanism for rhythmic cycling of *per* transcription is beginning to be understood, and many of the molecules involved are conserved in mammals and insects (27). Future models of *per* regulation also will need to explain the phenomenon reported here: increases in *per* mRNA levels in the bee brain that occur during behavioral maturation even as cycling persists. The increase in *per* mRNA in the bee brain was associated with foraging, but it cannot be caused solely, if at all, by increased chronological age, foraging, flight, or exposure to the sun. Bees in colonies confined to the laboratory also had high, forager-like, levels of *per*, and foragers isolated from their social environment and maintained in darkness for several days still maintained high *per* levels. We speculate that levels of brain *per* mRNA increase in anticipation of the challenges of foraging. Perhaps higher levels of *per* are required to engage clock-controlled systems in

the bee that are required for efficient social foraging, such as time-compensated sun compass navigation, dance communication, and the ability to time visits to flowers.

Our speculation is consistent with results showing that transgenic manipulations in *Drosophila* that result in changes in *per* mRNA dynamics also influence circadian rhythms (33, 34). The general notion that *per* is involved in a more diverse set of biological phenomena than previously recognized is supported by recent findings (35) of unexpected roles for *per* and other clock genes in cocaine sensitization and the similarity of a *Caenorhabditis elegans* developmental timing protein to proteins encoded by clock genes (36). A key first step in exploring the functional significance of the *per* increase in bees is to determine whether a similar increase occurs for the PERIOD protein, and if so, in what cells in the brain. Preliminary results (37) suggest that there is an age-related increase in the number of PERIOD-immunoreactive cells in the bee brain.

At present, it is not feasible to determine definitively the function of the *per* increase in bees because techniques to manipulate the honey bee genome are not yet available. However, it is possible to determine whether socially mediated mechanisms that control the onset of foraging, such as juvenile hormone, biogenic amines, and primer pheromones (3, 5), are associated with the expression of *per*. The only factors known to influence the expression of any clock gene were light, temperature, and other clock genes (27); recently a behaviorally induced phase advance of the circadian clock was shown to also cause rapid down-regulation of *per* expression (38). Our results demonstrate that new challenges to the understanding of clock genes emerge by studying animals that live in complex societies.

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